WHO Expert Committee on Biological Standardization

Sixty-third report



The World Health Organization was established in 1948 as a specialized agency of the United Nations serving as the directing and coordinating authority for international health matters and public health. One of WHO's constitutional functions is to provide objective and reliable information and advice in the field of human health, a responsibility that it fulfils in part through its extensive programme of publications.

The Organization seeks through its publications to support national health strategies and address the most pressing public health concerns of populations around the world. To respond to the needs of Member States at all levels of development, WHO publishes practical manuals, handbooks and training material for specific categories of health workers; internationally applicable guidelines and standards; reviews and analyses of health policies, programmes and research; and state-of-the-art consensus reports that offer technical advice and recommendations for decision-makers. These books are closely tied to the Organization's priority activities, encompassing disease prevention and control, the development of equitable health systems based on primary health care, and health promotion for individuals and communities. Progress towards better health for all also demands the global dissemination and exchange of information that draws on the knowledge and experience of all WHO's Member countries and the collaboration of world leaders in public health and the biomedical sciences.

To ensure the widest possible availability of authoritative information and guidance on health matters, WHO secures the broad international distribution of its publications and encourages their translation and adaptation. By helping to promote and protect health and prevent and control disease throughout the world, WHO's books contribute to achieving the Organization's principal objective – the attainment by all people of the highest possible level of health.

The WHO Technical Report Series makes available the findings of various international groups of experts that provide WHO with the latest scientific and technical advice on a broad range of medical and public health subjects. Members of such expert groups serve without remuneration in their personal capacities rather than as representatives of governments or other bodies; their views do not necessarily reflect the decisions or the stated policy of WHO.

For further information, please contact: WHO Press, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (tel. +41 22 791 3264; fax: +41 22 791 4857; e-mail: bookorders@who.int; order online: http://www.who.int/bookorders).

WHO Expert Committee on Biological Standardization

Sixty-third report

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization



WHO Library Cataloguing-in-Publication Data

WHO Expert Committee on Biological Standardization, sixty-third report.

(WHO technical report series: no. 980)

- 1. Biological products standards. 2. Vaccines standards. 3. Reference standards.
- $4. \ Guideline. \ I. World \ Health \ Organization. \ II. WHO \ Expert \ Committee \ on \ Biological \ Standardization \ (2012: Geneva, Switzerland). \ III. Series.$

ISBN 978 92 4 1209802 ISSN 0512-3054 (NLM classification: QW 800)

© World Health Organization 2014

All rights reserved. Publications of the World Health Organization are available on the WHO web site (www.who.int) or can be purchased from WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland (tel.: +41 22 791 3264; fax: +41 22 791 4857; e-mail: bookorders@who.int).

Requests for permission to reproduce or translate WHO publications – whether for sale or for noncommercial distribution – should be addressed to WHO Press through the WHO web site (http://www.who.int/about/licensing/copyright_form/en/index.html).

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

All reasonable precautions have been taken by the World Health Organization to verify the information contained in this publication. However, the published material is being distributed without warranty of any kind, either expressed or implied. The responsibility for the interpretation and use of the material lies with the reader. In no event shall the World Health Organization be liable for damages arising from its use.

This publication contains the collective views of an international group of experts and does not necessarily represent the decisions or the policies of the World Health Organization.

Printed in Italy

Contents

Abl	Abbreviations						
1.	Introduction						
2.	General						
	2.1	Currer	nt directions	2			
		2.1.1	Strategic directions in biological standardization: WHO priorities	4			
		2.1.2	Vaccines and biological therapeutics: recent and planned activities in				
			biological standardization .	4			
		2.1.3	Blood products and related in vitro diagnostics: recent and planned				
			activities in biological standardization	6			
	2.2	Repor		6			
		2.2.1	Report from the WHO Blood Regulators Network	6			
	2.2	2.2.2	Report from the WHO collaborating centres for biological standards	,			
	2.3	Issues 2.3.1	Scientific issues identified by custodians of WHO biological reference	8			
		2.3.1	preparations	8			
		2.3.2	Issues shared with the WHO Expert Committee on Specifications for	(
		2.3.2	Pharmaceutical Preparations	11			
	2.4	Feedb	ack from other WHO committees	12			
		2.4.1	Request from the Strategic Advisory Group of Experts (SAGE) on				
			Immunization for guidance on off-label use of vaccines	12			
		2.4.2	Request from the WHO Immunization Practices Advisory Committee (IPAC)				
			to establish harmonized standards for the labelling of vaccines	13			
3.	Inte	ernatio	onal Recommendations, Guidelines and other matters				
	related to the manufacture and quality control of biologicals						
	3.1	Vaccin	nes and related substances	14			
		3.1.1	Recommendations to assure the quality, safety and efficacy of poliomyelitis				
			vaccines (oral, live, attenuated)	14			
		3.1.2	Guidelines on the quality, safety and efficacy of recombinant				
			malaria vaccines targeting the pre-erythrocytic and blood stages of				
			Plasmodium falciparum	15			
		3.1.3	Recommendations to assure the quality, safety and efficacy of diphtheria				
		244	vaccines (adsorbed)	16			
		3.1.4	Recommendations to assure the quality, safety and efficacy of tetanus	17			
		3.1.5	vaccines (adsorbed) Recommendations to assure the quality, safety and efficacy of DT-based	17			
		3.1.3	combined vaccines	19			
		3.1.6	Recommendations to assure the quality, safety and efficacy of Japanese	٠.			
		3.1.0	encephalitis vaccines (live, attenuated) for human use	19			
		3.1.7	In vitro assay system to replace the histamine sensitization test for				
			acellular pertussis vaccines	20			
	3.2	Blood	products and related substances	21			
		3.2.1	Strategies to promote the availability and safety of blood products	21			
		3.2.2	Blood components as essential medicines	22			
		3.2.3	Residual risk in recovered plasma to be used as an active pharmaceutical	_			
			ingredient for fractionation	24			

		3.2.4	National strategies for plasma-derived medicinal products	25			
		3.2.5 3.2.6	Calibration of secondary reference materials Assessment of commutability in WHO collaborative studies	26 26			
			·				
4.	Inte		onal reference materials – vaccines and related substances	28			
	4.1		WHO International Standards and Reference Reagents – vaccines and related substances				
		4.1.1	First WHO International Standard for anti-human papillomavirus				
			type 18 serum	28			
		4.1.2	First WHO Reference Reagent for bacille Calmette–Guérin vaccine of Moreau-RJ substrain	28			
		4.1.3	First WHO International Standard for human diphtheria antitoxin	29			
		4.1.4	Second WHO International Standard for antibody to influenza				
			H1N1pdm virus	30			
5.	Inte	ernati	onal reference materials – blood products and				
	rela	ated su	ubstances	31			
	5.1	WHO International Standards and Reference Reagents – blood products and					
		relate	d substances Fourth WHO International Standard for factor II and factor X concentrates	31 31			
		5.1.2		31			
		5.1.3	Second WHO International Standard for fibrinogen concentrate	32			
		5.1.4	First WHO Reference Reagent for activated blood coagulation factor XI				
		5.1.5	(human) Second WHO International Standard for high-molecular-weight urokinase	33 34			
		5.1.6	Third WHO International Standard for low molecular weight heparin	34			
6.	Inte	ernati	onal reference materials – in vitro diagnostic				
٥.			agents	35			
	6.1	6.1 WHO International Standards and Reference Reagents – in vitro diagnostic					
		device 6.1.1	e reagents Second WHO Subtype Reference Panel for HIV-1 NAT-based assays	35 35			
_				<i>J</i> .			
7.			onal reference materials – biotherapeutics other than oducts	36			
	7.1	•	International Standards and Reference Reagents – biotherapeutics other	50			
	7.1		plood products	36			
		7.1.1	Third WHO International Standard for erythropoietin (recombinant)				
			for bioassay	36			
		7.1.2	Fifth WHO International Standard for follicle-stimulating hormone and luteinizing hormone (human, urinary) for bioassay	36			
		7.1.3	Second WHO International Standard for interleukin-2 (human, rDNA-derived)	37			
		7.1.4	First WHO Reference Reagent for interleukin-29 (human, rDNA-derived)	38			
8.	Inte	International reference materials – antibiotics					
	8.1	WHO	International Standards and Reference Reagents – antibiotics	39			
		8.1.1	Second WHO International Standard for neomycin B	39			
		8.1.2	Third WHO International Standard for neomycin	39			
9	Pro	nnsar	I projects for endorsement	40			

Annex 1				
WHO Recommendations, Guidelines and other documents related to the manufacture and quality control of biological substances used in medicine	43			
Annex 2				
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated) Replacement of Annex 1 of WHO Technical Report Series, No. 904, and Addendum to Annex 1 of WHO Technical Report Series, No. 910	49			
Annex 3				
Guidelines on the quality, safety and efficacy of recombinant malaria vaccines targeting the pre-erythrocytic and blood stages of <i>Plasmodium falciparum</i>	141			
Annex 4				
Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed) Replacement of Annex 2 of WHO Technical Report Series, No. 800, and Annex 5 of WHO Technical Report Series, No. 927	211			
Annex 5				
Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed) Replacement of Annex 2 of WHO Technical Report Series, No. 800, and Annex 5 of WHO Technical Report Series, No. 927	271			
Annex 6				
Recommendations to assure the quality, safety and efficacy of DT-based combined vaccines Replacement of Annex 2 of WHO Technical Report Series, No. 800	335			
Annex 7				
Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use Replacement of Annex 3 of WHO Technical Report Series, 910	407			
Annex 8				
Biological substances: WHO International Standards and Reference Reagents	487			

WHO Expert Committee on Biological Standardization

15 to 19 October 2012

Members¹

Professor K. Cichutek, Paul-Ehrlich-Institut, Langen, Germany

Dr J. Epstein, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, United States of America (USA) (also Blood Regulators Network representative)

Dr E. Griffiths, Kingston-upon-Thames, England (Chairman)

Mrs T. Jivapaisarnpong, Division of Biological Products, Ministry of Public Health, Nonthaburi, Thailand

Dr H. Klein, National Institutes of Health, Bethesda, MD, USA (Vice Chairman)

Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England

Dr F.M. Moftah, National Blood Transfusion Service, Ministry of Health, Giza, Egypt

Dr J. Petricciani, Palm Springs, CA, USA (Rapporteur)

Dr L.S. Slamet,² National Agency of Drug and Food Control, Jakarta, Indonesia

Dr Y. Sohn, Korea Food & Drug Administration, Chungcheongbuk-do, Republic of Korea

Dr P. Strengers, Sanquin, Amsterdam, the Netherlands

Professor H. Yin, State Food and Drug Administration, Beijing, China

Representatives of other organizations

American Association of Blood Banks (AABB)
Ms K. Shoos, Bethesda, MD, USA

Alliance of Blood Operators

Dr G. Sher, Ontario, Canada

Center for Biologics Evaluation and Research

Dr J.P. Weir, Bethesda, MD, USA

Chinese Pharmacopoeia Commission

Ms G. Zhongpin, Beijing, China

¹ The decisions of the Committee were taken in closed session with only members of the Committee present. Each Committee member had completed a Declaration of Interests form prior to the meeting. These were assessed by the WHO Secretariat and no declared interests were considered to be in conflict with full meeting participation.

² Unable to attend.

Council of Europe, European Directorate for the Quality of Medicines & HealthCare
Dr K.H. Buchheit and Dr E. Charton, Official Medicines Control Laboratories Network and
HealthCare, Strasbourg, France

Developing Country Vaccine Manufacturers' Network

Dr S. Pagliusi, Nyon, Switzerland

Dr M. Bhalgat and Mr P.V.V.S. Murthy, Biological E. Limited, Hyderabad, India

Dr S. Gairola, Serum Institute of India, Pune, India

Institut national de la santé et de la recherche médicale (INSERM)

Dr P. Lenting, Le Kremlin Bicêtre, France

International Alliance of Biological Standardization

Dr W. Egan, Colombia, MD, USA

Mr A. Mire-Sluis, Amgen Inc., Thousand Oaks, CA, USA

International Federation of Clinical Chemistry and Laboratory Medicine

Professor P. Gillery, Reims, France

International Federation of Pharmaceutical Manufacturers & Associations

Dr C. Saillez, Dr M-C Uwamwezi, and Dr P. Vandoolaeghe, GlaxoSmithKline Biologicals, Wavre, Belgium

Dr D. Schmalzing, San Francisco, CA, USA

International Plasma Fractionation Association

Dr R. Perry, Amsterdam, the Netherlands

International Society on Thrombosis and Haemostasis

Professor K. Mertens, Sanguin, Amsterdam, the Netherlands

Plasma Protein Therapeutics Association

Dr I. von Hoegen, Brussels, Belgium

Ms M. Gustafson, Annapolis, MD, USA

Pharmaceutical and Medical Device Regulatory Science Society of Japan (formerly Society of Japanese Pharmacopoeia)

Dr T. Murai, Osaka, Japan

United States Pharmacopeia

Dr T.S. Morris, Rockville, MD, USA

Participants

Professor J.P. Allain, East Anglia Blood Centre, Cambridge, England

Dr P. Aprea, National Administration of Drugs, Food and Medical Technology, Ministry of Health, Buenos Aires, Argentina

Dr M. Baca-Estrada, Bacterial and Combination Vaccines Division, Health Canada, Ottawa, Ontario, Canada

Professor A. Barrett, University of Texas Medical Branch, Galveston, TX, USA (*Temporary Adviser*)

Dr R. J. Benjamin, American Red Cross Holland Laboratories, Rockville, MD, USA

Dr R. Biswas,³ Center for Biologics Evaluation and Research, Rockville, MD, USA

Dr D.A. Bleijs, National Institute for Public Health and the Environment, Bilthoven, the Netherlands (*Temporary Adviser*)

Dr A. Bristow, National Institute for Biological Standards and Control, Potters Bar, England

Dr T. Burnouf, Human Protein Process Sciences, Lille, France

Dr C. Burns, National Institute for Biological Standards and Control, Potters Bar, England

Dr L. Castanheira, Agência Nacional de Vigilância Sanitária – Anvisa, Brasilia, Brazil

Dr K. Chumakov,³ Food and Drug Administration, Rockville, MD, USA

Dr C. Conrad, Paul-Ehrlich-Institut, Langen, Germany

Dr R. Dobbelaer, Lokeren, Belgium (Temporary Adviser)

Dr R. Drew,³ Center for Biologics Evaluation and Research, Rockville, MD, USA

Dr L. Elmgren, Centre for Vaccine Evaluation, Biologic and Genetic Therapies Directorate, Health Canada, Ottawa, Ontario, Canada

Dr S. Fakhrzadeh, Pharmaceutical & Narcotic Affairs Division, Food and Drug Organization, Ministry of Health & Medical Education of Islamic Republic of Iran, Tehran, Islamic Republic of Iran

Dr A. V. Farahani, Biologic Department, Food and Drug Organization, Ministry of Health & Medical Education of Islamic Republic of Iran, Tehran, Islamic Republic of Iran

Dr I. Feavers, National Institute for Biological Standards and Control, Potters Bar, England

Dr J. Fryer,³ National Institute for Biological Standards and Control, Potters Bar, England

Dr P. Ganz, Centre for Blood and Tissues Evaluation, Health Canada, Ottawa, Ontario, Canada (*BRN representative*)

Dr I. Hamaguchi, National Institute of Infectious Diseases, Tokyo, Japan

Dr K. Haslov, Statens Serum Institute, Copenhagen, Denmark

Dr M. Heiden, Paul-Ehrlich-Institut, Langen, Germany (BRN representative)

Professor A du P. Heyns, South African National Blood Service, Garsfontein, South Africa

³ Participated via teleconference.

- Dr S. Hindawi, Saudi Society of Transfusion Medicine, King Abdalaziz University, Jeddah, Saudi Arabia
- Dr A. Hubbard, National Institute for Biological Standards and Control, Potters Bar, England (*Temporary Adviser/Rapporteur*)
- Dr C. Ilonze, National Agency for Food and Drug Administration and Control, Lagos, Nigeria
- Dr S. Inglis, National Institute for Biological Standards and Control, Potters Bar, England
- Dr H. Jung Oh, Korea Food and Drug Administration, Chungcheongbuk-do, Republic of Korea
- Dr M. Jutzi, Swiss Agency for Therapeutic Products, Berne, Switzerland (BRN representative)
- Dr A. Kato, National Institute of Infectious Diseases, Tokyo, Japan
- Dr E. Karilari-Boateng, Ghana Food & Drugs Board, Accra, Ghana
- Mr S. Kerby,⁴ Center for Biologics Evaluation and Research, Rockville, MD, USA
- Dr R. Le Blanc, ⁴ Center for Biologics Evaluation and Research, Baltimore, MD, USA
- Dr H. Lechner, Paul-Ehrlich-Institut, Langen, Germany
- Dr M. Lennon, Horning, England (*Temporary Adviser*)
- Dr B. Meade, Meade Biologics, Hillsborough, NC, USA (Temporary Adviser)
- Dr G. Michaud, Center for Biologics Evaluation and Research, Rockville, MD, USA (*BRN representative*)
- Dr S. Morgeaux, Agence Nationale de Sécurité du Médicament et des Produits du Santé, Lyons, France
- Dr C. Morris, National Institute for Biological Standards and Control, Potters Bar, England
- Dr P. Milligan,⁴ London School of Hygiene and Tropical Medicine, London, England
- Dr H. Nakhasi,⁴ Center for Biologics Evaluation and Research, Rockville, MD, USA
- Dr S. Nick,⁴ Paul-Ehrlich-Institut, Langen, Germany
- Dr M. Nübling, Paul-Ehrlich-Institut, Langen, Germany (Temporary Adviser/Rapporteur)
- Dr L. Nzumbu, Directorate of Product Evaluation and Registration, Pharmacy and Poisons Board, Nairobi, Kenya
- Dr M. Ochiai, National Institute of Infectious Diseases, Tokyo, Japan
- Dr W. Oualikene-Gonin, Agence Nationale de Sécurité du Médicament et des Produits de Santé, Saint Denis, France (*BRN representative*)

⁴ Participated via teleconference.

Dr M. Pfleiderer, Paul-Ehrlich-Institut, Langen, Germany

Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England (*Temporary Adviser*)

Mr R. Reddy, South African National Blood Programme, Weltevreden Park, South Africa

Dr I. Sainte-Marie, Agence Nationale de Sécurité du Médicament et des Produits de Santé, Saint Denis Cedex, France (*BRN representative*)

Dr C. Schärer, Swiss Agency for Therapeutic Products, Berne, Switzerland (BRN representative)

Professor R. Seitz, Paul-Ehrlich-Institut, Langen, Germany (BRN representative)

Dr T. Sesardic, National Institute for Biological Standards and Control, Potters Bar, England (*Temporary Adviser*)

Mr S. Shani, Ministry of Health & Family Welfare, New Delhi, India

Dr S. Singh, Ministry of Health & Family Welfare, Uttar Pradesh, India

Dr J. Southern, Adviser to Medicines Control Council of South Africa, Cape Town, South Africa

Dr P. Stickings, National Institute for Biological Standards and Control, Potters Bar, England

Dr D.W. Trent, University of Texas Medical Branch, Galveston, TX, USA (Temporary Adviser)

Dr A.R.T. Utami, National Agency for Food and Drug Control, Jakarta Pusat, Indonesia

Professor G.N. Vyas, University of California, San Francisco, CA, USA

Dr M. Wadhwa, National Institute for Biological Standards and Control, Potters Bar, England

Dr J. Wang, National Institutes for Food and Drug Control, Beijing, China

Dr M. Weinstein,⁵ Center for Biologics Evaluation and Research, Rockville, MD, USA

Dr D. Wood, World Health Organization, Geneva, Switzerland (Secretary to the Committee)

Dr N. Yasuda, Ministry of Health, Labour and Welfare, Tokyo, Japan (BRN representative)

Dr K. Zoon, National Institute for Allergies and Infectious Diseases, National Institutes of Health Bethesda, MD, USA

⁵ Participated via teleconference.

Abbreviations

rct40 reproductive capacity at elevated temperature (test)

AABB American Association of Blood Banks

ABO Alliance of Blood Operators

ACD active case detection

AESI adverse event of special interest

AMA-1 apical membrane antigen type 1

ATP adenosine triphosphate

BCG bacille Calmette–Guérin (vaccine)

BGTD Biologics and Genetic Therapies Directorate

BHK baby hamster kidney

BRN Blood Regulators Network

C core (protein)

CBER Center for Biologics Evaluation and Research

CCID₅₀ cell culture infectious dose (median)

CD circular dichroism (spectroscopy)

CMI cell-mediated immunity

CNS central nervous system

CPE cytopathic effect

CRF2 corticotropin-releasing factor receptor 2

CSP circumsporozoite protein

rcDNA residual cellular DNA

DT diphtheria and tetanus

DTaP diphtheria-tetanus-acellular pertussis

DTP diphtheria, tetanus and pertussis

DTwP diphtheria-tetanus-whole-cell pertussis

DU D-antigen unit (of inactivated poliomyelitis vaccine)

E envelope (protein)

ED₅₀ effective dose (median)

EDQM European Directorate for the Quality of Medicines &

HealthCare

EIR entomological inoculation rate

ELISA enzyme-linked immunosorbent assay

ELISPOT enzyme-linked immunosorbent spot

EPI Expanded Programme on Immunization

EPO erythropoietin

ERA environmental risk assessment

ERL Essential Regulatory Laboratory

EU endotoxin unit

FDA United States Food and Drug Administration

FSH follicle-stimulating hormone

FXIa activated factor XI

GCP good clinical practice

GLP good laboratory practice

GMC geometric mean concentration

GMO genetically modified organism

GMP good manufacturing practice

GMT geometric mean titre

GPEI Global Polio Eradication Initiative

HAI haemagglutination inhibition

HBsAg hepatitis B surface antigen

Hib Haemophilus influenzae type b

HIV human immunodeficiency virus

HPLC high-performance liquid chromatography

HPV human papillomavirus

ICDRA International Conference of Drug Regulatory Authorities

IgG immunoglobulin G

IL interleukin

IPAC WHO Immunization Practices Advisory Committee

IPV inactivated poliomyelitis vaccine

IU International Unit

JE Japanese encephalitis

JE-CV Japanese encephalitis chimeric virus

JEV Japanese encephalitis virus
LAL Limulus amoebocyte lysate
Lf limit for flocculation units

LH luteinizing hormone

LLIN long-lasting insecticide-treated nets

M membrane (protein)

MALVAC Malaria Vaccine Committee

MAPREC mutant analysis by polymerase chain reaction and restriction

enzyme cleavage

MCB master cell bank

MNVT monkey neurovirulence test

MPL monophosphoryl lipid A MSP merozoite surface protein

NAT nucleic acid amplification technique

NCL national control laboratory

NIBSC National Institute for Biological Standards and Control

NIFDS National Institute of Food and Drug Safety Evaluation

NIH National Institutes of Health

NIID National Institute of Infectious Diseases

OLSS Office of Laboratories & Scientific Services

NRA national regulatory authority

NS non-structural

OMPC outer membrane protein complex

OPV oral poliomyelitis vaccine

aP acellular pertussis

wP whole-cell pertussis

WHO Technical Report Series, No. 980, 2014

PAGE polyacrylamide gel electrophoresis

PCD passive case detection

PCR polymerase chain reaction

PEI Paul Ehrlich Institut

PFU plaque-forming unit

Pfs25 Plasmodium falciparum mosquito stage antigen

PHK primary hamster kidney

PrM premembrane

PRNT plaque-reduction neutralization test

PRP polyribosylribitol phosphate

PT pertussis toxin

RCD reverse cumulative distribution

mRNA messenger RNA

RSO RNA-derived Sabin original type-3 virus

RTase reverse transcriptase

RT-PCR reverse transcription-polymerase chain reaction

SAGE Strategic Advisory Group of Experts

SAP statistical analysis plan

SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SHD single human dose

SO Sabin original

SOB Behringwerke stock of SO

SOJ Japanese stock of SO

SOM Sabin original Merck

SOP standard operating procedure

SOR Russian stock of SO

TgmNVT transgenic mice neurovirulence test

TgPVR21 transgenic mice expressing the human poliovirus receptor

TNT toxin neutralization test

ToBI toxin-binding inhibition

TRAP thrombospondin-related adhesion protein
TSEs transmissible spongiform encephalopathies

UNICEF United Nations Children's Fund

VN virus neutralization

VPPAG Vaccine Presentation and Packaging Advisory Group

VAPP vaccine-associated paralytic poliomyelitis

cVDPV circulating vaccine-derived poliovirus

VMS virus master seed

VWF von Willebrand factor

WCB working cell bank

WHA World Health Assembly

WPV wild poliovirus

YF yellow fever

1. Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 15 to 19 October 2012. The meeting was opened jointly by Dr Jean-Marie Okwo-bele, Director of the Department of Immunization, Vaccines, and Biologicals, and by Dr Kees de Joncheere, Director of the Department of Essential Medicines and Health Products.

Dr Okwo-bele noted that the Committee is one of the oldest at WHO, having been founded in 1947. According to the WHO Constitution, the Committee's goal is:

... to develop, establish and promote international standards for biological products.

The Committee's agenda was ambitious, and included a new initiative from WHO to network the six collaborating centres that already support its work in the area of standards and regulatory science for vaccines. WHO aims to develop synergies among collaborating centres to better support its mandate, with an additional four laboratories in the process of becoming collaborating centres. Expanding the number of collaborating centres providing support in the area of vaccine standardization has been a long-term goal of the biological standardization programme.

In 2012 the World Health Assembly (WHA) adopted a Global Vaccine Action Plan aimed at averting millions of preventable deaths by 2020 by ensuring more equitable access to vaccines for all people. Because appropriate regulatory oversight is crucial in ensuring that vaccines used in national immunization programmes are safe, effective and of assured quality, the Committee's work is essential in helping to update the tools used by regulators – such as guidance documents and reference preparations – to reflect current knowledge of the benefits and risks of new and existing vaccines.

In 2012, the Committee was asked to consider approving a new guidance document for malaria vaccines, and significant updates to the existing guidance for oral poliomyelitis vaccines; live-attenuated Japanese encephalitis vaccines for human use; and diphtheria and tetanus vaccines, as well as combined vaccines using diphtheria, tetanus and pertussis toxoids. The Committee also reviewed proposals to establish 11 new or replacement reference preparations.

Dr de Joncheere discussed activities in the field of blood products. In 2010, resolution WHA63.12 requested that WHO provide additional support to Member States to help improve the availability, safety and quality of blood products; ensure the sustainable production of WHO biological reference preparations and their provision to those who need them; and improve access

by developing countries to the scientific information obtained through their validation. Much of the work of the Committee relates directly to the support requested in resolution WHA63.12.

The Committee was asked to discuss the issue of adding whole blood and red blood cells to WHO's Model Lists of Essential Medicines. Essential medicines are defined by WHO as:

... those that satisfy the priority health care needs of the population.

The public-health relevance of whole blood and red blood cell concentrate is well established. The concept of blood as a medicine, its regulatory status and the benefits for countries and for the patients who need transfusions were discussed by the Committee and would also be considered by the WHO Blood Regulators Network (BRN) and at the Fifteenth International Conference of Drug Regulatory Authorities (ICDRA).

The Committee also considered a proposal to develop a guidance document to support low- and middle-income countries in making decisions about testing strategies to use when preparing recovered plasma for fractionation. The scope of the document would include evaluating the residual risk of bloodborne pathogens, and a cost-benefit analysis and risk-benefit analysis of the use of blood components. A substantial and increasing volume of recovered plasma potentially available in low- and middle-income countries is wasted. Deficient systems render the plasma unsuitable for fractionation for plasma derivatives, and it is destroyed. There is a fundamental need in these countries to build local capacity for the production of safe plasma for use as an active pharmaceutical ingredient.

Six new reference standards and one reference panel were proposed to the Committee in 2012, together with six new projects for endorsement. The WHO collaborative study carried out to validate a reference panel to assess the ability of diagnostic tests for human immunodeficiency virus (HIV) to detect relevant subtypes in different regions of the world is especially noteworthy because it will help the manufacturers of in vitro diagnostic devices and regulatory authorities to improve the relative efficiency of tests in their regions.

Dr de Joncheere expressed thanks to the Committee, to WHO's collaborating centres, and to the experts, institutions and professional societies working in this area whose efforts provide vital support to WHO programmes.

The Secretary to the Committee, Dr David Wood, described the organization of the meeting and outlined the issues to be discussed.

Dr Elwyn Griffiths was elected as Chairman and Dr John Petricciani was elected as Rapporteur for the plenary sessions, and for the track considering vaccines and biological therapeutics. Dr Harvey Klein was elected as Chairman and Dr Anthony Hubbard and Dr Micha Nübling as Rapporteurs for the track

considering blood products and in vitro diagnostic device reagents. Dr Klein was also elected as Vice-Chairman for the plenary sessions of the Committee. The Committee adopted the proposed agenda (WHO/BS/2012.2212), and noted that item number 42 on the proposed WHO Reference Panel for procoagulant activity in intravenous immunoglobulin had been withdrawn.

2. General

2.1 Current directions

2.1.1 Strategic directions in biological standardization: WHO priorities

The Global Vaccine Action Plan constitutes a catalyst and provides an opportunity for regulators worldwide to propose and develop a global agenda for regulatory science for vaccines. A collaborative process was used to develop an agenda that includes laboratory-based regulatory science. Some areas that might be explored as part of this agenda include correlates of immunity, correlates of safety, methods for improving the characterization of products and methods for improving potency assays. The regulatory science agenda also addresses regulatory processes, such as innovative trial designs and novel methods for conducting pharmacovigilance.

The importance of investing in regulatory science was highlighted for both developed and developing countries. There was support for extending the principle of investing in regulatory science to other biologicals. Safety surveillance is an important aspect of regulation, and new and improved methods are evolving rapidly.

Creating links with programmes that use biological medicines is important; enquiries have been received by the Committee in relation to the use of thiomersal in pharmaceutical products; the off-label use of vaccines; and the potential standardization of the layout and content of vaccine labels. An additional area of interest is the evaluation of commutability during the establishment of WHO reference preparations, and its impact on the harmonization of laboratory results. A review of the processes for evaluating commutability has been initiated with the aim of improving clinical laboratory testing through harmonization. The Committee was informed that an assessment of commutability in WHO collaborative studies is being undertaken, and that the development of a guidance document has been proposed.

In future, WHO aims to capitalize more effectively on its leadership position in global health, to retain the flexibility to adapt to a changing environment and to be more selective in setting priorities. Among the priorities under consideration by WHO are promoting universal access to health products; strengthening national regulatory authorities (NRAs); and prequalifying vaccines, selected medicines and diagnostic devices.

2.1.2 Vaccines and biological therapeutics: recent and planned activities in biological standardization

Dr Ivana Knezevic outlined activities undertaken in the area of vaccines and biological therapeutics, which include the development and implementation of written standards for vaccines. During 2010–2012, 13 written standards were

developed. Of these 13 documents, five are Recommendations for viral vaccines (dengue vaccine, hepatitis B vaccine, live-attenuated Japanese encephalitis vaccine, oral poliomyelitis vaccine and yellow fever vaccine); five are Recommendations for bacterial vaccines (acellular pertussis vaccine, bacille Calmette–Guérin vaccine, diphtheria vaccine, tetanus vaccine, and vaccines based on some combination of diphtheria, tetanus and pertussis toxoids), and one is a new set of guidelines on a vaccine to protect against the malaria parasite. In addition, there is one guidance document focusing on lot release that is applicable to all vaccines, along with Recommendations on the use of cell substrates for manufacturing biologicals and on the characterization of cell banks. For the period 2013–2014, five additional written standards are being developed or revised.

The priorities of the biological standardization programme must be adjusted to reflect developments in relation to specific vaccines, and existing guidance updated in light of scientific advances. In response to operational needs in the field, a proposal has been made to update guidelines to allow specific vaccines to be kept and administered under controlled temperatures outside the cold chain. In response to advances in vaccine development, a proposal has also been made to develop new guidance on typhoid conjugate vaccines.

The issue of conducting risk assessments as part of the regulatory process had previously been discussed by the Committee. Follow-up activities were in progress, including the development of case studies to help assess risk when adventitious agents are detected in licensed vaccines. These activities will be discussed at a meeting to be held in China in 2013.

A summary was provided of the implementation workshops conducted during 2008–2012, and plans for 2013 presented. A number of lessons had been learnt, including the need to ensure that participants in workshops had a basic understanding of the science and the issues being discussed; such lessons should be helpful in making future workshops more effective.

Planned activities in relation to the standardization of biotherapeutics were described, including the revising of guidelines for products using recombinant DNA, reviewing the concept of international standards for biotherapeutic products, and the convening of a workshop that would include case studies on assessing the regulatory risk of products licensed when there is only insufficient or inappropriate data. In addition, the implementation of WHO guidelines on similar biotherapeutic products ("biosimilars") would continue to be monitored.

Strategic issues to be addressed included setting priorities, developing synergies in the standardization and regulatory evaluation of vaccines, linking networks of regulators with one another, and determining the roles of organizations responsible for setting standards in the regulating of vaccines.

The Committee made several suggestions on how to improve the quality of planned guidelines and workshops, and on how to evaluate those that had already been implemented.

2.1.3 Blood products and related in vitro diagnostics: recent and planned activities in biological standardization

Dr Ana Padilla provided an overview of activities in the area of blood and blood products. WHO's strategic direction in relation to the biological standardization of blood products and related in vitro diagnostics was based on a plan for 2007–2012 that was previously presented to the Committee. During that period, 45 WHO reference standards and panels were established for use in the quality control of blood products and for ensuring the safety of blood through the standardization of in vitro diagnostic devices. In addition, important work was conducted in relation to snake antivenoms.

Dr Padilla reminded the Committee of resolution WHA63.12 (2010) and of the need to strengthen the regulation of blood and blood products. The WHO Achilles project aims to build capacity for ensuring that good manufacturing practice (GMP) is followed by blood establishments. Among the objectives of the project are training staff to strengthen regulatory systems, coordinating international experts to offer advice and increasing the level of support offered by WHO regional offices.

A report on improving access to safe blood products by implementing local production and transferring technology among blood establishments had been prepared and would be made available.

New projects proposed to the Committee included developing strategies to enhance the availability and safety of blood products, assessing the commutability of WHO reference preparations (initially in the area of standards for in vitro diagnostic devices), and preparing and calibrating secondary reference preparations (including standards for in vitro diagnostic devices). Proposals made to the Committee in 2011 for seven reference preparations were reviewed, and six new projects were presented for endorsement by the Committee. The Committee was also informed that a workshop on the use of blood as a medicine was to be held in conjunction with the 2012 meeting of the ICDRA.

2.2 Reports

2.2.1 Report from the WHO Blood Regulators Network

Dr Peter R Ganz reiterated the objectives of the Blood Regulators Network (BRN) and reviewed its activities during 2011–2012. These activities included:

- reviewing the scientific basis for excluding as donors men who have sex with other men;
- preparing and publishing a position paper on regulatory considerations for screening male donors who have sex with other men;

- endorsing the proposal to consider blood components as essential medicines;
- preparing workshops held before and during the ICDRA meeting;
- discussing regulatory convergence with the Alliance of Blood Operators (ABO);
- discussing blood cellular therapies;
- discussing pathogen-inactivation technologies.

Dr Ganz also highlighted the BRN agenda for 2012–2013.

2.2.2 Report from the WHO collaborating centres for biological standards

The history of the WHO collaborating centres for biological standards was reviewed by Dr Griffiths who pointed out that there were six centres working in the area of vaccines:

- National Institute for Biological Standards and Control (NIBSC), Health Protection Agency, Potters Bar, England;
- Center for Biologics Evaluation and Research (CBER), Food and Drug Administration, Silver Spring, MD, USA;
- Department of Bacterial Pathogenesis and Infection Control,
 National Institute of Infectious Diseases (NIID), Tokyo, Japan;
- Office of Laboratories & Scientific Services (OLSS), Therapeutic Goods Administration, Woden, Australia;
- National Institute of Food and Drug Safety Evaluation (NIFDS),
 Korea Food & Drug Administration, Chungcheongbuk-do, Republic of Korea;
- Biologics and Genetic Therapies Directorate (BGTD), Health Canada, Ottawa, Canada.

Representatives of these collaborating centres met in April 2012 and agreed to operate as a network in the areas of vaccine standardization and regulatory science. The network's terms of reference and operational details were under consideration. Four additional vaccine collaborating centres will be established during 2012–2014. The relationship between the network of WHO collaborating centres for biological standards and the WHO Expert Committee on Biological Standardization required further discussion, with good communication between all parties recognized as the key to ensuring success.

The Committee welcomed the establishment of the network, and requested that progress reports on the operation of the network be presented at future Committee meetings.

2.3 Issues

2.3.1 Scientific issues identified by custodians of WHO biological reference preparations

The Committee was informed of issues identified by the following custodians of WHO biological reference preparations.

National Institute for Biological Standards and Control (NIBSC), Potters Bar, England

Dr Stephen Inglis provided an overview of activities and developments at the NIBSC in relation to the WHO programme for biological standardization. In 2012, a total of 11 replacement standards and five new standards had been submitted to the WHO Expert Committee on Biological Standardization for consideration. For 2013, five replacement and six new standards have been proposed, along with three new collaborative projects with other institutions.

Overall, the institute has been responsible for developing more than 90% of all WHO international standards. Developing a biological standard is a complex process. The strengths and weaknesses of the process were presented, as was the need for a long-term plan to ensure that appropriate standards continue to be developed. The Committee agreed with the proposal to develop such a plan.

The increasing importance of biologicals in medicine was also emphasized, along with the challenges associated with their standardization. Standardizing biosimilars and variations of the same protein was particularly challenging, as was the standardizing of cell-based medicines.

The institute faced heavy demands in terms of preparing both new and replacement materials, with increasing demand for work on reference materials in new fields and for new purposes. Strategic issues that needed to be addressed included the commutability of the values assigned to reference preparations, standardizing innovative product classes and technologies, and sustaining the standardization programme in the long term.

Other contributions made by the institute include assisting WHO in preparing guidelines for product development and quality control, testing products for prequalification, inspecting manufacturing facilities, reviewing dossiers for vaccines, engaging in consultations and policy development with WHO, and the provision of training in biological standardization and medicines control. In addition, from November 2011 to October 2012 the institute shipped more than 20 000 vials and ampoules of reference materials. Although largely uneventful, there had been several instances of delays and other problems occurring during transit owing to issues with customs authorities.

During 2013, the institute will be merged with the Medicines and Healthcare Products Regulatory Agency; it was hoped that the institute's activities and programmes would benefit from being part of a regulatory organization and

that its roles in control and standardization, especially in the international arena, would not be restricted.

European Directorate for the Quality of Medicines & HealthCare (EDQM), Strasbourg, France

Dr Karl-Heinz Buchheit described EDQM activities in the area of biological standardization. The directorate's goals include establishing European Pharmacopoeia biological reference preparations, standardizing assay methods for biologicals, adapting the 3R concept – Refining, Reducing and Replacing the use of animals – to the standardization of assays for biological products, and implementing the international harmonization of regulatory decision-making through collaboration with WHO and with non-European partners.

The directorate's achievements in biological standardization included 122 projects that had either been initiated or concluded. In addition, participation in a collaborative effort had led to the replacement of an endotoxin standard. Discussions are also continuing within the scientific community on the development of alternative tests for pertussis toxin, and the directorate will continue to collaborate in this effort. The progress made in standardizing hepatitis A vaccine assays was outlined, along with the potential replacement of the United States National Institutes of Health (NIH) potency assay for rabies vaccine, and areas for future research highlighted.

EDQM has made a strong commitment to finding alternative methods to animal experiments, and requested that WHO consider incorporating the 3R initiative in its written standards, where appropriate. Including such alternative methods in WHO guidelines would provide a very strong impetus in ensuring their global acceptance and adoption.

The development of regional standards for measuring the potency of hepatitis B immunoglobulin and hepatitis A virus RNA for nucleic acid amplification technique (NAT) assays using commercial kits was identified as potentially problematic and WHO advice on how best to proceed was requested. The Committee considered that if WHO developed additional guidance on secondary reference preparations for in vitro diagnostic tests then it would have an opportunity to address the issues raised.

Center for Biologics Evaluation and Research (CBER), Rockville, MD, USA

Dr Jerry Weir outlined CBER activities in the area of vaccines and detailed several contributions including:

- serving as an Essential Regulatory Laboratory (ERL) within the Pandemic Influenza Preparedness Framework;
- participating in the drafting of a global regulatory science agenda for vaccines in accordance with WHO leadership in this area;

- enhancing regulatory capacity in low- and middle-income countries to support the introduction of influenza vaccines;
- developing the resource document: Guidance for Industry: General Principles for the Development of Vaccines to Protect Against Global Infectious Diseases;
- conducting public workshops on developing and evaluating vaccines against human cytomegalovirus and on developing a universal influenza vaccine;
- supporting WHO efforts to promote innovation in the designing of clinical trials of vaccines, and participating in WHO pharmacovigilance activities;
- ongoing efforts with other collaborating centres and ERLs in the selecting of vaccine strains and the preparing of vaccine reagents for seasonal influenza vaccine development;
- initiating work to develop a reference reagent to standardize neutralization assays for human respiratory syncytial virus, and working to develop reference reagents for rabies antibody to be used in serological assays.

CBER has been redesignated as a collaborating centre for 2012–2016.

Dr Jay Epstein then reviewed ongoing CBER work on the following reference materials related to blood safety for in vitro diagnostic testing:

- international standards for dengue virus RNA;
- reference reagent for Chikungunya virus RNA;
- reference reagent for West Nile virus RNA;
- reference panel for anti-human T cell lymphotropic virus type 1 and type 2;
- reference panels for malaria antibodies to *Plasmodium falciparum* and *Plasmodium vivax*;
- blood reference reagent for variant Creutzfeldt–Jakob disease.

An update was also given on the status of the inventory of WHO coagulation standards.

Paul Ehrlich Institut (PEI), Langen, Germany

Dr Klaus Cichutek reported on the work of the PEI which was first designated as a WHO collaborating centre in 2005. Its responsibilities include vaccines and biomedicines (such as blood products and monoclonal antibodies) as well as cell-therapy and gene-therapy products. The institute also engages in activities to

assure the quality and safety of blood products, and participates in the activities of the BRN.

Dr Cichutek outlined a number of projects undertaken by the institute including:

- developing an international reference panel for parvovirus B19 genotypes for NAT-based assays;
- developing an international standard for hepatitis E virus RNA for NAT-based assays;
- developing a hepatitis E virus genotype panel;
- developing a repository for transfusion-relevant bacterial strains;
- developing hepatitis B virus genotype reference preparations for DNA assays and hepatitis B surface antigen tests;
- developing a standard for diagnosing hepatitis B e-antigens;
- establishing a standard for mycoplasma NAT-based assays;
- providing training courses for assessors working for regulatory authorities;
- contributing to the development of relevant WHO guidelines and recommendations;
- establishing the First WHO International Standard for hepatitis D virus RNA;
- conducting preparatory work to establish the First WHO International Standard for factor XIII concentrate.

Dr Cichutek then outlined a number of research areas with potential future implications for regulatory activities.

2.3.2 Issues shared with the WHO Expert Committee on Specifications for Pharmaceutical Preparations

Proposed Third WHO International Standard for endotoxin

An international collaborative study was undertaken to harmonize replacement standards for endotoxins. The study was organized by WHO, NIBSC, the United States Pharmacopeial Convention and EDQM. Thirty-five laboratories worldwide, including Official Medicines Control Laboratories and manufacturers' laboratories, participated in the study.

Three candidate preparations were produced using the same material and formulation as the current reference standards. The objective was to generate a Third WHO International Standard with the same potency (10 000 IU/vial) as the current Second WHO International Standard for endotoxin (94/580). The study

evaluated the suitability of the candidate preparations for use as the reference standard in assays for endotoxins performed according to compendial methods.

The potency of each candidate preparation was calibrated against the Second WHO International Standard for endotoxin, with gelation and photometric methods producing similar results for each of the preparations. Overall, the results were in line with those generated to establish the current reference standard preparations. Evidence of the long-term stability of the three candidate preparations was obtained by accelerated degradation testing of vials stored at elevated temperatures.

The Committee considered the report that was submitted (WHO/BS/12.2193), and recommended that in order to avoid drift during the calibration of future replacement standards, preparation 10/178 should be established as the Third WHO International Standard for endotoxin, with an assigned unitage of 10 000 IU/vial.

Progress on a global legally binding instrument on mercury: implications for pharmaceuticals

Dr Wood reviewed the status of negotiations on the global legally binding instrument on mercury. There is growing concern about the effects of mercury on human health, and a general trend towards reducing and ultimately eliminating exposure to it. Sources of potential mercury exposure in health products include thermometers and devices used to measure blood pressure. Thiomersal (ethyl mercury) is widely used as a preservative in multi-dose vials of vaccines in both developed and developing countries. Data suggest that such vaccines are safe. Alternative preservatives are not available for all vaccines, and it would probably be too costly to produce small-dose vials.

The advice of WHO to countries during international treaty discussions has been that vaccines containing thiomersal are essential for public health purposes, and that the continued availability of multi-dose vials of vaccines containing thiomersal is necessary for the safe and effective prevention of serious infectious diseases worldwide.

In the long term WHO aims to develop and articulate an agreed vision for future vaccines that facilitates the delivery of effective and affordable vaccines to populations who are most at risk, in addition to securing short-term to medium-term access to pharmaceutical-grade thiomersal for global public health use.

2.4 Feedback from other WHO committees

2.4.1 Request from the Strategic Advisory Group of Experts (SAGE) on Immunization for guidance on off-label use of vaccines

Dr Wood noted that in April 2012 issues concerning the off-label use of vaccines were discussed by SAGE in the context of its public health recommendation

to use a single dose of hepatitis A vaccine. This issue had also been raised during discussions on the use of influenza vaccines in pregnant women and in discussions on removing age restrictions on the use of rotavirus vaccines.

SAGE requested that a paper be developed to define the circumstances under which the off-label use of any vaccine may be recommended, and to clarify the differences between regulatory decisions and public health recommendations. The legal and programmatic implications of recommending off-label use, and the need for clear communication, would need to be considered.

National immunization technical advisory groups may also issue recommendations that differ from the labelled indications for specific products. Such advisory groups have highlighted that vaccines supplied in a controlled temperature chain could be stored and administered at an elevated temperature for a single period of time immediately before administration. In addition, when the same vaccine type is produced by more than one manufacturer (for example, influenza vaccines) the printed indications and contraindications for specific vaccines may differ (for example, concerning their use during pregnancy).

The Committee agreed that WHO should be requested to provide advice on regulatory pathways and to facilitate studies to evaluate uses that are currently considered to be off-label. The Committee emphasized that collecting product-specific data is the most feasible approach to addressing the issue of whether to support changes to current labelling.

2.4.2 Request from the WHO Immunization Practices Advisory Committee (IPAC) to establish harmonized standards for the labelling of vaccines

Ms Simona Zipursky informed the Committee that two groups working on vaccine labelling issues had requested their assistance. The two groups are IPAC, which provides advice on operational practices related to immunization, and the Vaccine Presentation and Packaging Advisory Group (VPPAG) which provides a forum for vaccine manufacturers and the public to reach consensus on optimal presentation and packaging. VPPAG is hosted by WHO and chaired by the United Nations Children's Fund (UNICEF).

Current labelling requirements are described in Annex 1 of WHO Technical Report Series, No. 822 (1992). Questions raised in recent years by national immunization programmes relate to the specific content and language requirements for labels, the format for expiry dates, the use of generic names, the minimum acceptable font size and type, and the minimum area of the label.

In order to move forward, IPAC have recommended that WHO consider a VPPAG proposal covering labels for vaccine containers. In addition, IPAC would develop a detailed proposal for consideration by the Committee that would specify the content and layout requirements for label design. After reviewing the Committee's comments, the two groups would finalize a proposal to revise the current labelling requirements (1992) and present this to the Committee.

3. International Recommendations, Guidelines and other matters related to the manufacture and quality control of biologicals

All Recommendations and Guidelines established at the meeting are listed in Annex 1, which provides an updated listing of all current WHO Recommendations, Guidelines and other documents related to the manufacture and quality control of biological substances used in medicine.

3.1 Vaccines and related substances

3.1.1 Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated)

Written specifications for the quality, safety and efficacy of oral poliomyelitis vaccine (OPV) were previously known as Requirements but are now known as Recommendations, and were last fully revised in 1999. Since then, there have been advances in scientific knowledge, in the availability of novel laboratory techniques and in the use of new vaccine formulations such as monovalent OPV and bivalent OPV. In addition, new quality-control tests are available and their significance needs to be better explained and rationalized. In 2008, the Committee recommended that the 1999 Requirements for OPV be revised with sections included on the nonclinical and clinical evaluation of candidate OPVs.

To facilitate the revision process, WHO convened a group to begin work on revising the Recommendations for the production and quality control of OPV (WHO Technical Report Series, No. 904) and the subsequent Addendum (WHO Technical Report Series, No. 910). Experts from academia, NRAs, national control laboratories (NCLs) and industry involved in the research, manufacture, authorization, testing and release of OPV from countries around the world met during July 2010 to identify and discuss the issues to be considered for revision.

The major changes suggested include:

- updating information on the origin of the different strains used for OPV production, and inclusion of a new Appendix 1;
- updating the section on international standards and reference preparations;
- updating the section on general manufacturing recommendations and quality-control tests;
- updating information on neurovirulence tests, and mutant analysis by polymerase chain reaction and restriction enzyme cleavage (MAPREC) tests – the use of which is to be extended to all three viral types for seeds and bulks;

- developing a new Appendix 2 that provides the rationale for choosing a mouse neurovirulence test or a monkey neurovirulence test;
- considering new vaccine formulations for monovalent OPV and bivalent OPV;
- developing new text to address issues related to levels of residual cellular DNA in OPV produced in Vero cells;
- updating terminology so that "bulk suspension" is replaced by "monovalent bulk", and the term "virus submaster seed lot" is introduced, which applies only to master seed supplied by WHO;
- developing a new section on nonclinical evaluation, which addresses the requirements necessary in different situations;
- developing a new section on clinical evaluation that provides guidance based on approaches used to obtain regulatory approval of new monovalent and bivalent OPV formulations;
- updating Appendix 4 on the cell-culture techniques used to determine the virus content of OPV;
- updating information on the transgenic mouse neurovirulence test, the standard operating procedures for MAPREC and a new monkey neurovirulence protocol developed in light of current techniques.

The Committee reviewed the suggested revisions presented in document WHO/BS/2012.2185. After making a number of changes, the Committee recommended that the revised Recommendations be adopted and annexed to its report (Annex 2).

3.1.2 Guidelines on the quality, safety and efficacy of recombinant malaria vaccines targeting the pre-erythrocytic and blood stages of *Plasmodium falciparum*

These new Guidelines provide guidance on the following aspects of recombinant malaria vaccines produced in yeast that target the pre-erythrocytic and blood stages of *P. falciparum*:

- the quality of vaccines (including production, quality-control and stability issues);
- regulatory expectations for the nonclinical evaluation of vaccine candidates prior to their licensure;
- regulatory expectations for the clinical evaluation of vaccine candidates prior to their licensure.

A variety of approaches are being used to develop malaria vaccines. These approaches use different production platforms and target different stages of the

life-cycle of malaria parasites. The three aspects listed above differ somewhat in their scope to reflect the different stages of vaccine development and the diversity of production platforms and vaccine targets.

Only one candidate vaccine (RTS,S/AS01) is currently in phase III clinical trials. This recombinant *P. falciparum* malaria vaccine is produced in yeast and targets the pre-erythrocytic stage of the malaria parasite. In early trials, the vaccine has demonstrated some degree of efficacy in reducing all episodes of clinical malaria. However, no vaccine is presently licensed for malaria.

Careful statistical analysis is essential to accurately assess the efficacy of a malaria vaccine; such analysis must take into account the effect of natural immunity and the impact of waning immunity. In order to facilitate a series of consultations on the clinical evaluation of efficacy, WHO convened a Study Group on Measures of Malaria Vaccine Efficacy. It is the outcomes of these consultations that form the basis of the clinical section of the new Guidelines.

In addition, a number of methodological considerations are provided in the appendices to the Guidelines based upon the protocols used by the manufacturer of the most advanced candidate vaccine. These are provided for information only and should not be considered to constitute an endorsement of any candidate vaccine.

The Committee reviewed the Guidelines presented in document WHO/BS/2012.2186. After making a number of changes, the Committee recommended that the new Guidelines be adopted and annexed to its report (Annex 3).

3.1.3 Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed)

These Recommendations apply to the production and quality control of adsorbed diphtheria vaccines and are an update to the 1989 revision of the Requirements for diphtheria, tetanus, pertussis and combined vaccines (WHO Technical Report Series, No. 800), as well as to the amended Recommendations of 2003 (WHO Technical Report Series, No. 927). The updated Recommendations highlight advances in the production and testing of diphtheria vaccines and related intermediates, and include testing and quality-control guidance based on currently licensed vaccines. Other products, such as those containing a new type of antigen or produced using novel technology, may require additional considerations.

Although these Recommendations apply to the production and quality control of diphtheria vaccines, the final formulation of most diphtheria-vaccine products includes at least one other component. Therefore, in addition to monovalent diphtheria vaccine these Recommendations also apply to the diphtheria component of combination vaccines, and the tests recommended for the final bulk or final fill also apply to the combined vaccine where appropriate.

The main changes in this latest revision include:

- changing the title from Requirements to Recommendations;
- updating the section on international standards and reference preparations and moving it to the section on general considerations;
- updating the section on general manufacturing recommendations and quality-control tests;
- amending the minimum requirements for the potency of diphtheria vaccine, which now applies the lower limit of the 95% confidence interval;
- developing new sections to provide advice on the clinical and nonclinical evaluation of diphtheria vaccines to assess their safety, quality and efficacy.

The Committee reviewed the revision of the current Requirements presented in document WHO/BS/2012.2188. After making a number of changes, the Committee recommended that the revised Recommendations be adopted and annexed to its report (Annex 4).

3.1.4 Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed)

These Recommendations apply to the production and quality control of adsorbed tetanus vaccines, and are an update to the 1989 revision of the Requirements for diphtheria, tetanus, pertussis and combined vaccines (WHO Technical Report Series, No. 800), and to the 2003 Amendments (WHO Technical Report Series, No. 927). The updated Recommendations highlight advances in producing and testing tetanus vaccines and related intermediates, and include quality-control guidance based on currently licensed vaccines. Other products, such as those containing a new type of antigen or produced using novel technology, may require additional considerations.

Although these Recommendations apply to the production and quality control of tetanus vaccines, the final formulation of most tetanus-vaccine products includes at least one other component. Therefore, in addition to monovalent tetanus vaccine these Recommendations also apply to the tetanus component of combination vaccines, and the tests recommended for the final bulk or final fill also apply to the combined vaccine where appropriate.

The main changes in this latest revision include:

- changing the title from Requirements to Recommendations;
- updating the section on international standards and reference preparations, and moving it to the section on general considerations;

- updating the section on general manufacturing recommendations and quality-control tests;
- amending the minimum requirements for the potency of tetanus vaccine, which now applies the lower limit of the 95% confidence interval;
- provision for using the mouse International Unit (mouse IU) in mouse assays of potency;
- new sections providing guidance on the clinical and nonclinical evaluation of tetanus vaccines to assess their safety, quality and efficacy.

A working group was established at the request of WHO to discuss the possibility of transferability among different assay models, and the use of mouse potency assays for the expression of vaccine potency in IUs. The outcome of these discussions was reported to the Committee.

The working group noted that a number of studies had highlighted the lack of agreement between guinea-pig assays and mouse assays in evaluations of the potency of tetanus vaccines when expressed in IUs. The group also noted that mouse assays are routinely used for the quality control of tetanus vaccines and are often used to calibrate secondary standards.

The working group acknowledged that the WHO minimum requirement for tetanus vaccine potency was originally based on results obtained from guinea-pig challenge assays, using standards calibrated in IUs in guinea-pigs. However, the working group also acknowledged that vaccines with demonstrated clinical safety and efficacy had been licensed and released where in vivo potency had been determined using the mouse-challenge model. The group therefore proposed that specifications on the minimum requirements for potency assays should be maintained but referred to as "mouse IU" for assays performed using the mouse model.

It was also proposed that manufacturers should make an effort to define product-specific requirements for potency using consistency limits established during routine testing. This approach is broadly consistent with the 2010 recommendation of the Committee that mouse units should be used to demonstrate production consistency and to allow product monitoring in cases where compliance may be jeopardized if guinea-pig assays were required.

The working group also proposed that standards for tetanus vaccines (including international standards, and regional, national and other secondary standards) could be calibrated using a mouse-challenge assay and assigned mouse IU values. Based on the results obtained from the international collaborative study, the Committee therefore recommended that the Fourth WHO International Standard for Tetanus Toxoid Adsorbed (NIBSC code 08/218) should be assigned a potency of 260 mouse IU/ampoule for use in mouse-potency assays.

The Committee reviewed the revision of the current Recommendations presented in document WHO/BS/2012.2189. After making a number of changes, the Committee recommended that the revised Recommendations be adopted and annexed to its report (Annex 5).

3.1.5 Recommendations to assure the quality, safety and efficacy of DT-based combined vaccines

In 2011, in light of a number of major developments in this area, WHO convened two meetings to discuss revising its 1989 Requirements for diphtheria, tetanus, pertussis and combined vaccines (WHO Technical Report Series, No. 800) and 2003 Amendments (WHO Technical Report Series, No. 927).

The revised guidance was based upon experience with currently licensed products, and includes the following main changes:

- changing the title from Requirements to Recommendations;
- using abbreviations for vaccines containing some combination of diphtheria, tetanus or pertussis toxoids, and their components;
- including information on all combined vaccines that included diphtheria, tetanus or pertussis toxoids developed at the time this revision was drafted (for example, those combined with hepatitis B vaccine, *Haemophilus influenzae* type b conjugate vaccine, or inactivated poliomyelitis vaccine);
- including new sections on the clinical and nonclinical evaluation of combined vaccines containing diphtheria, tetanus or pertussis toxoids;
- revising the summary protocol for the information to be provided for lot release of combined vaccines containing diphtheria, tetanus or pertussis toxoids.

The Committee reviewed the revised Recommendations presented in document WHO/BS/2012.2187. After making a number of changes, the Committee recommended that the revised Recommendations be adopted and annexed to its report (Annex 6).

3.1.6 Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use

These Recommendations update Annex 3 in WHO Technical Report Series, No. 910 and cover the use of live-attenuated Japanese encephalitis vaccines in humans. Other types of Japanese encephalitis vaccines are thus outside the scope of these Recommendations which have been based upon experience gained with licensed live-attenuated vaccines. The major changes made include:

- updating the protocols for monitoring the health of animals from which primary hamster kidney cells are prepared;
- updating information on test methods and specifications for primary hamster kidney cell cultures used in vaccine production in accordance with revised recommendations on cell substrates;
- updating information on testing for attenuation for SA14-14-2 vaccine;
- adding recommendations for evaluating lot-release potency specifications, and emphasizing the need for monitoring the upper limit of potency in addition to monitoring the minimum potency specification for the immunizing dose;
- adding information on the relationship between passage levels of vaccine seeds and production in current production schemes for live Japanese encephalitis vaccines;
- adding new specifications for the manufacturing and quality control of live Japanese encephalitis vaccines grown in Vero cell cultures using an attenuated strain of yellow fever virus as a viral vector;
- adding guidelines for the nonclinical and clinical evaluation of new live-attenuated Japanese encephalitis vaccines;
- adding guidelines for conducting an environmental risk assessment for live-attenuated Japanese encephalitis vaccine seed;
- adding a model lot-release summary protocol and a model batchrelease certificate for use by NRAs.

The Committee reviewed the revised Recommendations presented in document WHO/BS/2012.2199. After making a number of changes, the Committee recommended that the revised Recommendations be adopted and annexed to its report (Annex 7).

3.1.7 In vitro assay system to replace the histamine sensitization test for acellular pertussis vaccines

An in vitro test has been developed at the NIBSC as a potential alternative to the in vivo histamine sensitization test. An international collaborative study was undertaken to assess the methods and transferability of the in vitro test, and its suitability for testing three different types of acellular pertussis products. Sixteen laboratories in nine countries participated in the study.

The reported results (WHO/BS/2012.2198) indicated good agreement among laboratories in respect to estimates of the carbohydrate-binding activity and enzymatic activity of the vaccine samples included in the study. However, direct correlation could not be established between the in vitro assay system and

the in vivo histamine sensitization test for each individual vaccine lot due to the large variation in results from the histamine sensitization test. Further evaluation is required before the in vitro system can be used routinely as an alternative to the histamine sensitization test, particularly since only a limited number of vaccine samples were evaluated in the study.

The Committee noted that a workshop on alternatives to the murine histamine sensitization test for acellular pertussis vaccines was held in November 2012 at the United States NIH. The Committee expressed its interest in being updated on future developments.

3.2 Blood products and related substances

3.2.1 Strategies to promote the availability and safety of blood products

Resolution WHA63.12, adopted in 2010, mandated that efforts be made to improve access to safe blood products globally. A primary objective of WHO in this area is to provide support to low- and middle-income countries to enable them to prepare recovered plasma for fractionation, so that essential plasmaderived medicines (such as blood coagulation factors, and polyvalent and specific immunoglobulins) can be produced for their populations. Efforts have been made to determine whether the local production of plasma-derived medicines would improve access to safe blood components for transfusion.

In June 2012 a workshop for stakeholders was held at WHO headquarters. The extent of plasma wastage in low- and middle-income countries was evaluated by reviewing the literature and by analysing blood-donation rates alongside the results of a questionnaire that had been distributed to selected countries. This evaluation estimated that approximately 9.3 million litres of non-transfused plasma are wasted annually. This estimate does not take into account plasma that is transfused inappropriately and is thus unavailable for fractionation. If current unmet needs for blood are also addressed through increased blood collection this could allow for the generation of additional recovered plasma.

As part of the workshop, representatives of countries in several WHO regions were asked to take part in a proof-of-concept test using risk estimates developed by WHO; they were also asked to consider the feasibility of implementing adequate quality control and safety measures. The goal of the exercise was to establish whether it would be possible to recover plasma for fractionation. Additionally, the feasibility of establishing or expanding fractionation across regions was explored – for example, by establishing fractionation facilities in South Africa that would supply the sub-Saharan area. During the workshop, a number of countries presented examples of initiatives that had reduced the costs of plasma derivatives by 40% after contract plasma fractionation programmes had been established.

Key elements affecting future plasma-recovery efforts include analysis of the gaps between demand and supply, ensuring governmental commitment to establishing the process in country, developing a favourable cost–benefit analysis, securing necessary investment, developing a strategy for managing the residual health risks associated with recovered plasma, and organizing national blood services to incorporate both regulatory oversight of blood establishments and the implementation and enforcement of GMP.

Methods for calculating plasma wastage were also discussed, including estimating the proportion of whole blood collections made into components, along with the need to apply the generalized experiences of countries taking part in pilot projects to other countries, the challenges faced in establishing plasma contract fractionation and local fractionation capability, and the steps that need to be taken by countries that discard plasma to fulfil quality requirements established by fractionation facilities.

To address the mandate of resolution WHA63.12, the Committee proposed providing guidance on estimating the residual health risk in blood components (including plasma for fractionation); developing guidelines or advocacy information (or both) as part of determining the effectiveness of contract plasma fractionation or local production in Member States; and including blood and blood components in the WHO Model Lists of Essential Medicines.

3.2.2 Blood components as essential medicines

Blood (whole blood and red blood cells) is a widely used life-saving treatment and should be processed and manufactured within defined quality-assurance systems. Blood thus appears to meet the WHO definition of an essential medicine. The committee that selects the medicines to be included in the WHO Model Lists of Essential Medicines meets once every two years, with the next meeting scheduled for April 2013.

Developments in blood transfusion – both technical and regulatory – now make it relevant to consider whole blood and red blood cells as biological medicines suitable for inclusion in the list of essential medicines. Several international standards apply to the manufacture of blood components (which are included in some national pharmacopoeias) while whole blood is regulated as a medicine in many national jurisdictions, including Australia, Canada, Germany, Japan, Switzerland and the United States.

The purposes of listing whole blood and red blood cells as essential medicines would be: (a) to increase awareness of the use of blood components, and the need for national commitment and oversight to improve the quality and safety of blood products; (b) to promote the global availability of safe blood for transfusions; and (c) to protect donors and patients by improving the standards used to screen donors.

The steps involved in manufacturing blood components include: (a) assessing and qualifying the raw materials (through the selection and testing of donors); (b) quarantining components while production is in process; (c) batch release; (d) implementing quality-control standards (for example by monitoring the storage temperature or visually inspecting the products); (e) setting expiration dates; and (f) ensuring that products are traceable so that any problems can be corrected. Additionally, products must be labelled for their intended use and the label should include information on whether they are to be provided on prescription.

Adding whole blood and red blood cells to the list of essential medicines would help meet the needs of many developing countries for effective treatments for haemorrhage and anaemia. For example, such products could be used to address bleeding caused by trauma, or during labour or delivery, or to treat anaemia caused by malaria. The addition of these essential products for protecting public health in all countries to the list of essential medicines will also promote the global availability of safe blood for transfusions. Such a proposal supports both resolution WHA63.12 and the Millennium Development Goals of reducing maternal mortality (Goal 5; Target 5.A), reducing childhood mortality (Goal 4; Target 4.A), and halting and reversing the spread of HIV/AIDS (Goal 6; Target 6.A).

The request for blood to be considered as an essential medicine was considered in light of the defined remit and scope of the Canadian Blood Services – Canada being the current chair of the BRN. Canada has a clear regulatory framework for blood and blood components, and national experience with tainted blood has emphasized the need to consider blood as an essential medicine. Strategically and operationally, blood and blood components must be manufactured effectively and efficiently to ensure that safe, relevant and high-quality products are delivered to those who need them. Adding blood to the list of essential medicines would encourage governments to invest in the required infrastructure and to provide oversight of blood systems, leading to the availability of safe and cost-effective therapies.

The AABB (formerly the American Association of Blood Banks) is one of the largest and oldest professional societies. As a sponsor of the application to include whole blood and red blood cells in the WHO Model Lists of Essential Medicines, the AABB outlined the main justifications for submitting an application. This application, submitted in December 2012, was initially restricted to whole blood and red blood cells, and contained the following statement by the WHO BRN:

At the meeting on 18 October 2012, the [BRN] members present expressed universal support for an initiative to establish whole blood and [red blood cells] in [the WHO Model Lists of Essential Medicines]. While mindful of

concerns regarding commercialization, the [BRN] members believe that listing by WHO of whole blood and [red blood cells] as essential medicines will promote global availability of these biological therapeutics as products that meet internationally recognized standards for their quality and safety, including oversight through effective regulation.

Although it too was mindful of potential unintended consequences, such as the commercialization of blood components as medicines, the Committee endorsed the application for listing whole blood and red blood cells as essential medicines.

3.2.3 Residual risk in recovered plasma to be used as an active pharmaceutical ingredient for fractionation

A proposed guidance document on the residual health risks associated with recovered plasma would aid policy-makers in choosing testing strategies for donated blood. By addressing the risks associated with blood components (including plasma and cellular components) and outlining the different testing strategies that may be used to reduce risks, it is intended that the document would help to improve the safety of all blood components, including the safety of components used for transfusion and as plasma for fractionation.

A draft model based upon estimates of the residual risks associated with blood products has been developed using practical experience with NAT gained throughout the world, especially in South Africa. Typically, estimates of residual risk use a model to calculate the incidence occurring during a window of time (known as the window period). However, this model is not easily applied in resource-limited countries where relevant information is often missing (such as the interval between donations for repeat donors), and where there is a relatively high proportion of first-time donors.

The proposed document will include estimates of the incidence of bloodborne viruses such as HIV, and hepatitis B and C viruses. The draft model is based on incidence and prevalence data from several countries. For example, for HIV, experience in different countries consistently shows that for repeat donors, the NAT yield rate is approximately nine-fold lower than the rate of seropositivity. The NAT yield rate in first-time donors is consistently 2.5 times higher than the rate in repeat donors. The residual risk of HIV can be estimated from the combined projected NAT yield rate in all donors using the known NAT window period for HIV positivity.

The proposed guidance document would also provide a method for estimating both the potential number of viral transmissions associated with different cellular blood components and the average viral load in pooled plasma. Different testing strategies will also be discussed, including NAT testing and antigen testing. Testing strategies and methods for estimating risk will be

combined with information on the actual incidence or prevalence of a disease, or both, in the relevant countries.

Worst-case scenarios rather than average scenarios should be used to estimate the degree of possible contamination of pooled plasma. These scenarios consider the highest possible titres that could be missed by screening assays. Furthermore, in areas where the risk is highest, NAT testing of single donations rather than of mini-pools of plasma may be more cost effective and logistically easier to implement as part of efforts to reduce the potential virus load in pooled plasma.

The Committee endorsed the project to develop the proposed guidance document.

3.2.4 National strategies for plasma-derived medicinal products

A discussion of national strategies for developing plasma-derived medicinal products encompassed two distinct proposals. First, a proposal was made to draft a four-page decisional framework document that would guide its users to a "Go" or "No-go" outcome. The document would deal with the potential direct engagement of low- and middle-income countries that may not be able to afford to import plasma derivatives in plasma fractionation activities (either locally or through a contracted facility). The proposed document would detail the potential challenges of the early stages of such an engagement process, and would bring together information already available in a number of advocacy documents developed by WHO.

A second proposal was made to develop a separate and more detailed guidance document to discuss the aspects to be considered prior to a decision being made either to enter into a contract with a facility to provide plasma fractionation or to develop domestic capability for this activity. The document would also detail the experiences of countries that have moved incrementally from receiving fractionation products from contract facilities towards eventual autonomous domestic production.

Guidance on developing domestic capability could cover national experiences in: (a) determining the minimum volumes of plasma needed; (b) assessing the costs of facilities; (c) evaluating the time frames for becoming an autonomous producer; (e) prioritizing the products that should be produced; (f) generating effective demand forecasts; (g) choosing the appropriate fractionation and viral-inactivation technologies; (h) entering into technical agreements with fractionators; and (i) developing a regulatory roadmap, which should incorporate national endorsement of the project and NRA involvement.

The primary aim of such guidance would be to help national decision-makers in Member States avoid the technical and economic problems, and missteps, experienced by other countries, while clearly setting out a number of technical options.

The Committee endorsed the project to develop both the proposed decisional framework and guidance document.

3.2.5 Calibration of secondary reference materials

Secondary standards are calibrated by various parties against WHO international standards in order to preserve the primary standard and respect its limited availability. Organizations that take part in calibration activities include manufacturers of in vitro diagnostic tests, regional and national institutions, and quality-control laboratories. An important task in calibrating secondary standards is to assure continuity in terms of their IUs. In principle, the same calibration procedures are undertaken when replacement standards for primary materials are established and calibrated, with the exception that the applicability of the secondary standard may be narrow when compared with the international standard, thus justifying a more-limited validation study.

The requirements and steps to be undertaken to establish an international standard are described in Annex 2 of WHO Technical Report Series, No. 932 (2005). Different approaches are followed by different parties in establishing, characterizing and calibrating secondary standards. The development of a guidance document on the steps and issues that should be considered when establishing secondary standards was proposed (WHO/BS/2012.2211). This document could include information on selecting and initially characterizing candidate materials, as well as information on processing and comparing a candidate material against the primary material. Guidance on the statistical evaluation of calibration studies would include estimates of uncertainty that can be used when assigning unitage, and advice on storing the reference materials and on record-keeping. The proposed document would also discuss various analytes (such as inactivated pathogens or nucleic acids, antigens and antibodies), and the different technologies or platforms used during in vitro diagnostic procedures.

The Committee endorsed the project to develop the proposed guidance document.

3.2.6 Assessment of commutability in WHO collaborative studies

WHO and its collaborating centres are intending to conduct an evaluation of the commutability of WHO biological reference preparations. Such an evaluation would be an important component in ensuring that the same numerical relationship is found among the results for any given analyte in a sample irrespective of the assay method used, and would initially focus on the in vitro detection of markers of infectious diseases.

Commutability could either be assessed as part of a multicentre collaborative study undertaken to assign values to a proposed standard or during a separate dedicated study after such values have been established –

with both options having logistical and resource implications. One recognized difficulty lies in defining a cut-off point for differentiating the commutability and noncommutability of any given preparation. There is agreement that the topic of commutability should be closely and actively monitored and that such an effort should include different stakeholders (for example manufacturers and users of in vitro diagnostic devices, and academics) in addition to the collaborating centres already working on the standardization of in vitro diagnostic procedures.

An NIBSC position document had been drafted (WHO/BS/2012.2211) that provides a good basis for discussion and for assessing options. This document would be circulated within the network of WHO collaborating centres for biological standards for comments or amendments before being shared with external stakeholders. Including blood products (such as clotting factors) in the same document could potentially provide the opportunity to use similar approaches for different products when assessing the commutability of international standards.

Circulation of the position document may also be combined with the distribution of a questionnaire to determine the experiences of users with respect to the commutability or noncommutability of WHO international standards, and to evaluate potential options for assessing commutability.

The Committee discussed whether international standards for in vitro diagnostic procedures for infectious diseases should serve as the basis of a pilot project, and if international standards from other fields such as haematology should be included as a parallel exercise.

The Committee recommended that WHO convene a meeting of stakeholders to consider the issues raised in relation to commutability.

4. International reference materials – vaccines and related substances

All reference materials established at the meeting are listed in Annex 8.

WHO International Standards and Reference Reagents – vaccines and related substances

4.1.1 First WHO International Standard for anti-human papillomavirus type 18 serum

A worldwide collaborative study was undertaken to evaluate a candidate international standard for antibodies to human papillomavirus type 18 (HPV18) for use in enzyme immunoassays and pseudovirion neutralization assays. The candidate (NIBSC code 10/140) was obtained from a pool of serum collected from women naturally infected with HPV18, and dispensed into ampoules in 0.5 ml aliquots and freeze-dried for long-term stability. Fourteen laboratories in 10 countries participated in the study to evaluate the fitness for purpose and potency of the candidate standard using their serology assays for antibodies to HPV18 and, where possible, assays for other HPV types. The freeze-dried candidate standard was evaluated alongside the liquid bulk of the candidate preparation, a coded duplicate of the candidate standard and negative serum – as well as serum from naturally infected or vaccinated women. Both enzyme immunoassays and pseudovirion neutralization assays were used. Study results indicated that the candidate is suitable to serve as an international standard.

The Committee considered the study report (WHO/BS/2012.2191) and recommended that preparation 10/140 be established as the First WHO International Standard for anti-human papillomavirus type 18 serum, with an assigned potency of 8 IU when reconstituted in 0.5 ml of water.

4.1.2 First WHO Reference Reagent for bacille Calmette-Guérin vaccine of Moreau-RJ substrain

An international collaborative study involving 16 laboratories in 13 countries was carried out to evaluate a candidate reference reagent for the bacille Calmette–Guérin (BCG) vaccine Moreau-RJ substrain. The lyophilized preparation (NIBSC code 10/272) was dispensed in ampoules and quantified using the viable count method for cultures and a modified adenosine triphosphate (ATP) assay; substrain identity was confirmed using multiplex polymerase chain reaction (PCR).

It was intended that once established the reference reagent would act as a comparator for monitoring the consistency of several aspects of viability assays, including counts of viable cells in culture and modified ATP assays, residual

virulence and local reactogenicity as well as monitoring the consistency of protection assays in animal models. In addition, it could be used as a reference BCG substrain in identity assays that use molecular biology techniques, such as multiplex PCR. Its use as a comparator or reference in preclinical studies for the development and evaluation of new tuberculosis vaccines would also be important.

Based upon the reported study results (WHO/BS/2012.2200), the Committee recommended that the substrain preparation 10/272 be established as the First WHO Reference Reagent for bacille Calmette–Guérin vaccine of Moreau-RJ substrain, with assigned values of 3.1 million colony-forming units and 24.69 ng of ATP per ampoule. A PCR fingerprint for use in multiplex PCR should also be defined as described in the study report.

4.1.3 First WHO International Standard for human diphtheria antitoxin

A report (WHO/BS/2012.2192) was presented to the Committee on the preparation and characterization of a proposed international standard for human diphtheria antitoxin (NIBSC code 10/262) and on its relative calibration. Calibration was performed by in vivo and in vitro toxin-neutralization testing using a Vero cell assay, with potency expressed relative to the international standard for equine diphtheria antitoxin.

A total of eight participants from eight different countries provided the data used to assign unitage to the proposed standard. The results suggested that 10/262 has a diphtheria antitoxin potency of 2 IU/ampoule. Stability was assessed by measuring the potency of samples stored at elevated temperatures for up to 12 months. Although no prediction of long-term stability could be made, the absence of any significant loss of activity suggested that the proposed standard was likely to have satisfactory stability. Further stability testing would be conducted once the standard had been established.

A follow-up study was performed to assess the impact of the proposed standard relative to results obtained from routine assays used to measure diphtheria antibodies in human serum. The follow-up study also allowed commutability to be assessed. It was conducted as an external quality assessment, modified to include the proposed standard. A total of 16 laboratories in 15 countries participated and performed an in vitro serological assay to determine levels of anti-diphtheria antibodies in a panel of 148 human serum samples. The results suggested that the proposed standard showed comparable behaviour to native human serum samples in the majority of the assays compared, and was likely to be suitable for use as a reference preparation in assays used to measure the level of anti-diphtheria antibodies in human serum.

The Committee considered the report and recommended that preparation 10/262 be established as the First WHO International Standard for human diphtheria antitoxin, with an assigned potency of 2 IU/ampoule.

4.1.4 Second WHO International Standard for antibody to influenza H1N1pdm virus

A freeze-dried international standard for antibody to pandemic influenza A virus subtype H1N1pdm (NIBSC code 09/194) was established by WHO in 2010, and has been distributed to many laboratories conducting influenza H1N1pdm serology tests. Due to the urgent need to make this material available and limits on the number of vials that could be prepared, stocks were completely depleted by the end of 2012. A freeze-dried candidate replacement international standard (NIBSC code 10/202) was therefore prepared from pooled plasma collected from patients who had received a split influenza H1N1pdm vaccine prepared from the reassortant virus NYMC X-179A (derived from A/California/7/2009 virus). Eighteen laboratories in 11 countries tested the candidate preparation along with a panel of human plasma collected from people who had also been vaccinated with the A/California/7/2009 pandemic vaccine.

The reported results (WHO/BS/2012.2190) indicated that the candidate preparation 10/202 would be useful in standardizing haemagglutination inhibition (HAI) assays and virus neutralization (VN) assays of antibody to influenza H1N1pdm virus vaccines. It was also shown that both HAI and VN titres differed between the first international standard 09/194 and the candidate second international standard 10/202.

The Committee considered the report provided and recommended that preparation 10/202 be established as the Second WHO International Standard for antibody to influenza H1N1pdm virus, with an assigned potency for use in HAI assays of 1200 IU/ampoule (that is, 2400 IU/ml when reconstituted as directed with 0.5 ml water). The consensus VN titre for 10/202 should be stated in the instructions for use.

5. International reference materials – blood products and related substances

All reference materials established at the meeting are listed in Annex 8.

5.1 WHO International Standards and Reference Reagents – blood products and related substances

5.1.1 Fourth WHO International Standard for factor II and factor X concentrates

This international standard is used to estimate the activity of factor II and factor X in concentrates of prothrombin complex, and in high-purity factor IX concentrates.

A report (WHO/BS/2012.2210) was presented to the Committee of a collaborative study undertaken by 28 laboratories in 14 countries to assign values to a proposed Fourth WHO International Standard for factor II and factor X concentrates by assaying the candidate preparation (NIBSC code 11/126) relative to the current Third WHO International Standard for factor II and factor X concentrates. The inter-laboratory variability of estimates for both factors was low (with a variability measured by geometric coefficient of variation of < 5%. Small but significant differences were observed between clotting methods and chromogenic methods for both factors; however, these were considered not to have practical significance, and a single overall combined value was proposed for each factor. Potency estimates for a second test concentrate, calculated relative to both the current and proposed replacement international standards (and using the same proposed values) gave identical results.

The Committee considered the report and recommended that preparation 11/126 be established as the Fourth WHO International Standard for factor II and factor X concentrates, with assigned values of 9.4 IU/ampoule for factor II and 8.1 IU/ampoule for factor X.

5.1.2 Second WHO International Standard for factor VII concentrate

This international standard is used to label the potency of therapeutic factor VII concentrates, and to estimate residual factor VII in concentrates of prothrombin complex.

A report (WHO/BS/2012.2204) was presented to the Committee of a collaborative study undertaken by 24 laboratories in 11 countries to assign values to a proposed Second WHO International Standard for factor VII concentrate. Two candidate materials – A (NIBSC code 10/250) and B (NIBSC code 10/252) – were assayed relative to the current First WHO International Standard for factor VII concentrate using the one-stage clotting method and the chromogenic method.

For both candidate materials the estimates produced using clotting and chromogenic methods were significantly different, with the discrepancy being largest for candidate A. For candidate A there was also a significant difference in estimates from the clotting assay when using recombinant or natural thromboplastin reagents; this did not occur with candidate B which was thus proposed as the replacement international standard. The observed difference between the clotting-method and chromogenic-method estimates was considered to be too large to assign a mean value by consensus. In the interests of maintaining continuity in product labelling, it was proposed that separate values be assigned for each method.

The Committee considered the report and recommended that preparation 10/252 (candidate B) be established as the Second WHO International Standard for factor VII concentrate, with an assigned potency of 9.8 IU/ampoule for the chromogenic method and 10.6 IU/ampoule for the clotting method.

5.1.3 Second WHO International Standard for fibringen concentrate

The assignment of values to a proposed Second WHO International Standard for fibrinogen concentrate – for both clottable protein and total protein – was undertaken as part of a collaborative study involving 27 laboratories in 12 countries.

Two candidate concentrates – B (NIBSC code 09/242) and C (NIBSC code 10/100) – were assessed. Good agreement was found between laboratories for estimates of total protein for both candidates. The geometric coefficient of variation for candidate B was 4.4% (with mean potency = 15.04 mg/ml) and for candidate C was 7.7% (with mean potency = 12.10 mg/ml). Estimates for clottable protein by Clauss assay were significantly higher and more variable than estimates using the clot-removal method, with the observed mean values differing by 18% for candidate B and by 80% for candidate C. Estimates of clottable protein for candidate B determined using absolute methods (for example, the Kjeldahl method) were consistent with the results obtained using the clot-removal method.

Candidate B was thus proposed as the Second WHO International Standard for fibrinogen concentrate given its lower interlaboratory variability and smaller degree of discrepancy in the results between the Clauss assay and the clot-removal method. This discrepancy was however too large to allow for the assignment of a combined mean value for clottable protein. It was proposed that the value obtained using the clot-removal method be assigned to the international standard since this value was less variable than those obtained using the Clauss assay, and because they were in agreement with the absolute estimates. Furthermore, the Clauss-assay estimates were anomalous as the estimate for candidate C exceeded the estimate for total protein. Most manufacturers and NCLs use the clot-removal method.

The Committee considered the report of the study (WHO/BS/2012.2208) and recommended that preparation 09/242 (candidate B) be established as the Second WHO International Standard for fibrinogen concentrate, with assigned values of 10.9 mg/ampoule for clottable protein and 15.0 mg/ampoule for total protein. Furthermore, the instructions for use should indicate that the assigned value for clottable protein was based upon the use of a clot-removal method, and that the Clauss assay should always be carefully validated prior to its routine use for testing fibrinogen concentrates.

5.1.4 First WHO Reference Reagent for activated blood coagulation factor XI (human)

Activated factor XI (FXIa) has been identified as a primary cause of procoagulant activity in intravenous immunoglobulin products associated with thromboembolic events. There is thus an urgent need for a reference material for FXIa to support the testing of immunoglobulin products for associated procoagulant activity. Such a reference material would also allow intralaboratory and interlaboratory variability to be evaluated, and for laboratory testing to be optimized and harmonized.

A freeze-dried candidate preparation of purified FXIa (NIBSC code 11/236) with an arbitrary assigned value of 10 u/ampoule was sent to 11 laboratories for evaluation, with results from six of these having been received at the time of the meeting. All laboratories used functional methods for FXIa either linked to FXa generation or based on the direct cleavage of chromogenic substrate by FXIa. The direct-cleavage methods exhibit less specificity for FXIa and may be affected by other proteases. The proposed reference preparation was tested as a coded duplicate against itself, with the resulting intralaboratory variability ranging from 2% to 14%. The overall mean value of 9.88 u/ampoule was in good agreement with the expected value of 10 u/ampoule.

Valid assays for endogenous FXIa in freeze-dried samples of intravenous immunoglobulin were obtained relative to the proposed reference preparation. Expected levels were recovered when the reference preparation was spiked into local samples of intravenous immunoglobulin, thus indicating no interference from the matrix. These initial results were considered promising, and provide support for the future development of an international standard.

The Committee considered the report of the study (WHO/BS/2012.2206) and recommended that preparation 11/236 be established as the First WHO Reference Reagent for activated blood coagulation factor XI (human), with an assigned value of 10 u/ampoule. The Committee requested that feedback on the use of the preparation be provided at its next meeting, and indicated that the final report should include information on its stability after reconstitution.

5.1.5 Second WHO International Standard for high-molecular-weight urokinase

A collaborative study involving 14 laboratories in 10 countries was undertaken to assign a value to a proposed second international standard for high molecular weight urokinase (NIBSC code 11/184) relative to the first international standard. All laboratories used clot-lysis assays, mainly in microtitre-plate format, to measure fibrin turbidity. Ten of the laboratories used purified systems to generate the clots, with the remaining four using a human-plasma system. No significant variations were observed between the different methods.

The overall combined mean potency of 3238 IU/ampoule was associated with a low geometric coefficient of variation (7.1%). Accelerated degradation studies did not detect any potency loss in the candidate after six months' storage at temperatures of up to $45\,^{\circ}\text{C}$.

The Committee considered the report of the study (WHO/BS/2012.2205) and recommended that preparation 11/184 be established as the Second WHO International Standard for high molecular weight urokinase, with an assigned potency of 3200 IU/ampoule.

5.1.6 Third WHO International Standard for low molecular weight heparin

Declining stocks of the Second WHO International Standard for low molecular weight heparin have necessitated the preparation of a replacement. Two candidate materials were therefore included in a collaborative study involving 22 laboratories in 13 countries. These materials underwent testing for anti-IIa and anti-Xa activities relative to the Second WHO International Standard for low molecular weight heparin.

There were no outlying results for the candidate materials using either the anti-IIa assay or the anti-Xa assay. The interlaboratory variability of estimates was lowest for candidate B (NIBSC code 11/176), with generalized cross-validation of 3.1% for the anti-Xa assay and 3.2% for the anti-IIa assay. There was no significant difference between laboratories using the methods of the European Pharmacopoeia or the United States Pharmacopeial Convention.

The Committee considered the report of the study (WHO/BS/2012.2207) and recommended that preparation 11/176 be established as the Third WHO International Standard for low molecular weight heparin, with assigned values of 1068 IU/ampoule for anti-Xa activity and 342 IU/ampoule for anti-IIa activity.

6. International reference materials – in vitro diagnostic device reagents

All reference materials established at the meeting are listed in Annex 8.

6.1 WHO International Standards and Reference Reagents – in vitro diagnostic device reagents

6.1.1 Second WHO Subtype Reference Panel for HIV-1 NAT-based assays

HIV reference panels consisting of different HIV subtypes are an important tool for assessing the relative efficiency of different assays in detecting different subtypes, and can be used to generate data to help improve such assays. In 2001, the WHO First Subtype Reference Panel for HIV-1 NAT-based assays – representing subtypes A, B, C, D, AE, F, G, AG–GH, and groups N and O – was established as frozen liquid material. By 2012, this material had been completely depleted. However, the original virus stocks were still available in liquid nitrogen, and were used to develop a subsequent candidate reference panel that included these same HIV subtypes with similar RNA concentrations to those used in the previous panel.

The viruses were grown on human peripheral blood mononuclear cells, and underwent heat inactivation for 1 hour at 60 °C before dilution into negative plasma. The efficacy of heat inactivation was confirmed by a reverse transcriptase activity assay. After freeze-drying, the panel was evaluated as part of a global collaborative study involving a total of 21 laboratories. The specific protocols to be used for quantitative assays and qualitative assays were defined, and the Third WHO International Standard for HIV-1 for NAT-based assays included as an additional coded specimen.

Results were obtained using 11 commercial assays and 7 in-house NAT-based assays (three quantitative and four qualitative). There was good agreement between assay results for most of the panel, but results for HIV-1 group N and HIV-1 group O members differed between two commercial assays. Despite a number of such divergent results, it was apparent that NAT-based assays had improved during the past 10 years when the results of the first panel validation study were compared with the results of the current study. Initial stability data confirmed that the appropriate storage temperature was –20 °C with degradation or inefficient reconstitution being observed after several weeks of storage at 45 °C.

The Committee considered the report of the study (WHO/BS/2012.2209) and recommended that the candidate panel be established as the Second WHO Subtype Reference Panel for HIV-1 NAT-based assays. It was also urged that efforts to establish reference materials for prevalent circulating recombinant forms of HIV should continue.

7. International reference materials – biotherapeutics other than blood products

All reference materials established at the meeting are listed in Annex 8.

7.1 WHO International Standards and Reference Reagents – biotherapeutics other than blood products

7.1.1 Third WHO International Standard for erythropoietin (recombinant) for bioassay

Erythropoietin (EPO) is a glycoprotein hormone produced in the kidneys that plays a major part in regulating the production of red blood cells. Recombinant preparations of EPO are widely used therapeutically to treat anaemia. An international standard is used to define the IU for EPO activity and is thus an essential requirement for the correct labelling of potency for therapeutic products. Stocks of the second international standard had become depleted and a replacement international standard was thus required. An international collaborative study was carried out involving 15 laboratories in seven countries to evaluate a candidate international standard for EPO (NIBSC code 11/170).

In addition to the primary aim of establishing a replacement international standard, the study had the secondary aim of calibrating a national EPO standard on behalf of the National Institutes for Food and Drug Control in China. Therefore, participants were also invited to evaluate the corresponding preparation (NIBSC code 11/172). Ensuring that national secondary reference materials are calibrated correctly is crucial to maintaining the quality of therapeutic preparations. The in vivo nature of these calibration exercises also means that every effort should be made to keep the number of assays performed to a minimum, hence the request to include this national standard in the study.

The Committee considered the report of the study (WHO/BS/2012.2195) and recommended that preparation 11/170 be established as the Third WHO International Standard for erythropoietin (recombinant) for bioassay, with an assigned value of 1650 IU/ampoule.

7.1.2 Fifth WHO International Standard for follicle-stimulating hormone and luteinizing hormone (human, urinary) for bioassay

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are glycoprotein hormones produced in the anterior pituitary gland that have a major role in regulating reproductive processes and pubertal maturation. Human urinary FSH and urinary LH (known as menotrophin) are widely used together therapeutically to stimulate ovulation in women, and to achieve controlled ovarian hyperstimulation as part of assisted reproductive technologies. They are also used to treat male infertility caused by hypogonadotropic hypogonadism.

The Fourth WHO International Standard for follicle-stimulating hormone and luteinizing hormone, human, urinary was established in 2000. Following its widespread use to calibrate preparations of human urinary FSH and urinary LH, stocks had now been completely depleted, and there was an urgent requirement for a replacement standard. A new preparation of human urinary FSH and urinary LH (NIBSC code 10/286) was dispensed into ampoules in accordance with WHO-recommended procedures. An international collaborative study involving 11 laboratories in 10 countries was carried out to assign a value to the proposed new standard.

The Committee considered the report of the study (WHO/BS/2012.2196) and recommended that preparation 10/286 be established as the Fifth WHO International Standard for follicle-stimulating hormone and luteinizing hormone (human, urinary) for bioassay, with an assigned bioactivity of 183 IU FSH and 177 IU LH/ampoule.

7.1.3 Second WHO International Standard for interleukin-2 (human, rDNA-derived)

Interleukin-2 (IL-2) is approved for the treatment of patients with metastatic renal cell carcinoma and metastatic melanoma. Following an international collaborative study involving 18 laboratories, the current international standard for IL-2 was established in 1987 (WHO Technical Report Series, No. 771). This first international standard consisted of a highly purified preparation of glycosylated IL-2 derived from Jurkat cells, and was assigned a potency of 100 IU/ampoule.

This defined potency was derived following evaluation in a wide range of bioassays, most of which used either mouse cell lines or human T cell lines and, in rare instances, lectin-stimulated blast cells. The first international standard has proved suitable for its intended purpose of providing a basis for the potency labelling of approved products. However, stocks were almost completely depleted and a replacement was required.

In 2011, the Committee recognized the need to replace the international standard for IL-2, and agreed that lyophilized candidate preparations from the study conducted to establish the first standard should be evaluated and, subject to their suitability, be considered as potential replacements. Thus, an international collaborative study was carried out to evaluate two candidate preparations (NIBSC codes 86/500 and 86/564) of human sequence recombinant IL-2 as an international standard. Eight laboratories in four countries participated in the study.

The Committee considered the report of the study (WHO/BS/2012.2194) and recommended that preparation 86/500 be established as the Second WHO International Standard for interleukin-2 (human, rDNA-derived), with an assigned IL-2 activity value of 210 IU/ampoule.

7.1.4 First WHO Reference Reagent for interleukin-29 (human, rDNA-derived)

Interleukin-29 (IL-29) is the prototypic member of a small family of three closely related cytokines (IL-28A, IL-28B and IL-29) that share common functional features and structural features with a class of cytokines that act through corticotropin-releasing factor receptor 2 (CRF2). The activities of IL-29 are the same as those documented for type I interferons, namely antiviral activity (both in vitro and in vivo), immunostimulatory activity and antiproliferative activity – though all of these activities are more selective and weaker than those found in type I interferons. Additionally, IL-29 has been shown to inhibit the replication of several viruses, including hepatitis C virus in vitro.

Two preparations of human sequence recombinant IL-29 – one expressed in murine NS0 cells, the other in *Escherichia coli* – were formulated and lyophilized at the NIBSC for evaluation in a collaborative study of their suitability as a reference standard. The preparations were tested by six laboratories using in vitro bioassays. On the basis of the results, both the NS0-derived preparation (NIBSC code 07/212) and the *E. coli*-derived preparation (NIBSC code 10/176) were judged sufficiently active and stable to serve as a reference standard.

The Committee considered the report of the study (WHO/BS/2012.2197) and – given that the production of IL-29 from *E. coli* is more likely to be used for clinical applications – recommended that preparation 10/176 be established as the First WHO Reference Reagent for interleukin-29 (human, rDNA-derived), with an assigned unitage of 5000 u/ampoule.

8. International reference materials – antibiotics

All reference materials established at the meeting are listed in Annex 8.

8.1 WHO International Standards and Reference Reagents – antibiotics

8.1.1 Second WHO International Standard for neomycin B

Neomycin B (also called framycetin) is a broad-spectrum aminoglycoside antibiotic derived from *Streptomyces decaris* or *Streptomyces fradiae* and is a major component of neomycin. Generally, neomycin B is combined with other antibacterial agents and used in topical preparations to treat infections of the skin, nose, ears and eyes.

Because stocks of the First WHO International Standard for neomycin B were becoming depleted, EDQM had taken appropriate steps to establish a new batch (EDQM code ISA_46104). An international collaborative study involving seven laboratories in seven different countries was carried out to evaluate the use of this preparation as a replacement international standard for neomycin B.

The Committee considered the report of the study (WHO/BS/2012.2201) and recommended that EDQM preparation ISA_46104 be established as the Second WHO International Standard for neomycin B, with an assigned antimicrobiological activity of 17 640 IU/vial.

8.1.2 Third WHO International Standard for neomycin

Neomycin is an aminoglycoside antibiotic found in many topical medicines or given orally, mainly prior to gastrointestinal surgery.

Because stocks of the Second WHO International Standard for neomycin were becoming depleted, EDQM had taken appropriate steps to establish a new batch (EDQM code ISA_49163). An international collaborative study involving ten laboratories in ten different countries was carried out to evaluate the use of this preparation as a replacement international standard for neomycin. Potencies of the candidate material were estimated by microbiological assays with sensitive microorganisms. To ensure continuity between consecutive batches, the Second WHO International Standard for neomycin was used.

The Committee considered the report of the study (WHO/BS/2012.2202) and recommended that EDQM preparation ISA_49163 be established as the Third WHO International Standard for neomycin, with an assigned antimicrobiological activity of 19 050 IU/vial.

9. Proposed projects for endorsement

The considerations used to assign priorities to the development of international biological measurement standards or reference reagents were published by WHO in 2005 (Annex 2, Appendix 1 of WHO Technical Report Series, No. 932). These considerations are used to guide the Secretariat and WHO collaborating centres in developing a proposed programme for future work. To facilitate the priority-setting process and improve transparency, a simple tool was developed that describes the salient features of each new project proposal.

This tool provides a means for the Committee and other stakeholders to review and comment on proposals that are under consideration. The proposals made in document WHO/BS/12.2203 included requests to initiate projects in the areas of: vaccines and related substances (Appendix 1); and cytokines, growth factors and endocrinological substances (Appendix 2). No requests were received to initiate new projects for antibiotics.

The proposals presented in document WHO/BS/12.2211, and its addendum WHO/BS/2012.2211, include requests to initiate projects in the areas of blood products and in vitro diagnostic devices.

The Committee considered the following proposals for projects and approved them all, with the exception of the proposal for monoclonal-antibody-based biotherapeutics. The Committee requested further definition of the scope and intent of this proposed project to ensure that it considers the current regulatory environment. The Committee agreed only to an exploratory project since this request was to initiate work on a whole new class of reagents, rather than on a single material.

- Haemophilus influenzae type b polysaccharide (WHO/ BS/2012.2203);
- Anti-enterovirus 71 serum (WHO/BS/2012.2203);
- Enterovirus 71 inactivated vaccine (WHO/BS/2012.2203);
- Anti-Japanese encephalitis SA14-14-2 serum (WHO/BS/2012.2203);
- Japanese encephalitis vaccine (SA14-14-2, live) (WHO/BS/2012.2203);
- Japanese encephalitis vaccine (yellow fever 17D-vectored, live) (WHO/BS/2012.2203);
- Tumour necrosis factor antagonist (WHO/BS/2012.2203);
- Tumour necrosis factor-alpha (WHO/BS/2012.2203);
- Recombinant human granulocyte colony-stimulating factor, pegylated (WHO/BS/2012.2203);
- Standardization of bioassays for monoclonal-antibody-based biotherapeutics (WHO/BS/2012.2203);

- Factor IX concentrate (WHO/BS/2012.2211);
- Procoagulant activity of human intravenous immunoglobulin (WHO/BS/2012.2211);
- Disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, plasma (WHO/BS/2012.2211);
- Antihuman neutrophil antigen 3a (WHO/BS/2012.2211);
- Hepatitis B surface antigen (WHO/BS/2012.2211);
- Parvovirus B19 for NAT-based assays (WHO/BS/2012.2211);
- West Nile virus for NAT-based assays (WHO/BS/2012.2211 Addendum).

Annex 1

WHO Recommendations, Guidelines and other documents related to the manufacture and quality control of biological substances used in medicine

The Recommendations (previously called Requirements) and Guidelines published by WHO are scientific and advisory in nature but may be adopted by an NRA as national requirements or used as the basis of such requirements.

These international Recommendations are intended to provide guidance to those responsible for the production of biologicals as well as to others who may have to decide upon appropriate methods of assay and control to ensure that products are safe, reliable and potent.

Recommendations concerned with biological substances used in medicine are formulated by international groups of experts and are published in the WHO Technical Report Series¹ as listed below. A historical list of Requirements and other sets of Recommendations is available on request from the World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland.

Reports of the WHO Expert Committee on Biological Standardization published in the WHO Technical Report Series can be purchased from:

WHO Press World Health Organization 20 avenue Appia 1211 Geneva 27 Switzerland

Telephone: +41 22 791 3246

Fax: +41 22 791 4857

E-mail: bookorders@who.int

Web site: http://www.who.int/bookorders

Individual Recommendations and Guidelines may be obtained free of charge as offprints by writing to:

Technologies, Standards and Norms
Department of Essential Medicines and Pharmaceutical Policies
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland

¹ Abbreviated in the following pages to TRS.

Recommendations, Guidelines and other documents	Reference
Animal cells, use of, as in vitro substrates for the production of biologicals	Revised 2010, TRS 978 (2013)
BCG vaccines (dried)	Revised 2011, TRS 979 (2013)
Biological products: good manufacturing practices	Adopted 1991, TRS 822 (1992)
Biological products prepared by recombinant DNA technology	Adopted 1990, TRS 814 (1991)
Biological standardization and control: a scientific review commissioned by the UK National Biologicals Standards Board (1997)	Unpublished document WHO/ BLG/97.1
Biological substances: International Standards and Reference Reagents	Revised 2004, TRS 932 (2006)
Biotherapeutic products, similar	Adopted 2009, TRS 977 (2013)
Blood, blood components and plasma derivatives: collection, processing and quality control	Revised 1992, TRS 840 (1994)
Blood establishments: good manufacturing practices	Adopted 2010, TRS 961 (2011)
Blood plasma (human) for fractionation	Adopted 2005, TRS 941 (2007)
Blood plasma products (human): viral inactivation and removal procedures	Adopted 2001, TRS 924 (2004)
Blood regulatory systems, assessment criteria for national	Adopted 2011, TRS 979 (2013)
Cholera vaccines (inactivated, oral)	Adopted 2001, TRS 924 (2004)
Dengue tetravalent vaccines (live, attenuated)	Revised 2011, TRS 979 (2013)
Diphtheria, tetanus, pertussis (whole cell), and combined (DTwP) vaccines	Revised 2012, TRS 980 (2014)
Diphtheria vaccines (adsorbed)	Revised 2012, TRS 980 (2014)
DNA vaccines: assuring quality and nonclinical safety	Revised 2005, TRS 941 (2007)
Haemophilus influenzae type b conjugate vaccines	Revised 1998, TRS 897 (2000)

Recommendations, Guidelines and other documents	Reference
Haemorrhagic fever with renal syndrome (HFRS) vaccines (inactivated)	Adopted 1993, TRS 848 (1994)
Hepatitis A vaccines (inactivated)	Adopted 1994, TRS 858 (1995)
Hepatitis B vaccines prepared from plasma	Revised 1987, TRS 771 (1988)
Hepatitis B vaccines made by recombinant DNA techniques	Revised 2010, TRS 978 (2013)
Human interferons prepared from lymphoblastoid cells	Adopted 1988, TRS 786 (1989)
Influenza, biosafety risk assessment and safe production and control for (human) pandemic vaccines	Adopted 2005, TRS 941 (2007)
Influenza vaccines (inactivated)	Revised 2003, TRS 927 (2005)
Influenza vaccines (live)	Revised 2009, TRS 977 (2013)
Influenza vaccines, human, pandemic, regulatory preparedness	Adopted 2007, TRS 963 (2011)
Japanese encephalitis vaccines (inactivated) for human use	Revised 2007, TRS 963 (2011)
Japanese encephalitis vaccines (live, attenuated) for human use	Revised 2012, TRS 980 (2014)
Louse-borne human typhus vaccines (live)	Adopted 1982, TRS 687 (1983)
Malaria vaccines (recombinant)	Adopted 2012, TRS 980 (2014)
Measles, mumps and rubella vaccines and combined vaccines (live)	Adopted 1992, TRS 848 (1994); Note TRS 848 (1994)
Meningococcal polysaccharide vaccines	Adopted 1975, TRS 594 (1976); Addendum 1980, TRS 658 (1981); Amendment 1999, TRS 904 (2002)
Meningococcal A conjugate vaccines	Adopted 2006, TRS 962 (2011)
Meningococcal C conjugate vaccines	Adopted 2001, TRS 924 (2004); Addendum (revised) 2007, TRS 963 (2011)
Monoclonal antibodies	Adopted 1991, TRS 822 (1992)
Papillomavirus vaccines (human)	Adopted 2006, TRS 962 (2011)
Pertussis vaccines (acellular)	Revised 2011, TRS 979 (2013)

Recommendations, Guidelines and other documents	Reference
Pertussis vaccines (whole-cell)	Revised 2005, TRS 941 (2007)
Pharmaceutical products, storage and transport of time- and temperature-sensitive	Adopted 2010, TRS 961 (2011)
Pneumococcal conjugate vaccines	Revised 2009, TRS 977 (2013)
Poliomyelitis vaccines (inactivated)	Revised 2000, TRS 910 (2002); Amendment 2003, TRS 926 (2004)
Poliomyelitis vaccines (inactivated): guidelines for the safe production and quality control of inactivated poliovirus manufactured from wild polioviruses	Adopted 2003, TRS 926 (2004)
Poliomyelitis vaccines (oral)	Revised 2012, TRS 980 (2014)
Quality assurance for biological products, guidelines for national authorities	Adopted 1991, TRS 822 (1992)
Rabies vaccines for human use (inactivated) produced in cell substrates and embryonated eggs	Revised 2005, TRS 941 (2007)
Regulation and licensing of biological products in countries with newly developing regulatory authorities	Adopted 1994, TRS 858 (1995)
Rotavirus vaccines (live-attenuated, oral)	Adopted 2005, TRS 941 (2007)
Smallpox vaccines	Revised 2003, TRS 926 (2004)
Snake antivenom immunoglobulins	Adopted 2008, TRS 964 (2012)
Sterility of biological substances	Revised 1973, TRS 530 (1973); Amendment 1995, TRS 872 (1998)
Synthetic peptide vaccines	Adopted 1997, TRS 889 (1999)
Tetanus vaccines (adsorbed)	Revised 2012, TRS 980 (2014)
Thiomersal for vaccines: regulatory expectations for elimination, reduction or removal	Adopted 2003, TRS 926 (2004)
Thromboplastins and plasma used to control oral anticoagulant therapy	Revised 2011, TRS 979 (2013)
Tick-borne encephalitis vaccines (inactivated)	Adopted 1997, TRS 889 (1999)
Transmissible spongiform encephalopathies in relation to biological and pharmaceutical products, guidelines	Revised 2005, WHO (2006) http://www.who.int/biologicals/ publications/en/whotse2003.pdf

Recommendations, Guidelines and other documents	Reference
Tuberculins	Revised 1985, TRS 745 (1987)
Typhoid vaccines	Adopted 1966, TRS 361 (1967)
Typhoid vaccines, Vi polysaccharide	Adopted 1992, TRS 840 (1994)
Vaccines, clinical evaluation: regulatory expectations	Adopted 2001, TRS 924 (2004)
Vaccines, lot release	Adopted 2010, TRS 978 (2013)
Vaccines, nonclinical evaluation	Adopted 2003, TRS 926 (2004)
Vaccines, prequalification procedure	Adopted 2010, TRS 978 (2013)
Vaccines, stability evaluation	Adopted 2006, TRS 962 (2011)
Varicella vaccines (live)	Revised 1993, TRS 848 (1994)
Yellow fever vaccines	Revised 2010, TRS 978 (2013)
Yellow fever vaccines, laboratories approved by WHO for the production of	Revised 1995, TRS 872 (1998)
Yellow fever virus, production and testing of WHO primary seed lot 213-77 and reference batch 168-736	Adopted 1985, TRS 745 (1987)

Annex 2

Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated)

Replacement of Annex 1 of WHO Technical Report Series, No. 904, and Addendum to Annex 1 of WHO Technical Report Series, No. 910

Intro	duction	51
Gene	ral considerations	52
Scope	e of the Recommendations	58
Part A	A. Manufacturing recommendations	58
A A A A A A	A.1 Definitions A.2 General manufacturing recommendations A.3 Control of source materials A.4 Control of vaccine production A.5 Filling and containers A.6 Control tests on final lot A.7 Records A.8 Retained samples A.9 Labelling A.10 Distribution and transport	58 61 61 66 76 79 79 79
Α	A.11 Stability, storage and expiry date 3. Nonclinical evaluation of poliomyelitis vaccines	80
	live, attenuated)	81
В	Characterization of a new virus submaster seed from the WHO master seed Characterization of virus working seeds from an established master seed where passage level between master seed and working seed is increased Characterization following changes in the manufacturing process	81 82 82
Part C	C. Clinical evaluation of poliomyelitis vaccines (oral, live, attenuated)	82
Č	General considerations Safety and immunogenicity studies Post-marketing studies and surveillance	83 84 86
Part D	D. Recommendations for NRAs	87
_	D.1 General D.2 Release and certification by the NRA	87 88
	. Recommendations for poliomyelitis vaccines	
	live, attenuated) prepared in primary monkey kidney cells	88
E	E.1 Control of vaccine production	89

Authors and acknowledgements	95
References	100
Appendix 1	
Overview of virus seeds used in OPV production	104
Appendix 2	
In vivo tests for neurovirulence, and considerations in relation to assay choice	108
Appendix 3	
Preparation of poliomyelitis vaccines (oral, live, attenuated) using cell banks – example of a flowsheet	114
Appendix 4	
Cell-culture techniques for determining the virus content of poliomyelitis vaccines (oral, live, attenuated)	116
Appendix 5	
Model protocol for the manufacturing and control of poliomyelitis vaccines (oral, live, attenuated)	118
Appendix 6	
Model certificate for the release of poliomyelitis vaccines (oral, live, attenuated) by NRAs	137
Appendix 7	
Preparation of poliomyelitis vaccines (oral, live, attenuated) using primary monkey kidney cells – example of a flowsheet	139

Recommendations published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes recommendations for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that such modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments or examples intended to provide additional guidance to manufacturers and NRAs.

Introduction

WHO Requirements for oral poliomyelitis vaccine (OPV) were first formulated in 1962 (1), and revised in 1965 (2), and then again in 1971 (3), when an appendix describing the production of OPV in human diploid cells was added. The Requirements were further updated in 1982 (4) following an accumulation of data, particularly on the performance and evaluation of the monkey neurovirulence test (MNVT) and tests on the karyology of human diploid cells. The Requirements for poliomyelitis vaccine (oral) were updated in full in 1989 (5) to take account of the general requirements for the characterization of continuous cell lines for the preparation of biologicals, which were adopted in 1985 (6), and after a WHO study group concluded that, in principle, such cell lines are acceptable as substrates for the production of biologicals (7). An addendum was subsequently adopted (8) that introduced changes in the tests used to confirm freedom from detectable DNA sequences of simian virus 40 (SV40); introduced the mutant analysis by polymerase chain reaction (PCR) and restriction enzyme cleavage (MAPREC) assay as an optional additional in vitro test for poliovirus type 3; increased levels of laboratory containment for wild polioviruses (WPVs) (9); and provided guidance on additional antibody screening tests (for foamy viruses) for animals from closed primate colonies used as a source for primary monkey kidney cells.

The Requirements (now Recommendations) were last revised in full in 1999 (10) when the use of transgenic mice expressing the human poliovirus receptor (TgPVR21 mice) (11) as an alternative to the MNVT for type-3 virus was included in the revision, and the MAPREC test was introduced as the in vitro test of preference for the evaluation of filtered bulk suspensions for poliovirus type 3 (12). The previously mandated reproductive capacity at elevated temperature (rct40) test then became an optional, additional test. The studies with poliovirus types 1 and 2 in TgPVR21 mice were completed by June 2000, and an addendum to the WHO Recommendations for the production and control of poliomyelitis vaccine (oral) was adopted in 2000 (13) that included the neurovirulence test in TgPVR21 mice as an alternative to the MNVT for all three poliovirus serotypes.

Since then, advances in scientific knowledge have been made, novel laboratory techniques have become available and new vaccine formulations (such as monovalent and bivalent OPV) are being used. In 2008, the WHO Expert Committee on Biological Standardization advised that the Recommendations for OPV should be revised. In addition, various tests are now applicable to all three types of polioviruses, and their significance needs to be better explained and rationalized. Sections on the nonclinical and clinical evaluation of new candidate OPVs are also required. To facilitate this process, WHO convened a working group to initiate the revision of the Recommendations for the production and control of OPV, as outlined in WHO Technical Report Series No. 904 and

No. 910. Experts from academia, national regulatory authorities (NRAs), national control laboratories (NCLs) and industry involved in the research, manufacture, authorization and testing or release of OPV from countries around the world met from 20–22 July 2010 to identify and discuss the issues to be considered in revising Technical Report Series No. 904 and No. 910 (14).

The major issues addressed during this revision process included:

- updating information on the origin of different strains for OPV production, and the addition of a new Appendix 1;
- updating the section on international standards and reference preparations;
- updating the section on general manufacturing recommendations and control tests;
- updating information on neurovirulence tests in monkeys (MNVTs) and in transgenic mice (TgmNVTs), and on the MAPREC test, which is extended to all three types of seeds and bulks;
- a new Appendix 2, giving rationales for the choice of monkey or mouse neurovirulence tests;
- consideration of new vaccine formulations (monovalent OPV and bivalent OPV);
- an update on terminology, and the introduction of the "virus submaster seed lot", which is applicable only to the master seed supplied by WHO;
- inclusion of new sections on the nonclinical and clinical evaluation of OPV;
- updating the appendices;
- updating the standard operating procedures (SOPs) for TgmNVTs and MAPREC assays, and for new MNVTs in light of technical developments.

Additional changes have been made to bring the document into line with other WHO Recommendations published since the last revision.

General considerations

Poliomyelitis is an acute communicable disease of humans caused by three distinct poliovirus serotypes (types 1, 2 and 3) distinguishable by a neutralization test (15). Poliovirus is a species C human enterovirus of the *Picornaviridae* family, and is composed of a single-stranded, positive-sense RNA genome and a protein capsid.

Where sanitation is poor, these viruses are believed to be spread mainly by faecal-to-oral transmission, whereas the oral-to-oral mode of transmission probably dominates in settings with higher standards of sanitation. However, in most settings, mixed patterns of transmission are likely to occur. In the prevaccine era, roughly one in 200 susceptible individuals infected by polioviruses developed paralytic poliomyelitis (15).

Progress in polio control (and, since 1988, polio eradication) has occurred mainly due to the widespread use of vaccines. An inactivated poliomyelitis vaccine (IPV Salk vaccine) was licensed in 1955; live-attenuated OPV (Sabin vaccine) was licensed as a monovalent OPV (mOPV) in 1961, and as a trivalent OPV (tOPV) in 1963. The Sabin strains of poliovirus used in the production of OPV were shown to be both immunogenic and highly attenuated when administered orally to susceptible children and adults. Most countries that initially introduced vaccination with IPV later changed to OPV because OPV provided many advantages, including easier administration, suitability for mass vaccination campaigns, superior induction of intestinal mucosal immunity, and lower production costs. In 1974, OPV was recommended as part of the Expanded Programme on Immunization, and OPV was again the vaccine of choice in 1988 when the World Health Assembly resolved to eradicate polio globally by the year 2000. By 2010, three of the six WHO Regions had been certified as free of WPVs, and WPV2 has not been detected worldwide since 1999 (15).

In addition to tOPV, which is used in many countries for routine or supplementary vaccination, monovalent OPV against type 1 (mOPV1) and against type 3 (mOPV3), and bivalent OPV against type 1 and type 3 (bOPV) (15), as used by the Global Polio Eradication Initiative (GPEI) have been licensed for use in endemic countries or for outbreak control in situations where one or two types may re-emerge. In addition, mOPV against type 2 has been licensed but is expected to be used primarily for emergency response stockpiles. In 2012, the Strategic Advisory Group of Experts on Immunization was asked by WHO to consider the possibility of replacing tOPV with bOPV for routine immunization globally.

Following the introduction and widespread use of mOPV1 and mOPV3 in supplementary immunization activities in 2005, the GPEI reported substantial reductions in these poliovirus types. The last reported case of polio in India involved poliovirus type 1 and occurred in January 2011. Since polio is now considered to have been eradicated in India, the country has been removed from the list of endemic countries. However, the co-circulation of WPV1 and WPV3 in the three remaining polio-endemic countries requires that huge quantities of bOPV be used to supplement the tOPV given during routine immunization and mass immunization campaigns. A clinical trial to evaluate the immunogenicity of different OPV formulations (mOPV1, mOPV3 and bOPV) compared with tOPV in an Indian population was conducted by WHO. The seroconversion

rates to poliovirus type 1 and type 3 following immunization with bOPV were significantly higher than those induced by tOPV, and they were not lower than those induced by immunization with either mOPV1 or mOPV3 (16).

Although OPV is a safe vaccine, adverse events may occur on rare occasions (15) with vaccine-associated paralytic poliomyelitis (VAPP) being the most serious of these rare adverse events. Cases of VAPP are clinically indistinguishable from poliomyelitis caused by WPV, but can be distinguished by laboratory analysis. The incidence of VAPP has been estimated at 4 cases/1 000 000 birth cohort per year in countries using OPV (17). Sabin viruses can also spread in populations where the coverage of OPV is low. In such situations, Sabin viruses can acquire the neurovirulence and transmissibility characteristics of WPV, and can cause polio cases and outbreaks as circulating vaccine-derived poliovirus (cVDPV) (18).

Live vaccines prepared from the Sabin strains of poliomyelitis viruses types 1, 2 and 3 were introduced for large-scale immunization in 1957. In 1972, Albert Sabin proposed that WHO should be the custodian of his poliovirus seed strains. The Director-General of WHO agreed to assume responsibility for ensuring the proper use of the strains, and established a scientific committee, the Consultative Group on Poliomyelitis Vaccines, to advise WHO on all matters pertaining to their use. Detailed information on the work of the consultative group, and the preparation of the strains by Behringwerke of Marburg, Germany, has been published by Cockburn (19). NRAs should decide on which strains to use and on the appropriate procedures for preparing virus seed lots for OPV in their own countries.

The original poliovirus seeds produced by Sabin – Sabin original (SO) (20) – were sent to Merck, which generated seeds from them designated Sabin original Merck (SOM). Aliquots of SOM were supplied to other manufacturers to enable them to develop their own seeds. Some seed lots were contaminated with SV40, which had been present in the primary Rhesus kidney cells, the preferred cell-culture system at that time for virus propagation. OPV manufacturers used various strategies to reduce the contamination, including passage in the presence of a specific antibody, treatment with toluidine blue or thermal inactivation of SV40 in the presence of 1M magnesium chloride (MgCl₂), which stabilizes poliovirus. In 1974, Behringwerke generously agreed to produce SO+1 seeds for WHO free of charge. The Behringwerke type 1 and type 2 seeds have been widely used since the 1970s.

In the 1950s, it was established that, particularly for the type-3 strain, increases in the passage number correlated with an increase in reactivity in the MNVT. This finding led to the establishment of rigorous limits on the passage level for vaccine production for all types of OPV.

The type-3 vaccine was found to be less stable on passage than either type 1 or type 2; this was manifested in a higher number of type-3 vaccine lots

failing the MNVT. In order to develop a more stable strain, a new seed was prepared by Pfizer; susceptible cells were transfected with viral RNA extracted from poliovirus at the SO+2 level. One plaque, designated 457-III, was identified as having particularly favourable properties (21). Theoretically, vaccine derived from this stock was at passage SO+7 level. However, the purpose of tracking the passage history of seed viruses is to reduce the accumulation of mutations that takes place during the course of their serial propagation. Since plaque purification represents the cloning of a single infectious particle, it eliminates the heterogeneity of the viral population, and the passage level is effectively reset to zero. Thus the cloned stock 457-III was renamed RNA-derived Sabin original (RSO).

Two additional passages were used to prepare virus master seeds (RSO1) and working seeds (RSO2), and vaccines produced from this virus are at RSO3 level. Retrospectively, the RSO sequence has been shown to be the same as the consensus of SO (22), but more homogeneous and containing smaller quantities of mutant viruses.

The RSO seed was not used for the production of type-3 vaccine until the 1980s when it became clear that the stocks of material passaged from the SOM and other SO+1 seeds were inadequate. Since then, it has been widely used by European and American manufacturers because it is of lower virulence in laboratory tests than the SO+1 type-3 seed. The RSO seeds were bought from Pfizer by Sanofi Pasteur which donated them to WHO.

The virus seeds available from WHO (WHO master seeds) are types 1, 2 and 3 at SO+1 level produced by Behringwerke from SO seeds, and the type-3 RSO seed donated by Sanofi Pasteur. The seeds are kept at the National Institute for Biological Standards and Control (NIBSC) in England, and include a proportion of the stocks of the SO+1 seeds formerly held at Istituto Superiore di Sanità in Italy (19, 21).

In addition to vaccines based upon the RSO type-3 seed, a number of manufacturers in China, Japan and the Russian Federation have produced vaccines using their own purified seed stocks of the Sabin 3 strain derived by plaque purification (cloning). Sequencing of these seed viruses demonstrated that, although they had only a low content of neurovirulent mutants, there were differences among these strains and the consensus sequence of SO virus (22). However, there are no reports of any differences in clinical safety between OPV produced from Pfizer stocks and the alternative seeds of Sabin 3 virus. An overview of virus seeds used in OPV production is given in Appendix 1.

The MNVT, as described in the 1989 Requirements (5), has been used as a quality-control test, and is based on the level and the distribution of virus-specific lesions within the central nervous system produced by vaccine virus when compared with an appropriate reference preparation (23). Because nonhuman primates are used, efforts to complement and eventually replace the test are of

considerable importance. WHO has encouraged and supported research on various aspects of poliovirus biology, including the development of alternative animal models, as part of its initiative to promote the development of new norms and standards for vaccines. Two groups of scientists developed transgenic mice by introducing into the mouse genome the human gene encoding the cellular receptor for poliovirus (24, 25). This receptor, known as CD155, makes TgPVR mice susceptible to poliovirus infection with clinical signs of flaccid paralysis and with histological lesions in the central nervous system similar to those observed in monkeys.

In 1992, WHO initiated a project to evaluate the suitability of such transgenic mice for testing the neurovirulence of OPV, with the aim of replacing monkeys with mice. The advantages of a neurovirulence test in transgenic mice are:

- a reduction in the number of primates used for quality control of OPV;
- the use of animals with highly defined genetic and microbiological quality standards;
- a reduction in hazards to laboratory personnel through a reduced need to handle primates;
- in some countries, a reduction in the cost of quality-control tests for OPV.

Studies were carried out initially on mOPV3 vaccines using the TgPVR21 mouse line, provided free of charge by the Central Institute for Experimental Animals in Japan. Researchers at the Japan Poliomyelitis Research Institute and at the United States Food and Drug Administration Center for Biologics Evaluation and Research (CBER) developed an intraspinal inoculation method suitable for testing vaccine lots. This method was evaluated in an international collaborative study designed to establish a standardized TgmNVT test for OPV (26). Several laboratories participated in the study, and the results were assessed by WHO at meetings held in 1995, 1997, 1998 and 1999. As a result, the revised WHO Recommendations for the production and control of poliomyelitis vaccine (oral) (10) introduced the murine model as an alternative to the MNVT for type-3 poliovirus, and further studies demonstrated that this test was also suitable as an alternative to the MNVT for poliovirus type 1 and type 2 (13). Laboratories must comply with specifications for containment of the transgenic animals (27). As with the MNVT, the TgmNVT can also provide evidence of the consistency of production.

The molecular mechanisms and genetic determinants of attenuation and of reversion to virulence of all three types of Sabin polioviruses used to manufacture OPV have been well studied. Evidence strongly suggests that mutations in the 5' noncoding region of the poliovirus genome, especially for the Sabin type-3

strain, are critical in determining the attenuated phenotype (28). A molecular biological test, known as the MAPREC assay, was developed by researchers at CBER to quantify reversion at the molecular level (29). Studies showed that all analysed batches of type-3 OPV contained measurable amounts of revertants, with C instead of U at nucleotide 472. Batches that failed the MNVT contained significantly higher quantities of 472-C than batches that passed the test. Studies with coded samples at CBER identified 100% of lots that failed the MNVT (30).

In 1991, WHO initiated a series of international collaborative studies to evaluate the MAPREC assay for all three types of poliovirus, and to validate appropriate reference materials. Several laboratories participated in the collaborative studies, and the results were assessed by WHO at meetings held in 1995 and 1997 in Geneva, Switzerland. It was concluded that the MAPREC assay was a sensitive, robust and standardized molecular biological assay suitable for use by manufacturers and NRAs for monitoring the consistency of the production of type-3 OPV. The revised WHO Recommendations for the production and control of poliomyelitis vaccine (oral) (10) introduced MAPREC as the preferred in vitro test for type 3 poliovirus in place of the rct40 test. Reference materials for the MAPREC assay for comparable positions in type 1 and type 2 have now been established. While the results do not correlate with neurovirulence in the range studied, they provide a measure of production consistency. The quantity of other mutants (such as 2493-U in Sabin 3 virus) can also be used to identify types of seed virus, and to monitor the consistency of manufacturing. After appropriate validation, quantitative profiles of other mutations in stocks of OPV could be used for this purpose.

The manufacturer of the final lot must be responsible for ensuring conformity with all of the recommendations applicable to the final vaccine (see Part A, sections A.5–A.11), even where manufacturing involves only the filling of final containers with vaccine obtained in bulk from another manufacturer. The manufacturer of the final lot must also be responsible for any production and control tests performed, with the approval of the NRA, by an external contract laboratory, if applicable.

OPV has been in worldwide use since the 1960s, and although vaccines produced from human diploid cells or continuous cell lines have been used to a lesser extent than those produced in cultures of primary monkey kidney cells, experience has indicated that all three cell substrates produce safe and effective vaccines.

In 1986, a WHO study group (7) stated that the risks for residual cellular DNA (rcDNA) in vaccines produced in continuous cell lines should be considered negligible for preparations given orally. This conclusion was based on the finding that polyomavirus DNA was not infectious when administered orally (31). For such products, the principal requirement is the elimination of potentially contaminating viruses. Additional data on the uptake of DNA via the oral route

have been published (32). These studies demonstrated that the efficiency of the uptake of DNA introduced orally was significantly lower than that of DNA introduced intramuscularly. Nevertheless, the specifics of the manufacturing process and the formulation of a given product should be considered by NRAs (33) and, where possible, data should be accumulated on the levels of rcDNA in OPV produced in Vero cells.

There is increasing interest in developing alternative strains of poliovirus for use in OPV production using molecular-manipulation techniques. The poliovirus-specific quality evaluation of such strains – e.g. for neurovirulence testing or for the MAPREC assay – as described in these Recommendations and associated SOPs, may not be appropriate. The testing of such vaccines – which is likely to include extensive preclinical and clinical studies to demonstrate attenuation, genetic stability, and the safety and transmissibility of the proposed strains – will need to be considered on a case-by-case basis, and may differ fundamentally from the approaches described in the current document.

Scope of the Recommendations

The scope of the present Recommendations encompasses poliomyelitis vaccines (oral, live, attenuated) derived from the original Sabin strains, some by simple passage and others by more complex routes, including plaque purification. This document is intended to apply to all Sabin poliovirus strains regardless of their history. It does not necessarily apply to other strains that may be developed.

This document should be read in conjunction with other relevant WHO Guidelines, such as those on the nonclinical (34) and clinical evaluation (35) of vaccines.

Part A. Manufacturing recommendations

A.1 **Definitions**

A.1.1 International name and proper name

The international name should be poliomyelitis vaccine (oral, live, attenuated) with additions to indicate the virus serotype or serotypes of the vaccine. The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 **Descriptive definition**

Poliomyelitis vaccine (oral, live, attenuated) is a preparation of live-attenuated poliovirus type 1, 2 or 3 grown in in vitro cultures of suitable cells containing any

one type or any combination of the three types of the Sabin strains, prepared in a form suitable for oral administration and satisfying all the recommendations formulated in this document.

A 13 International reference materials

A trivalent virus mixture is available as the Second WHO International Reference Reagent for live-attenuated poliovirus (Sabin) types 1, 2 and 3 for determination of virus titre.

Three monotypic virus suspensions of types 1, 2 and 3 have been established as WHO Reference Reagents for use in reference laboratories to measure the sensitivity of cell cultures for poliovirus infection.

International standards for MAPREC analysis of poliovirus types 1, 2 and 3 (Sabin) and international reference reagents for control of MAPREC assays of poliovirus type 1, 2 and 3 (Sabin) are available.

International standards for antipoliovirus types 1, 2 and 3 antibodies (human) are available for standardization of neutralizing antibody tests for poliovirus.

The reference materials listed above are available from the NIBSC, Potters Bar, England.

Reference preparations at the SO+2 passage level, designated WHO/I for type-1 virus, WHO/II for type-2 virus and WHO/III for type-3 virus are available upon request from WHO.¹ These reference preparations are for use in in vivo neurovirulence tests of homotypic vaccines. The relevant reference materials should be included in each test of vaccine (see section A.4.4.7.2).

A.1.4 **Terminology**

The definitions given below apply to the terms as used in these Recommendations. They may have different meanings in other contexts.

Adventitious agents: contaminating microorganisms of the cell substrate or source materials used in their cultures; these may include bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses that have been unintentionally introduced.

Cell culture infectious dose 50% (CCID $_{50}$): the amount of a virus sufficient to cause a cytopathic effect in 50% of inoculated replicate cell cultures, as determined in an end-point dilution assay in monolayer cell cultures.

¹ Contact the Coordinator, Quality, Safety and Standards, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).

Cell seed: a quantity of vials containing well-characterized cells derived from a single tissue or cell of human or animal origin, stored frozen in liquid nitrogen in aliquots of uniform composition, one or more of which may be used for the production of a master cell bank.

Comparator vaccine: an approved vaccine with established efficacy, or with traceability to a vaccine with established efficacy, that is tested in parallel with an experimental vaccine and serves as an active control in nonclinical or clinical testing.

Final bulk: the finished vaccine from which the final containers are filled. The final bulk may be prepared from one or more monovalent bulks, and may contain more than one virus type.

Final lot: a collection of sealed final containers of finished vaccine that is homogeneous with respect to the risk of contamination during the filling process. Therefore, all of the final containers must have been filled from a single vessel of final bulk in one working session.

Master cell bank (MCB): a quantity of fully characterized cells of human or animal origin derived from the cell seed and frozen in aliquots of uniform composition at -70 °C or below. The MCB is itself an aliquot of a single pool of cells that has been dispensed into multiple containers and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a replacement MCB – derived from the same cell clone or from an existing master or working cell bank – is the same as that for the initial MCB unless a justified exception is made.

Monovalent bulk: a pool of a number of single harvests of the same virus type.

Production cell culture: a cell culture derived from one or more ampoules of the working cell bank or from primary tissue, and used for the production of vaccines.

RNA-derived Sabin original type-3 virus (RSO) (*21*): All subsequent passages are designated by an additional number – e.g. RSO1 (master seed) is one passage on from RSO. The working seed passage level is therefore RSO2, and the vaccine is RSO3.

Single harvest: a quantity of virus suspension of one virus type harvested from cell cultures derived from the same working cell bank, and prepared from a single production run.

Sabin original virus (SO): as described by Sabin and Boulger in 1973 (20). All subsequent passages are designated by an additional number – e.g. SO+1 is one passage on from Sabin original.

Virus master seed lot: a quantity of virus suspension that has been processed at the same time to ensure a uniform composition, and that has been characterized to the extent necessary to support development of the virus working seed lot. The characterized virus master seed lot is used for the preparation of virus working seed lots or a virus submaster seed (if applicable).

Virus submaster seed lot (applicable only to master seed supplied by WHO): a quantity of virus suspension produced by a single passage from the virus master seed supplied by WHO, and made at a multiplicity of infection that ensures the development of a cytopathic effect within an appropriate time frame; the virus submaster seed lot must have been processed at the same time to ensure a uniform composition. The virus submaster seed lot should be characterized to the extent necessary to support the development of the virus working seed lot. The characterized virus submaster seed lot is used for the preparation of virus working seed lots (see section A.3.2.2 and Part B).

Virus working seed lot: a quantity of virus of uniform composition, fully characterized, derived from only one passage made at the multiplicity of infection, ensuring that a cytopathic effect develops within an appropriate time frame (e.g. three days), from a virus master seed lot or submaster seed lot by a method approved by the NRA.

Working cell bank (WCB): a quantity of cells of uniform composition derived from one or more ampoules of the MCB at a finite passage level, stored frozen in aliquots at $-70\,^{\circ}$ C or below, one or more of which may be used for vaccine production. All containers must be treated identically, and once removed from storage must not be returned to stock.

A.2 General manufacturing recommendations

The general manufacturing recommendations contained in WHO good manufacturing practices for pharmaceutical products: main principles (36) and Good manufacturing practices for biological products (37) should apply to establishments manufacturing OPV, with the addition of the following recommendations:

- The production of OPV should be conducted by staff who are healthy and who are examined medically at regular intervals. Steps should be taken to ensure that all persons in the production areas are immune to poliomyelitis. Personnel working in monkey quarters should also be examined for tuberculosis as outlined in Part A, section 2 of Recommendations to assure the quality, safety and efficacy of BCG vaccines (38).
- The establishment should be in compliance with current global recommendations for poliovirus containment.

A.3 Control of source materials

General production precautions, as formulated in Good manufacturing practices for biological products (37), should apply to the manufacture of OPV, with the additional recommendation that during production only one type of cell should

be introduced or handled in the production area at any one time. Vaccines may be produced in cell lines such as MRC-5 and Vero cells (see section A.3.1) or in primary monkey kidney cells (see Part E).

A.3.1 Cell lines

A.3.1.1 Master cell bank and working cell bank

The use of a cell line for the manufacture of OPVs should be based on the cell-bank system. The cell seed and cell banks should conform with the Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33). The cell bank should be approved by the NRA. The maximum number of passages (or population doublings) allowed between the cell seed, the MCB, the WCB and the production passage level should be established by the manufacturer, and approved by the NRA. Additional tests may include but are not limited to propagation of the MCB or WCB cells to or beyond the maximum in vitro age for production, and examination for the presence of retroviruses and tumorigenicity in an animal test system (33).

It is important to show that the cell banks (cell seed, MCB and WCB) are free from adventitious agents relevant to the species used in their derivation. Cell banks should be assessed for the absence of adventitious agents that may have been present during production.

The WHO Vero reference cell bank 10-87 is considered suitable for use as a cell seed for generating an MCB (39), and is available to manufacturers on application to the Coordinator, Quality, Safety and Standards, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland.

A.3.1.2 **Identity tests**

Identity tests on the MCB and WCB are performed in accordance with WHO Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33), and should be approved by the NRA.

The WCB should be identified by means of, inter alia, biochemical tests (e.g. isoenzyme analysis), immunological tests, tests for cytogenetic markers, and DNA fingerprinting or sequencing. The tests should be approved by the NRA.

A.3.1.3 Cell culture medium

Serum used for the propagation of cells should be tested to demonstrate that it is free from infectious viruses as well as from bacteria, fungi and mycoplasmas using appropriate tests as specified in Part A, sections A.5.2 (40) in the General requirements for the sterility of biological substances no. 6 (1973) and A.5.3 (41) in the General requirements for the sterility of biological substances no. 6

(amended 1995). Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the WHO Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33).

Validated molecular tests for bovine viruses may be used instead of cell culture tests of bovine serum if approved by the NRA. As an additional means of monitoring quality, serum may be examined to ensure it is free from bacteriophages and endotoxins. Gamma radiation may be used to inactivate potentially contaminating viruses, while recognizing that some viruses are relatively resistant to gamma radiation.

The source or sources of animal components used in the culture medium should be approved by the NRA. These components should comply with the WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (42).

Human serum should not be used. If human serum albumin is used at any stage of manufacturing, the NRA should be consulted regarding requirements because these may differ from country to country. As a minimum, the serum should meet the Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (43). In addition, human albumin and materials of animal origin should comply with current WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (42).

Penicillin and other beta-lactam antibiotics should not be used at any stage of manufacturing because of their nature as highly sensitizing substances.

Other antibiotics may be used at any stage of manufacturing provided that the quantity present in the final lot is acceptable to the NRA.

Nontoxic pH indicators may be added, such as phenol red at a concentration of 0.002%.

Only substances that have been approved by the NRA may be added.

Bovine or porcine trypsin used for preparing cell cultures should be tested and found free from cultivable bacteria, fungi, mycoplasmas and infectious viruses, as appropriate. The methods used to ensure this should be approved by the NRA.

In some countries, irradiation is used to inactivate potentially contaminating viruses. If irradiation is used, it is important to ensure that a reproducible dose is delivered to all batches and to the component units of each batch. The irradiation dose must be low enough for the biological properties of the reagents to be retained but high enough to reduce virological risk. Therefore, irradiation cannot be considered a sterilizing process (33).

Recombinant trypsin is available and its use should be considered; however, it should not be assumed to be free from the risk of contamination, and should be subject to the usual considerations for any reagent of biological origin (33).

The source or sources of trypsin of bovine origin, if used, should be approved by the NRA, and should comply with the current WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (42).

A.3.2 Virus seeds

A.3.2.1 Virus strains

Strains of poliovirus used in the production of OPV should be identified by historical records, which should include information on their origin. Producers of OPV can obtain virus master seeds from WHO. Manufacturers receiving this virus may prepare a submaster seed by a single passage, and then prepare their working seed. However, only virus strains that are approved by the NRA should be used (see General considerations in the Introduction).

A.3.2.2 Virus-seed lot system

Vaccine production should be based on the seed lot system. Virus-seed lots should not be purified. The virus master seed lot and virus working seed lot used for the production of vaccine batches should be prepared by a single passage from the virus strain and the virus master seed lot, respectively, using a method and a passage level from the original seed virus approved by the NRA. A virus submaster seed lot may be prepared by a single passage from WHO master seed, and the characterized virus submaster seed lot (see Part B) may be used for the preparation of virus working seed lots by a single passage.

Virus master seed lots, submaster seed lots and working seed lots should be stored in dedicated, monitored freezers at a temperature that ensures stability on storage – that is, \leq –60 °C. Guidance on the additional characterization of master and submaster seeds is provided in Part B.

A.3.2.3 Tests on virus master seed, submaster seed and working seed lots

The virus master seed is provided by WHO as well characterized seed material. The virus submaster seed lot and working seed lot used for the production of vaccine batches should be shown to be free from detectable extraneous viruses and from detectable SV40 DNA as determined by a validated nucleic acid amplification test; the submaster seed lot and the working seed lot should conform to the recommendations set out in Part A, sections A.4.3 (single

harvests) and A.4.4.1–A.4.4.4 (monovalent bulks). The control cell cultures should conform to section A.4.1 (control of cell cultures).

DNA from SV40 is widely used as a molecular biological reagent, and contamination of PCR assays is potentially a major problem. One approach is to identify separate genomic regions of SV40 for amplification, and to use one region for screening purposes and the other for the confirmation of repeatedly positive samples. It is useful if the genomic region used for confirmation varies between isolates from different sources because it is then possible to show that it has a unique sequence, and that positive results are not due to contamination with laboratory strains of SV40. The sensitivity of the PCR assays for the genomic regions used should be established.

A.3.2.4 Tests to monitor molecular characteristics of the virus

A.3.2.4.1 Tests in vitro

Seed viruses should be tested with MAPREC assays or temperature-sensitivity assays (such as the rct40 test) (see section A.4.4.7.1). If the NRA agrees, then at least three consecutive monovalent bulks prepared from the seed virus should meet the criteria for acceptability given in section A.4.4.7.1.

Historically, four consecutive monovalent bulks prepared from the seed virus have been tested to monitor the molecular characteristics of the virus and production consistency.

A.3.2.4.2 Neurovirulence tests

New virus working seeds should be evaluated for neurovirulence. Summaries of the MNVT and TgmNVT, including pass/fail criteria, are given in Appendix 2 along with considerations on the choice of assay. The test should be approved by the NRA for the specific product, and transgenic mice, nonhuman primates, or both, may be used.

The test for neurovirulence in nonhuman primates should be carried out as summarized in Appendix 2, and following the SOPs available from WHO² for neurovirulence tests for types 1, 2 or 3 live-attenuated OPV in monkeys.

The use of the TgmNVT should be approved by the NRA, and it should be carried out as summarized in Appendix 2, and described in detail in the SOPs available from WHO² for the neurovirulence tests for type 1, 2 or 3 live-attenuated OPV in transgenic mice susceptible to poliovirus.

² Contact the Coordinator, Technologies, Standards and Norms, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).

Under normal circumstances, a new virus working seed will be prepared using the same production protocol and from the same virus master seed as the currently approved virus working seed. If the TgmNVT has been approved by the NRA for the release of vaccine batches, and if the virus working seed is generated by the same production process, the new seed can be qualified using the TgmNVT and supporting in vitro data.

If there are any major changes in the production process for a new virus master seed, full characterization using tests in nonhuman primates and transgenic mice will be required (see Part B).

If the NRA agrees, then the neurovirulence of the virus working seeds and at least three consecutive monovalent bulks prepared from it should meet the criteria for acceptability given in section A.4.4.7.2 and the appropriate SOP before the working seed can be considered suitable for use in the production of OPV.

Historically, four consecutive monovalent bulks prepared from the seed virus have been tested in monkeys to monitor production consistency.

A.3.2.5 **Genotype characterization**

Advances have been made in the development and application of molecular methods such as deep sequencing. For any new virus working seed, it may be useful for information purposes to analyse the new virus working seed and at least three consecutive monovalent bulks for nucleotide sequence changes from the seed virus (deep genome sequence). If such tests are performed for regulatory purposes, they should be scientifically validated and approved by the NRA.

A.4 Control of vaccine production

Part E contains additional or alternative recommendations for OPV prepared in cultures of primary monkey kidney cells, and information on testing the cell substrate used for the production of the vaccine.

A.4.1 Control of production cell cultures

When human diploid or continuous cell lines are used to prepare cultures for the production of vaccine, a fraction equivalent to at least 5% of the total or 500 ml of cell suspension, or 100 000 000 cells, at the concentration and cell passage level employed for seeding vaccine production cultures, should be used to prepare control cultures. (See Appendix 3 for an example of a flowsheet for tests in cell cultures.)

If fermenter technology is used, the NRA should determine the size and treatment of the cell sample to be examined.

A.4.1.1 Tests of control cell cultures

The treatment of the cells set aside as control material should be similar to that of the production cell cultures but they should remain uninoculated so they can be used as control cultures for detecting adventitious agents.

These control cell cultures should be incubated for at least two weeks under conditions as similar as possible to the inoculated cultures, and they should be tested for the presence of adventitious agents as described below. For the test to be valid, 20% or fewer of the control cultures should have been discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cultures should be examined for degeneration caused by an extraneous agent. If this examination of a control culture, or any of the tests specified in this section, shows the presence of an adventitious agent, the poliovirus grown in the corresponding inoculated cultures should not be used for vaccine production.

A.4.1.2 Tests for haemadsorbing viruses

At the end of the observation period, 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea-pig red blood cells. If these cells have been stored, the duration of storage should not have exceeded seven days, and the storage temperature should have been in the range of 2–8 °C. In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

Some NRAs require that as an additional test for haemadsorbing viruses, other types of red cells – including cells from humans (blood group IV O), monkeys and chickens (or other avian species) – should be used in addition to guinea-pig cells.

A reading should be taken after 30 minutes' incubation at 2–8 °C, and after incubation for an additional 30 minutes at 20–25 °C.

If a test with monkey red blood cells is performed, readings should also be taken after a final incubation for 30 minutes at 34–37 °C.

A.4.1.3 Tests for other adventitious agents in cell fluids

At the end of the observation period, a sample of the pooled fluid from each group of control cultures should be tested for adventitious agents. For this purpose, 10 ml from each pool should be tested in the same cells, but not the same batch of cells, as those used for the production of vaccine.

A second indicator cell line should be used to test an additional 10 ml sample from each pool. When a human diploid cell line is used for production, a simian kidney cell line should be used as the second indicator cell line. When

a simian kidney cell line is used for production, a human diploid cell line should be used as the second indicator cell line (33).

The pooled fluid should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not fall below 1 part in 4. The area of the cell sheet should be at least 3 cm² per ml of pooled fluid. At least one bottle of each kind of cell culture should remain uninoculated to serve as a control.

The inoculated cultures should be incubated at 35–37 °C, and should be observed for at least 14 days.

Some NRAs require that at the end of this observation period a subculture is made in the same culture system and observed for at least an additional 14 days. Furthermore, some NRAs require that these cells be tested for the presence of haemadsorbing viruses.

For the tests to be valid, 20% or fewer of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period.

If any cytopathic changes caused by adventitious agents occur in any of the cultures, the virus harvests produced from the batch of cells from which the control cells were taken should be discarded.

Some selected viruses may be screened for by using specific validated assays that have been approved by the NRA, such as molecular techniques (e.g. nucleic acid amplification) (33).

If these tests are not performed immediately, the samples should be kept at -60 °C or below.

A.4.1.4 **Identity test**

At the production level, the cells should be identified by means of tests approved by the NRA. Suitable methods include but are not limited to biochemical tests (e.g. isoenzyme analyses), immunological tests, cytogenetic tests (e.g. for chromosomal markers) and tests for genetic markers (e.g. DNA fingerprinting or sequencing).

A.4.2 Cell cultures for vaccine production

A.4.2.1 Observation of cultures for adventitious agents

On the day of inoculation with the virus working seed lot, each cell culture or a sample from each culture vessel should be examined visually for degeneration caused by infective agents. If such examination of a cell culture shows evidence of any adventitious agent, the culture should not be used for vaccine production (see section A.4.1.3).

If animal serum is used for cell cultures before the inoculation of virus, the medium should be removed and replaced with serum-free maintenance medium after the cells have been washed with serum-free medium.

A.4.3 Control of single harvests

A.4.3.1 Single harvest

After inoculation of the production cells with the virus working seed lot, inoculated cell cultures and control cultures should be held at a fixed temperature that has been shown to be suitable and that falls within the range 33–35 °C for the relevant incubation periods. The temperature should not vary by more than 0.5 °C from the set temperature. The optimal range for pH, multiplicity of infection, cell density, virus recovery and time of incubation should be established for each manufacturer, and should be approved by the NRA.

The virus suspension should be harvested not later than four days after virus inoculation.

The inoculated cell cultures should be processed so that each virus suspension harvested remains identifiable as a single harvest and is kept separate from other harvests until the results of all tests have been obtained as described in Part A sections A.4.1.2–4.1.4, A.4.3.3.1–4.3.3.3, and A.4.3.3.4 and A.4.3.3.5.

A.4.3.2 **Sampling**

Samples required for testing single harvests should be taken immediately on harvesting. If the tests for adventitious agents described in Part A section A.4.3.3.3 are not performed immediately, the samples taken for these tests should be kept at -60 °C or lower and subjected to no more than one freeze–thaw cycle.

A.4.3.3 **Tests on single harvests**

A.4.3.3.1 *Identity*

Each single harvest should be identified as the appropriate poliovirus serotype by immunological assay on cell culture using specific antibodies or by a molecular method that has been validated and approved by the NRA.

Neutralization tests can distinguish the serotype of polioviruses. Molecular methods, such as sequencing or deep sequencing, can distinguish Sabin virus from wild-type virus.

Care should be taken to ensure that the serum samples used are monospecific by titrating them against homotypic and heterotypic viruses of known virus titre. Monoclonal antibodies may be useful in this test.

A.4.3.3.2 Titration for virus content

The virus titre per millilitre of single harvest should be determined for cell cultures by comparing them with an existing reference preparation (see Appendix 4).

A.4.3.3.3 Tests of neutralized single harvests for adventitious agents

Some selected viruses may be screened by using specific assays, such as molecular techniques (e.g. nucleic acid amplification) (33). For the recommendations set out in this section of Part A, the volume of each single harvest taken for neutralization and testing should be at least 10 ml, and should ensure that a total of at least 50 ml or the equivalent of 500 doses of the final vaccine, whichever is greater, has been withheld from the corresponding single harvest.

The antiserum used for neutralization should be of nonhuman origin, and should have been prepared in animals other than monkeys using virus cultured in cells from a species different from that used in the production of the vaccine. Samples of each virus harvest should be tested in human cells and at least one other sensitive cell system.

The neutralized suspensions should be inoculated into bottles of these cell cultures so that the dilution of the suspension in the nutrient medium does not fall below 1 part in 4. The area of the cell sheet should be at least 3 cm² per ml of neutralized suspension. At least one bottle of each kind of cell culture should remain uninoculated to serve as a control; it should be maintained using nutrient medium containing the same concentration of the specific antiserum used for neutralization.

Animal serum may be used to propagate the cells but the maintenance medium used after the test material has been inoculated should not contain any added serum other than the poliovirus neutralizing antiserum or fetal calf serum of controlled origin.

The inoculated cultures should be incubated at 35–37 °C, and should be observed for at least 14 days.

If adequately justified and validated, lower temperatures may be used.

For the tests to be valid, 20% or fewer of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period.

If any cytopathic changes caused by adventitious agents occur in any of the cultures, the virus harvest should be discarded.

New molecular methods with broad capabilities are being developed to detect adventitious agents. These methods include degenerate nucleic acid amplification testing for whole virus families that analyses the amplicons by hybridization, sequencing or mass spectrometry; nucleic acid amplification testing with random primers that is followed by analysis of the amplicons on large oligonucleotide microarrays of conserved viral sequencing or digital subtraction of expressed sequences; and high throughput sequencing. These methods may be used in the future to supplement existing methods, or as alternatives to both in vivo and in vitro tests after appropriate validation and approval by NRAs (33).

A.4.3.3.4 Sterility tests for bacteria, fungi and mycoplasmas

A volume of at least 10 ml of each single harvest should be tested for bacterial, fungal and mycoplasmal contamination using the appropriate tests specified in Part A, sections 5.2 and 5.3 of the General requirements for the sterility of biological substances (41) or by a method approved by the NRA.

Nucleic acid amplification techniques, used alone or in combination with cell culture and an appropriate detection method, may be used as alternatives to one or both of the compendial mycoplasma detection methods if they have been validated and the NRA agrees (33).

A.4.3.3.5 Test for mycobacteria

The virus harvest should be shown to be free from mycobacteria using an appropriate method approved by the NRA.

Molecular assays may be used as alternatives to microbiological culture tests for detecting mycobacteria after they have been validated and approved by the NRA (33).

With NRA approval, some manufacturers test for mycobacteria only at the monovalent bulk stage.

A.4.3.3.6 Tests for molecular consistency of production

Some manufacturers perform a test for the molecular consistency of production on single harvests using the MAPREC assay (see section A.4.4.7.1.1). If performed, the acceptance and rejection criteria for this test should be updated periodically and approved by the NRA.

A.4.4 Control of monovalent bulk

A.4.4.1 Preparation of monovalent bulk

The monovalent bulk may be prepared by pooling a number of single harvests of the same virus serotype into a single vessel. The filter used for this bulk should be able to retain cell debris.

The NRA may require further purification of harvests derived from continuous cell lines. However, if the harvests are derived from human diploid cells or monkey kidney cells, further purification is not required.

A.4.4.2 **Sampling**

Samples of the monovalent bulk prepared as described in section A.4.4.1 should be taken immediately, and if not tested immediately should be kept at -60 °C or below until the tests described in the following sections are performed.

A.4.4.3 **Identity test**

Each monovalent bulk should be identified as the appropriate poliovirus serotype by immunological assay on cell culture using specific antibodies, or by a molecular method that has been validated and approved by the NRA.

Neutralization tests can distinguish the serotype of polioviruses. Molecular methods, such as sequencing or deep sequencing, can distinguish Sabin virus from wild-type virus.

Care should be taken to ensure that the serum samples used are monospecific by titrating them against homotypic and heterotypic viruses of known virus titre. Monoclonal antibodies may be useful in this test.

A 4 4 4 Titration for virus content

The virus titre per millilitre of filtered monovalent bulk should be determined for cell cultures by comparing them with an existing reference preparation (see Appendix 4).

The virus titre as determined by this test should be the basis for the quantity of virus used in the neurovirulence tests in monkeys or in TgPVR mice (see Part A, section A.4.4.7.2), and for formulation of the final bulk (see Part A, section A.4.5).

The detailed procedures for carrying out this test and for interpreting the results should be approved by the NRA.

A.4.4.5 Sterility tests for bacteria and fungi

The final vaccine bulk should be tested for bacterial and fungal sterility as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances (40).

A.4.4.6 **Test for mycobacteria**

The virus harvest should be shown to be free from mycobacteria by an appropriate method approved by the NRA.

Molecular assays may be used as alternatives to microbiological culture tests for detecting mycobacteria after they have been validated and approved by the NRA (33).

A.4.4.7 Tests to monitor molecular characteristics of the virus (consistency)

The poliovirus in the filtered monovalent bulk, prepared as described in section A.4.4.1, should be compared with the seed lot or a reference virus preparation (see Part A, section A.1.3) to ensure that the vaccine virus has not undergone changes during its multiplication in the production cell culture.

A.4.4.7.1 Tests in vitro

The virus in the monovalent bulk should be tested by at least one in vitro test. The test used should be approved by the NRA. The MAPREC assay provides a sensitive and quantitative measure for consistency purposes. However, other assays are acceptable after they have been validated. Historically, the assay used tests the property of reproducing virus at temperatures of 36 °C and 40 °C in comparison with the seed lot or a reference virus preparation of poliovirus of the same type.

A.4.4.7.1.1 The MAPREC assay

The MAPREC assay is suitable for all three serotypes. Implementation of the assay should be fully validated by each manufacturer, and performed according to the WHO SOP for the MAPREC assay for oral poliovirus (Sabin) vaccine, which was developed from collaborative studies and is available from WHO,³ or according to a validated alternative procedure.

Once the test has been validated and normal values for the standards have been determined, the MAPREC assay should be used to establish the consistency of production. Depending on a laboratory's experience with the MAPREC test, an approach using "warning limits" of ± 2 standard deviations and "rejection limits" of ± 3 standard deviations may be appropriate. Acceptance and rejection criteria should be specific to each manufacturer and each working seed, and should be continually updated as each new bulk is prepared. An investigation of consistency should take place if a batch gives results that are inconsistent with previous production batches.

Results should be expressed as ratios relative to the type-specific International Standard for MAPREC analysis of poliovirus (Sabin). The acceptable variation of mutant content from batch to batch should be agreed with the NRA in light of experience with production and testing.

For type-3 OPV (with revertant 472-C), a batch should be rejected if the level of mutations is above 1.0% when normalized against the International Standard. The limits for type 1 and type 2 should be approved by the NRA.

Levels of mutations obtained by manufacturers who have implemented tests for type 1 and type 2 virus have been less than 2.0% for type-1 Sabin (for the sum of both mutations, 480-A and 525-C) and less than 1.5% for type-2 Sabin (481-G) (14).

Contact the Coordinator, Technologies, Standards and Norms, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).

If a filtered monovalent bulk fails a MAPREC assay, it cannot be used in the manufacturing of the finished product, and an evaluation of the manufacturing process, including the suitability of the virus working seed, should be undertaken and discussed with the NRA. Filtered monovalent bulks that pass the MAPREC assay should be tested subsequently for in vivo neurovirulence.

The assay for type-3 OPV is highly predictive of in vivo neurovirulence in animal models. No such correlation exists for type 1 and type 2 at the level of revertants present in vaccine bulks. For these types, the assay results provide a measure of consistency (14).

Nonradioactive methods for performing MAPREC analysis are available and may be introduced after being validated and approved by the NRA.

Alternative molecular biological methods that demonstrate an equivalent or better level of discrimination may be used after being validated and approved by the NRA.

A.4.4.7.1.2 Temperature sensitivity

The monovalent bulk may be tested for the property of reproducing at $36\,^{\circ}$ C and $40\,^{\circ}$ C in comparison with the seed lot or a reference virus preparation for the marker tests, and with appropriate rct/40– and rct40+ strains of poliovirus of the same type. The wild-type viruses (defined as field isolates or reference strains from polioviruses known or believed to have circulated persistently in the community), which are used as rct40+ controls in this test, should be maintained within the laboratory at progressively higher levels of containment in accordance with the GPEI global action plan and the timetable for the safe handling of WPVs. The incubation temperatures used in this test should be controlled to within $\pm 0.1\,^{\circ}$ C.

The monovalent bulk passes the test if, for both the virus in the monovalent bulk and that in the appropriate reference material, the titre determined at 36 °C is at least $5.0\log_{10}$ greater than that determined at 40 °C. If all of the titres obtained for the reference viruses are not in line with the expected values, the test should be repeated.

An additional specification that the virus titre must not exceed $10 \text{ CCID}_{50}/\text{ml}$ at the higher temperature may also be applied.

It is desirable that the temperatures used in the test should also include one in the region of $39.0\text{--}39.5\,^{\circ}\text{C}$, at which the titre of the reference material should be reduced by a factor in the range of $3.0\text{--}5.0\,\log_{10}$ of its value at $36\,^{\circ}\text{C}$. In one laboratory, a temperature of $39.2\,^{\circ}\text{C}$ was found to be suitable.

It is important to show that the behaviour of the monovalent bulk is comparable to that of the Sabin reference strain over a range of temperatures so that a more-accurate comparison can be made.

A.4.4.7.2 Neurovirulence tests

An appropriate in vivo test should be used to evaluate virus seeds and monovalent bulks. Summaries of the MNVT and TgmNVT, including pass and fail criteria, are given in Appendix 2, along with considerations on the choice of assay.

The test should be approved by the NRA for the specific product, and may use transgenic mice or nonhuman primates, or both. The test for neurovirulence in nonhuman primates should be carried out as summarized in Appendix 2 and described in the SOP on neurovirulence tests for types 1, 2 or 3 live-attenuated OPV in monkeys, available from WHO.⁴

Where the TgmNVT has been approved by the NRA, it should be carried out as summarized in Appendix 2 and described in detail in the SOP on neurovirulence tests for type 1, 2 or 3 live-attenuated OPV in transgenic mice susceptible to poliovirus, available from WHO.⁴ Its use for batch-release purposes should follow the appropriate validation and implementation processes, according to national and international regulations. This SOP has been validated for vaccines made from Behringwerke SO-derived seeds (type 1 and type 2) and RSO-derived seeds (type 3).

To qualify as competent to perform the TgmNVT test, there is a requirement for laboratories to complete a standard implementation process as detailed in the SOP. Once qualified as competent, each laboratory should continue to monitor its performance routinely.

A collaborative study organized by WHO demonstrated that the MNVT and TgmNVT are equivalent for testing vaccines prepared from RSO seeds, but lots prepared from derivative strains containing additional mutations may be found acceptable by the MNVT but fail the TgmNVT (26). Therefore, the TgmNVT can be used as a replacement for the MNVT for vaccines made from RSO Sabin 3 strain, but the TgmNVT may require further validation for other derivative strains. This validation may include developing an appropriate homologous reference.

A.4.5 Final bulk

Different final bulks can be formulated.

Final tOPV bulk, mOPV1 bulk, mOPV3 bulk and bOPV bulk (bOPV1+3) can be manufactured using a defined virus concentration of each component.

Contact the Coordinator, Technologies, Standards and Norms, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).

The operations necessary for preparing the final bulk should be conducted in such a manner as to avoid contaminating the product.

The dilution and mixing procedures involved in preparing the final vaccine bulk should be approved by the NRA.

A.4.5.1 Stabilizers

Any stabilizers that may be added to the final bulk should have been shown to the satisfaction of the NRA to improve the stability of the vaccine in the concentrations used, and not to impair the safety of the vaccine.

All of the tests described in Part A, sections A.4.3.3 and A.4.4 should be performed on samples taken before any stabilizers are added.

A.4.5.2 Sterility tests for bacteria and fungi

The final vaccine bulk should be tested for bacterial and fungal sterility, as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances (40).

A.5 Filling and containers

The requirements concerning filling and containers given in Good manufacturing practices for biological products (*37*) apply to vaccine filled in the final form.

Care should be taken that the material of which the container is made does not adversely affect the virus content of the vaccine under the recommended storage conditions.

A final filtration stage may be included just before the filling operations.

The manufacturer should provide the NRA with adequate data to prove that the product is stable under appropriate conditions of storage and shipping.

A.6 Control tests on final lot

Samples should be taken from each filling lot for the tests described in the following sections. The following tests should be performed on each final lot of vaccine (i.e. in the final containers). Unless otherwise justified and authorized, the tests should be performed on labelled containers taken from each final lot by means of validated methods approved by the NRA. The permissible limits for the different parameters listed under this section, unless otherwise specified, should be approved by the NRA.

A.6.1 Inspection of final containers

Every container in each final lot should be inspected visually or mechanically, and those showing abnormalities should be discarded.

A.6.1.1 Appearance

The appearance of the vaccine should be described with respect to its form and colour.

A.6.2 Extractable volume

Unless otherwise justified and authorized, the extractable volume (in ml) and the number of drops (using an approved dropper) should be determined in a minimum of five individual final containers.

A.6.3 **pH**

The pH of the final lot should be tested in a pool of final containers, and an appropriate limit set to guarantee virus stability.

A.6.4 **Identity**

Each final lot should be identified by immunological assay on cell culture using specific antibodies, or by a molecular method that has been validated and approved by the NRA.

Neutralization tests can distinguish the serotype of polioviruses. Molecular methods, such as sequencing or deep sequencing, can distinguish Sabin virus from wild-type virus.

Care should be taken to ensure that the serum samples used are monospecific by titrating them against homotypic and heterotypic viruses of known virus titre. Monoclonal antibodies may be used for this purpose.

A.6.5 Sterility tests for bacteria and fungi

Liquid vaccine should be tested for bacterial and fungal sterility, as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances (40), or by methods approved by the NRA.

A.6.6 **Potency**

At least three final containers should be selected at random from each final lot, and should be individually tested with a single assay. The poliovirus content of each serotype, and the total virus content, should be determined by assay as described in Appendix 4 of these Recommendations, using assays that include a reference preparation. When the vaccine contains more than one poliovirus type, each type should be titrated separately, using appropriate type-specific antiserum to neutralize each of the other types present. The NRA should specify the minimum virus titre per human dose.

An internal upper limit may be established by each manufacturer to monitor the consistency of production (e.g. based on the mean titre of the $CCID_{50}$ +3 standard deviations). The upper limit should be approved by the NRA.

It is recommended that as determined by assay described in Appendix 4, the estimated mean virus titres for a single human dose of tOPV should be: for type 1: not less than $10^{6.0}$ CCID₅₀; for type 2: not less than $10^{5.0}$ CCID₅₀; and for type 3: not less than $10^{5.5}$ CCID₅₀. The 95% confidence intervals for the assays should not differ by a factor of more than 0.3 \log_{10} from the estimated number of infectious units in the vaccine.

In 1986, the WHO Region of the Americas began using a trivalent formulation with $10^{5.8}$ CCID₅₀ of poliovirus type 3 (44) following a study in Brazil that demonstrated improved immunogenicity when the amount of type-3 virus in the trivalent vaccine was increased (45). The subsequent success in controlling poliomyelitis in the Americas using this formulation led the Global Advisory Group for the Expanded Programme on Immunization to recommend a formulation of tOPV for use worldwide with $10^{6.0}$ CCID₅₀ per dose for type 1, $10^{5.0}$ CCID₅₀ per dose for type 2, and $10^{5.8}$ CCID₅₀ per dose for type 3 (16, 46).

A.6.7 Thermal stability

Thermal stability should be considered as a vaccine characteristic that provides an indicator of the consistency of production. The thermal stability test is not designed to provide a predictive value of real-time stability but to evaluate whether the product complies with a defined specification. Additional guidance on the evaluation of vaccine stability is provided in WHO Guidelines on stability evaluation of vaccines (47).

Three final containers of the vaccine should be incubated at 37 °C for 48 hours. The total virus content in both exposed and unexposed containers should be determined concurrently with that of a suitable, validated reference preparation. For trivalent vaccines, the vaccine passes the test when the loss on exposure is not greater than a factor of $0.5 \log_{10} \text{CCID}_{50}$ per human dose.

Several OPV manufacturers have demonstrated that the thermal stability specification applied to tOPV formulations (loss on exposure is not greater than a factor of $0.5 \log_{10} \text{CCID}_{50}$ per human dose) is not applicable to some mOPVs and bOPVs. Some manufacturers have shown that mOPV formulations that failed to meet the specification of $0.5 \log_{10}$ have an acceptable stability profile throughout the product's shelf-life. Therefore, a specification of $0.6 \log_{10}$ has been accepted by NRAs and by the WHO prequalification programme on the basis of

documented evidence that mOPV1 is stable over two years when stored at -20 °C or below, and is stable for six months when stored at 2-8 °C.

A.6.8 Residual antibiotics (if applicable)

If any antibiotics are added during vaccine production, the content of the residual antibiotics should be determined and should be within limits approved by the NRA. This test may be omitted from routine lot release once the consistency of production has been established to the satisfaction of the NRA.

A.6.9 Stabilizer (if applicable)

If a stabilizer is added during vaccine production, the content of the stabilizer should be determined, and should be within limits approved by the NRA.

A.7 Records

The recommendations given in section 8 of Good manufacturing practices for biological products (37) apply.

A.8 Retained samples

The requirements given in section 9.5 of Good manufacturing practices for biological products (37) apply.

A.9 Labelling

The requirements given in section 7 of Good manufacturing practices for biological products (37) apply, but the following information should be added.

The label on the container or package should include:

- the designation(s) of the strain(s) of poliovirus contained in the vaccine:
- the minimum amount of each type of virus contained in one recommended human dose;
- the cell substrate used to prepare the vaccine, and the nature and amount of any stabilizer present in the vaccine;
- a statement that the vaccine is not to be injected;
- the number of doses in each vial;
- the volume of the dose.

It is desirable for the label to carry the names of both the producer and of the source of the bulk material if the producer of the final vaccine did not prepare it. The nature and amount of the antibiotics present in the vaccine, if any, may be included.

A.10 **Distribution and transport**

The requirements given in section 8 of Good manufacturing practices for biological products (37) apply. Further guidance is provided in WHO Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (48).

A.11 Stability, storage and expiry date

A.11.1 Stability testing

Adequate stability studies form an essential part of vaccine development. Guidance on the evaluation of vaccine stability is provided in WHO Guidelines on stability evaluation of vaccines (47). Stability testing should be performed at different stages of production, namely on single harvests, monovalent bulk, final bulk and final lot. Parameters that indicate stability should be defined or selected according to the stage of production. A shelf-life should be assigned to all in-process materials during vaccine production, particularly intermediates such as single harvests, monovalent bulk and final bulk.

The stability of the vaccine in its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRA on at least three consecutive lots of final product. Accelerated thermal stability tests may be undertaken to give additional information on the overall characteristics of a vaccine.

The formulation of vaccine should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with NRAs. Following licensure, continual monitoring of vaccine stability is recommended to support shelf-life specifications and to refine the stability profile (47). Data should be provided to the NRA in accordance with local requirements.

Where vaccine is to be stockpiled, manufacturers should conduct real-time stability studies on monovalent bulks at $-40\,^{\circ}$ C or below, or on finished monovalent, bivalent and trivalent compositions at $-20\,^{\circ}$ C.

Any extension of the shelf-life should be approved by the NRA.

The final stability testing programme should be approved by the NRA, and should include an agreed set of parameters, procedures for the continuing collection and sharing of data on stability, and criteria for the rejection of vaccines.

A.11.2 Storage conditions

Before being released by the manufacturer, all vaccines in final containers should be kept continuously frozen at a temperature below -20 °C.

The manufacturer should indicate the conditions for storage and shipping that will ensure the vaccine conforms to the requirements of potency until the expiry date stated on the label. These conditions must be approved by the NRA.

Although the recommended storage temperature is -20 °C, vaccine may be stored at 2-8 °C for six months. During shipment or in the field, the vaccine may be thawed and refrozen.

Manufacturers should demonstrate that multiple freeze—thaw cycles do not adversely affect the quality of the product. The number of freeze—thaw cycles permitted should be approved by the NRA.

The total storage period at 2–8 °C should not exceed six months. Stability data should be generated for each formulation of OPV to support storing the formulation at 2–8 °C following thawing, and these data should be approved by the NRA.

A.11.3 Expiry date

The expiry date should be based on the shelf-life, and should be supported by stability studies and approved by the NRA. The expiry date should relate to the date of filling or to the date of the first valid titration for virus content after filling (i.e. the date of the potency test), which should be performed as an assay of virus concentration as described in Appendix 4.

The label should specify only one storage temperature and expiry date.

Part B. Nonclinical evaluation of poliomyelitis vaccines (oral, live, attenuated)

The nonclinical evaluation of candidate poliomyelitis vaccines (oral, live, attenuated) should be based on the WHO guidelines on nonclinical evaluation of vaccines (34). In addition to the tests described in sections A.3.2.3 and A.3.2.4, the following specific issues should be considered in the context of a change in virus seed or manufacturing process for OPV.

8.1 Characterization of a new virus submaster seed from the WHO master seed

In the event that a new virus submaster seed is prepared by a single passage from the WHO master seed, it should be subjected to extensive characterization; this should include evaluation of the virus working seeds and at least three monovalent bulks derived from it, as described in section A.4.4.7. Characterization studies must include the evaluation of identity by complete nucleotide sequencing to prove that the new submaster seed consensus sequence is identical to conventional Sabin master seeds, and that the mutational composition is consistent (e.g. in a MAPREC assay). Massively parallel sequencing may also be undertaken to determine the distribution of mutants. These approaches have not yet been

formally validated, other than the MAPREC tests used for base positions in the 5′ noncoding region, which are described in section A.4.4.7.1.1. A new submaster seed should be tested for neurovirulence using the MNVT or the TgmNVT. Summaries of the MNVT and TgmNVT are given in Appendix 2, along with considerations on the choice of assay.

B.2 Characterization of virus working seeds from an established master seed where passage level between master seed and working seed is increased

The acceptable passage level of live polio vaccines relative to the original seeds is rigidly specified because there is evidence that for some seeds, increases in virulence have occurred with increases in passage. However, due to the limited stocks of master seeds, in the future it may be necessary for some manufacturers to prepare working seed lots by expanding current seed lots with an additional passage. Studies will be required that carefully compare new working seed lots with the previously approved working seed lot, and the new lots will need to meet the criteria outlined in sections A.3.2.3 and A.3.2.4. At least three monovalent bulks produced from the new virus working seed lot should also be tested and shown to meet the requirements of section A.4.4.7.

B.3 Characterization following changes in the manufacturing process

If the OPV manufacturing process is new or major changes are implemented in production – such as changing from primary monkey cells to cell lines – extensive assessment should be conducted to ensure that the mutational composition is not significantly altered by the new process. This evaluation may include the use of nucleotide sequencing and studies of mutant accumulation during passage in production cultures by MAPREC assay and other molecular methods, such as massively parallel sequencing. The new virus working seed lots will need to meet the criteria outlined in sections A.3.2.3 and A.3.2.4. In addition, at least three monovalent bulks produced from the new lots will need to be tested and shown to meet the requirements outlined in section A.4.4.7. In addition, clinical studies may be required, depending on the results of the genetic characterization and animal neurovirulence tests (see Part C).

Part C. Clinical evaluation of poliomyelitis vaccines (oral, live, attenuated)

Clinical trials should adhere to the principles described in the WHO Guidelines for good clinical practice for trials on pharmaceutical products (49) and

Guidelines on clinical evaluation of vaccines: regulatory expectations (35). All clinical trials should be approved by the relevant NRA.

Some of the issues that are specific to the clinical evaluation of OPVs are discussed in the following sections. These sections should be read in conjunction with the general guidance mentioned above. It is also recommended that manufacturers consult with the relevant NRAs regarding their overall clinical development programme.

Part C considers the provision of clinical data required for:

- new formulations based on licensed OPVs that are derived from Sabin poliovirus strains, including monovalent, bivalent and trivalent vaccines;
- situations where there have been major changes to the manufacturing process of an established vaccine (e.g. changing from primary monkey kidney cells to a cell line).

Clinical evaluation is not required for a vaccine manufactured using a new virus working seed lot, provided that the passage level is not more than one from the master seed lot, the working seed has been characterized, and the consistency of the manufacturing process has been demonstrated (see sections A.3.2.3 and A.3.2.4). Generating a new submaster seed requires extensive characterization but not clinical trials (see Part B).

Vaccine formulations containing one or two poliovirus serotypes have been licensed based on the findings from clinical trials in endemic countries. The results of clinical trials in Egypt and northern India have demonstrated that the efficacy of mOPV1 is superior to that of tOPV in terms of inducing immunity against poliovirus type 1 (16, 50). Health authorities have recommended widespread use of this vaccine to eliminate transmission of poliovirus type 1 in India. In addition, studies on bOPV containing type 1 and type 3 have demonstrated that bOPV is noninferior to mOPV1 and mOPV3 individually, and superior to tOPV. As a result of these findings, the Advisory Committee on Poliomyelitis Eradication recommended that bOPV should be used as a complement to tOPV in routine immunization programmes and to complement tOPV and mOPVs during supplementary immunization activities.

C1 General considerations

The Global Polio Eradication Initiative was prompted by World Health Assembly resolution WHA41.28 in 1988, and has led to a dramatic decrease in poliomyelitis cases globally (15). As a result, efficacy studies for poliovirus vaccines are not feasible, and clinical evaluations and seroprevalence studies should compare the safety and immunogenicity of candidate vaccines with a licensed vaccine (comparator vaccine). The assessment of seroconversion

should be based on the elicitation of neutralizing antibodies, which are the basis of protection (15). The approval of a candidate OPV should be based on a clear demonstration of noninferiority compared with licensed OPVs. The relative risk of VAPP for a new candidate vaccine when compared with approved vaccines cannot be estimated from pre-approval studies but should be addressed as part of post-marketing surveillance.

Safety and immunogenicity studies

C.2.1 Assessment of the immune response

A serum neutralizing antibody titre of 1/4–1/8 is considered to be a marker of protection against poliovirus (51). The demonstration of an immune response to OPV vaccination should be based on the pre-vaccination and post-vaccination measurement of neutralizing antibody titres. Geometric mean titres (GMTs), seroconversion rates and reverse cumulative distributions should be provided. Seroconversion for polio antigen is defined as:

- for subjects who are seronegative at the pre-vaccination time point, antibody titres above the cut-off titre (1/4-1/8);
- for subjects who are seropositive at the pre-vaccination time point, antibody titres that are four-fold above the expected titre for maternal antibodies (based on the pre-vaccination titre declining with a half-life of 28 days) (52);
- in populations with high antibody titres, a change from below the highest dilution tested (< 8192) to above the highest dilution tested (> 8192) will also indicate seroconversion.

WHO has made an effort to standardize polio virology methods, leading to the publication in 1990 of the Manual for the virological investigation of polio (53). It is recommended that a standardized technique for measuring neutralizing antibodies, involving standard cell lines and other standard reagents, should be used, such as International Standards for antipoliovirus sera for types 1, 2 and 3, and that the results should be expressed in IUs of neutralizing antibody (54, 55).

C.2.2 Immunogenicity studies

Candidate OPVs manufactured using different vaccine compositions (e.g. monovalent or bivalent) should be compared with a licensed formulation. Candidate vaccines should be compared with at least one well established and licensed OPV. The comparator vaccine or vaccines selected should have been in use for a number of years so that some data on effectiveness are available in addition to a reliable description of the safety profile.

C.2.3 Population

The evaluation of new OPV formulations based on Sabin strains, including monovalent, bivalent and trivalent vaccines, may be conducted in infants and neonates since safety profiles in these populations have already been established.

The study exclusion criteria should reflect contraindications to the administration of OPVs.

C.2.4 End-points and analyses

The clinical study protocol should state the primary objectives of the study. The neutralizing antibody response to the candidate vaccine should be demonstrated to be noninferior when compared with an appropriate, licensed OPV using primarily GMTs or seroconversion rates, or both. The primary end-point should be selected according to the study population and the anticipated immune response. For example, very high seroprevalence rates are expected in highly immunized populations, and this has implications for the selection of the noninferiority margin and therefore the calculation of the sample size. Further details on demonstrating noninferiority are given in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (35).

Other immunological parameters should be compared by using planned secondary analyses (e.g. percentages reaching predefined titres).

C.2.5 **Dose-ranging studies**

As of 2012, all licensed OPV formulations (monovalent, bivalent and trivalent) contained the recommended dose for each poliovirus type – namely, for type 1: not less than $10^{6.0}$ CCID₅₀; for type 2: not less than $10^{5.0}$ CCID₅₀; and for type 3: not less than $10^{5.5}$ CCID₅₀. However, the development of novel formulations with improved stability (owing to the addition of stabilizers or excipients) or immunogenicity (used in combination with an adjuvant) may require dose-ranging studies to determine the minimum dose of virus required in the CCID₅₀ to provide adequate immune responses (35). These data could also be used to support the derivation of the minimum viral titre that should be present in the vaccine at the end of its shelf-life.

C.2.6 Vaccine virus shedding and transmission

Although the primary objective of immunization with OPV is to provide individual protection against paralytic disease, information on virus shedding is desirable for a better understanding of the underlying biological mechanisms of protection. Therefore guidance is provided for optional studies on virus shedding.

As changes in vaccine composition may impact virus replication in the intestinal tract, and may influence the ability to induce an immune response

manufacturers may undertake studies to determine the profile of the vaccine virus (if applicable, by serotype) excreted in the stools of vaccinees, and the duration of shedding. Evaluation of the virus excretion of new vaccine formulations containing one, two or three serotypes (i.e. monovalent, bivalent or trivalent) should be compared, if done, with the licensed trivalent formulation (16).

For evaluation of modified strains (intentionally containing additional mutations compared to Sabin strains) then virus excretion studies may be required rather than being optional.

C.2.7 Challenge studies with attenuated Sabin poliovirus

Although the primary objective of immunization with OPV is to provide individual protection against paralytic disease, information on mucosal immunity is desirable for a better understanding of the underlying biological mechanisms of protection. Therefore guidance is provided for optional studies on mucosal immunity.

Induction of mucosal immunity by candidate and comparator vaccines may be determined by assessing virus excretion after administering a challenge dose of mOPV. Excretion of poliovirus in stool specimens is determined immediately before the challenge (day 0) and on days 7, 14, 21 and 28 thereafter (50).

For evaluation of modified strains (intentionally containing additional mutations compared to Sabin strains) then studies of mucosal immunity may be required rather than being optional.

C2.8 Concomitant administration with other vaccines

An evaluation of the effects of co-administration of an OPV with other vaccines should be considered, taking into account which vaccines are most likely to be given concomitantly in different age groups and populations.

When OPVs are used in the Expanded Programme on Immunization simultaneously with other vaccines, it is particularly important that the effects of co-administration should be evaluated (e.g. studies may evaluate co-administration with rotavirus vaccines, which are also administered by the oral route).

Immune responses to all other antigens co-administered with a new OPV should be measured at least in subsets. While a study will usually be powered only to demonstrate noninferiority with respect to neutralizing antibodies against the different poliovirus types used in the vaccine, the protocols should at least include planned secondary analyses of antigen-specific responses. If these analyses indicate that immune responses are lower on co-administration with a new OPV compared with a licensed vaccine, NRAs will need to consider the potential clinical consequences on a case by case basis.

C.2.9 Prelicensure safety data

The general approach to assessing the safety of a new OPV during clinical studies should follow WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (35). Planned safety studies should be supported by a clear, scientific rationale. Given the long history of the use of vaccines based on Sabin strains, an NRA may decide that additional prelicensure safety studies are not required. In cases in which a new vaccine formulation, which has not been used previously, is investigated, larger-scale studies will be needed.

An appropriate pharmacovigilance plan should be developed and approved by the NRA prior to licensure.

Post-marketing studies and surveillance

Enhanced safety surveillance, particularly for detecting VAPP, should be undertaken during the initial post-approval years in collaboration with NRAs. Manufacturers and health authorities should collaborate with the Global Polio Laboratory Network to monitor new vaccines once they are introduced into immunization programmes. These laboratories have extensive experience in poliovirus surveillance, and can provide excellent surveillance and post-marketing support.

The total duration of enhanced surveillance should be regularly reviewed by NRAs. If particular issues arise during prelicensure studies or during post-licensure safety surveillance, it may be necessary to conduct specific safety studies after licensure.

Part D. Recommendations for NRAs

D.1 General

The general recommendations for NRAs and NCLs given in the Guidelines for national authorities on quality assurance for biological products (56) and the Guidelines for independent lot release of vaccines by regulatory authorities (57) should apply.

The detailed production and control procedures, as well as any significant changes in them that may affect the quality, safety and efficacy of live-attenuated OPV, should be discussed with and approved by the NRA.

For control purposes, the International Standards currently in force should be obtained for the purpose of calibrating the national, regional and working standards (58). The NRA may obtain the product-specific working reference from the manufacturer and use this for lot release until the international standard preparation or national standard preparation has been established.

Only a monovalent bulk approved by the NRA with regard to the neurovirulence test can be used by the manufacturer for the formulation of a final bulk.

If the NCL does not perform the MNVT itself, it should carry out a second reading of the histological sections provided by the manufacturer for each monovalent bulk. In addition, the NCL should perform a second reading of at least four neurovirulence tests on the reference preparations using the MNVT in order to obtain the necessary baseline data for comparison with the neurovirulence of test vaccines.

The NCL should encourage the use of the standard form for reporting data on virus activity in the sections taken for histopathological examination.

If the NCL performs the TgmNVT itself, it should complete the standard implementation process.

If the NCL does not perform the TgmNVT, it should carry out a clinical scoring of mice in parallel with the manufacturer for each monovalent bulk at least at days 3 and 4, plus day 14. Moreover, once a year the injection of mice should be observed by the NCL. Only appropriately trained staff from a competent NCL can carry out a clinical scoring of mice in parallel with the manufacturer.

In one region of the world 1 in 10 bulks is also independently tested by an NCL certified as competent to carry out the test. Countries or other regions that implement the TgmNVT may wish to follow this approach.

Consistency of production has been recognized as an essential component in the quality assurance of live-attenuated OPV. In particular, NRAs should carefully monitor production records and quality control test results for clinical lots, as well as a series of consecutive lots of the vaccine.

D.2 Release and certification by the NRA

A vaccine should be released only if it fulfils all national requirements or satisfies Part A of these Recommendations, or both (*57*).

A protocol based on the model given in Appendix 5, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for release of the vaccine for use.

A statement signed by the appropriate official of the NRA should be provided to the manufacturing establishment, and should certify that the lot of vaccine in question meets all national requirements, as well as Part A of these Recommendations. The certificate should provide sufficient information about the vaccine lot. A model certificate is given in Appendix 6. The official national release certificate should be provided to importers of the vaccines. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

Part E. Recommendations for poliomyelitis vaccines (oral, live, attenuated) prepared in primary monkey kidney cells

The following additional or alternative recommendations are for OPV prepared in cultures of primary monkey kidney cells, and concern the testing of the cell substrate used for the production of the vaccine. They should therefore be added to – or used as an alternative to – the appropriate sections in Part A.4 as follows:

- sections E.1.1.1, E.1.3.1, E.1.4.1 and E.1.4.2 are additions to the corresponding Part A.4 sections as indicated below;
- sections E.1.2.1, E.1.2.2 and E.1.2.3 are replacements for the corresponding Part A.4 sections as indicated below.

All the other recommendations given in Parts A and B of this document are also applicable to this type of vaccine.

E.1 Control of vaccine production

E.1.1 Control of source materials

E.1.1.1 Monkeys used for preparation of kidney-cell cultures and for testing of virus

Addition to section A.4.1

If vaccine is prepared in monkey kidney-cell cultures, animals should be from a species approved by the NRA, and the animals should be in good health and not previously have been used for experimental purposes.

Manufacturers should use animals from closed or intensively monitored colonies.

The monkeys should be kept in well constructed and adequately ventilated animal rooms in cages separated in such a way as to prevent cross-infection among cages. Cage-mates should not be interchanged. The monkeys should be kept in the country where the vaccine will be manufactured in quarantine groups⁵ for a period of not less than six weeks before use. If at any time during the quarantine period the overall death rate of a shipment consisting of one or more groups reaches 5% (excluding deaths from accidents or where the cause was specifically determined not to be an infectious disease), monkeys from that entire shipment should continue in quarantine for a further period of not less than six weeks. The monkeys used should be free from infection. At the end of

A quarantine group is a colony of selected healthy monkeys kept in one room, with feeding and cleaning facilities separate from those of other groups; each group should have no contact with other monkeys during the quarantine period.

the extended quarantine period, and following thorough investigations, if any additional monkeys die from the same infectious disease, the entire group must be discarded from production.

The groups should be kept continuously in isolation, as in quarantine, even after completion of the quarantine period, until the monkeys are used. After the last monkey in a group has been taken, the room that housed the group should be thoroughly cleaned and decontaminated before being used for a new group.

In countries in which the kidneys from near-term monkeys are used, the mother should be quarantined for the term of pregnancy.

All actions taken by working personnel should be based on the assumption that a great potential hazard exists at all times in the quarantine area. Personnel should be provided with protective clothing, including gloves, footwear, and masks or visors. Street clothes should not be permitted to be worn in the rooms where the animals are kept. Smoking, eating and drinking should be forbidden while personnel are in the rooms where the animals are kept.

A supervisor should be responsible for reporting unusual illnesses among employees, and for ensuring that all injuries are properly treated. No worker who has cuts or abrasions on exposed areas of the body should enter the animal area. Any unexplained febrile illness, even one that occurs while off duty, should be considered as potentially related to the employee's occupation.

Monkeys from which kidneys are to be removed should be anaesthetized and thoroughly examined, particularly for evidence of tuberculosis and herpes B virus infection.

Before the preparation of a seed lot or vaccine, if a monkey shows any pathological lesion relevant to the use of its kidneys, the animal should not be used, nor should any of the remaining monkeys in the same quarantine group be used unless it is evident that their use will not impair the safety of the product.

All the operations described in this section should be conducted outside the areas where the vaccine is made.

The monkeys should be free from antibodies to SV40 and simian immunodeficiency virus.

It is desirable that kidney-cell cultures are derived from monkeys shown to be free from antibodies to foamy viruses. In some countries, monkeys are tested for antibodies to herpes B virus.

E.1.2 Production precautions

The general production precautions called for by Good manufacturing practices for biological products (37) apply to the manufacture of vaccines, with the addition of the following tests.

E.1.2.1 Monkey kidney-cell cultures for vaccine production

Replacement of section A.4.2.1 – in conjunction with section E.1.2.2 (below).

Cultures of monkey kidney cells should be prepared from kidneys that have no pathological signs. Virus for the preparation of vaccine should be grown by aseptic methods in such cultures. If animal serum is used in the propagation of the cells, the maintenance medium used after virus inoculation should contain no added serum.

To reduce animal use, the virus may be grown in serially passaged monkey kidney-cell cultures from primary monkey kidney cells.

Each group of cell cultures derived from a single monkey, or from no more than 10 near-term monkeys, should be prepared and tested as a group.

E.1.2.2 Tests of cell cultures used for vaccine production (see Appendix 7)

Replacement of section A.4.2.1 – in conjunction with section E.1.2.1 (above).

On the day of inoculation with virus working seed lot, each cell culture should be examined for degeneration caused by an infective agent. If during this examination evidence is found in a cell culture of any adventitious agent, the entire group of cultures should not be used for vaccine production.

On the day of inoculation with the virus working seed lot, a sample of at least 30 ml of the pooled fluid removed from the cell cultures of the kidneys of each single monkey, or from no more than 10 near-term monkeys, should be divided into two equal portions. One portion of the pooled fluid should be tested in monkey kidney-cell cultures prepared from the same species but not the same animal as that used for vaccine production. The other portion of the pooled fluid should be tested in kidney-cell cultures from another species of monkey, provided that the tests are done in cell cultures from at least one species known to be sensitive to SV40. The pooled fluid should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not fall below 1 part in 4. The area of the cell sheet should be at least 3 cm² per ml of pooled fluid. At least one bottle of each kind of cell culture should remain uninoculated to serve as a control.

When the monkey species used for vaccine production is known to be sensitive to SV40, a test in a second species may be omitted if the NRA approves.

Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody or other inhibitors, but the maintenance medium used after inoculation of the test material should contain no added serum except as described below.

The cultures should be incubated at 35–37 °C, and should be observed for at least four weeks. During this observation period, and after not less than two weeks' incubation, from each of these cultures at least one subculture of fluid should be made in the same tissue-culture system. The subculture should also be observed for at least two weeks.

Serum may be added to the original culture at the time of subculturing, provided that the serum does not contain SV40 antibody or other inhibitors. Immunochemical techniques may be useful for detecting SV40 and other viruses in the cells.

A further sample of at least 10 ml of the pooled fluid should be tested in rabbit kidney-cell cultures for the presence of herpes B virus and other viruses. Serum used in the nutrient medium of these cultures should be free from inhibitors. The sample should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not fall below 1 part in 4. The area of the cell sheet should be at least 3 cm² per ml of pooled fluid. At least one bottle of the cell cultures should remain uninoculated to serve as a control.

The cultures should be incubated at 35–37 °C, and should be observed for at least two weeks.

It is suggested that in addition to these tests, a further sample of 10 ml of pooled fluid removed from the cell cultures on the day of inoculation with the seed lot virus should be tested for the presence of adventitious agents by inoculation into cell cultures sensitive to measles virus.

For the tests to be valid, 20% or fewer of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the respective test periods.

If during these tests evidence is found of an adventitious agent, the single harvest from the whole group of cell cultures should not be used for vaccine production.

If the presence of herpes B virus is demonstrated, the manufacturing of the vaccine should be discontinued and the NRA should be informed. Manufacturing should not be resumed until a thorough investigation has been completed and precautions have been taken against any reappearance of the infection; manufacturing should be resumed only with the approval of the NRA.

⁶ Human herpesvirus (herpes simplex) has been used as an indicator of freedom from B-virus inhibitors because of the danger of handling herpes B virus.

If these tests are not carried out immediately, the samples of pooled cell-culture fluid should be kept at $-60\,^{\circ}\text{C}$ or below, with the exception of the sample for the test for herpes B virus, which may be held at $4\,^{\circ}\text{C}$, provided that the test is done not more than seven days after the sample has been taken.

E.1.2.3 Test of control cell cultures

Replacement of section A.4.1.

Cultures prepared on the day of inoculation with the virus working seed lot from 25% of the cell suspension (but not more than 2.5 litres of suspension) obtained from the kidneys of each single monkey, or from not more than 10 near-term monkeys, should remain uninoculated to serve as controls. These control cell cultures should be incubated under the same conditions as the inoculated cultures for at least two weeks, and should be examined during this period for evidence of cytopathic changes. For the tests to be valid, 20% or fewer of the control cultures should have been discarded for nonspecific, accidental reasons. At the end of the observation period, the control cultures should be examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section shows evidence in a control culture of any adventitious agent, the poliovirus grown in the corresponding inoculated cultures from the same group should not be used for vaccine production.

E.1.2.3.1 *Tests for haemadsorbing viruses*

At the time of harvest, or not more than four days after the day of inoculation of the production cultures with the virus working seed lot, a sample of 4% of the control cell cultures should be tested for haemadsorbing viruses. At the end of the observation period, the remaining control cultures should be similarly tested. The tests should be carried out as described in Part A, section A.4.1.2.

E.1.2.3.2 Tests for other adventitious agents

At the time of harvest, or no more than seven days after the day of inoculation of the production cultures with the virus working seed lot, a sample of at least 20 ml of the pooled fluid from each group of control cultures should be taken and tested in two kinds of monkey kidney-cell cultures as described in section E.1.2.2.

At the end of the observation period for the original control cultures, similar samples of the pooled fluid should be taken, and the tests referred to in this section in the two kinds of monkey kidney-cell cultures and in the rabbit-cell culture should be repeated as described in section E.1.2.2.

If the presence of herpes B virus is demonstrated, the production cell cultures should not be used, and the measures concerning vaccine production described in section E.1.2.2, should be taken.

In some countries, fluids are collected from the control cell cultures at the time of virus harvest and at the end of the observation period. Such fluids may then be pooled before being tested for adventitious agents.

E.1.3 Control of single harvests

Addition to section A.4.3.

E.1.3.1 Tests for neutralized single harvests in monkey kidney-cell cultures

A sample of at least 10 ml of each single harvest should be neutralized by type-specific poliomyelitis antiserum prepared from animals other than monkeys. In preparing antiserum for this purpose, the immunizing antigens used should be prepared in nonsimian cells.

Care should be taken to ensure that the antiserum used is monospecific. This may be demonstrated by titration of the antiserum against homotypic and heterotypic viruses of known virus titres, using the same dilution of the antiserum as that used for neutralization.

Half of the neutralized suspension (corresponding to at least 5 ml of a single harvest) should be tested in monkey kidney-cell cultures prepared from the same species but not the same animal as that used for vaccine production. The other half of the neutralized suspension should be tested in monkey kidney-cell cultures from another species, provided that the tests are done in cell cultures from at least one species known to be sensitive to SV40.

The neutralized suspensions should be inoculated into bottles of these cell cultures in such a way that the dilution of the suspension in the nutrient medium does not fall below 1 part in 4. The area of the cell sheet should be at least 3 cm² per ml of neutralized suspension. At least one bottle of each kind of cell culture should remain uninoculated to serve as a control; it should be maintained using nutrient medium containing the same concentration of the specific antiserum used for neutralization.

Animal serum may be used to propagate the cells provided that it does not contain inhibitors, but the maintenance medium used after the test material has been inoculated should not contain any added serum other than the poliovirus neutralizing antiserum, except as described below.

The cultures should be incubated at 35–37 °C, and should be observed for at least four weeks. During this observation period, and after no less than two weeks' incubation, at least one subculture of fluid should be made from each of these cultures in the same tissue-culture system. The subcultures should also be observed for at least two weeks.

Serum may be added to the original cultures at the time of subculturing, provided that the serum does not contain inhibitors. Immunohistochemical techniques may be useful for detecting SV40 and other viruses in the cells.

It is suggested that in addition to these tests, a further sample of the neutralized single harvest should be tested by inoculating 10 ml into human cell cultures sensitive to measles virus.

For the tests to be valid, 20% or fewer of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the respective test periods.

If any cytopathic changes occur in any of the cultures, the causes of these changes should be investigated. If the cytopathic changes are due to unneutralized poliovirus, the test should be repeated. If there is evidence of the presence of SV40 or other adventitious agents attributable to the single harvest, that single harvest should not be used for vaccine production.

E.1.4 Control of monovalent bulk

Addition to section A.4.

E.1.4.1 Monovalent bulk (before filtration)

E.1.4.1.1 Tests in rabbits

A sample of the monovalent bulk should be tested for the presence of herpes B virus and other viruses by injection into at least 10 healthy rabbits, each weighing between 1.5 kg and 2.5 kg. The sample should consist of at least 100 ml. Each rabbit should receive not less than 10 ml and not more than 20 ml, of which 1 ml is given intradermally at multiple sites and the remainder is given subcutaneously. The rabbits should be observed for between three and five weeks for signs of illness or death.

It is suggested that the sample should consist of at least 1% of monovalent bulk, provided that this is not less than 100 ml and is not more than 500 ml.

All rabbits that die after the first 24 hours of the test should be examined by necropsy, with the brain and organs removed for detailed examination to establish the cause of death. Animals showing signs of illness should be humanely killed and subjected to a similar necropsy.

The monovalent bulk passes the test if 20% or fewer of the inoculated rabbits show signs of intercurrent infection during the observation period, and if none of the rabbits shows evidence of infection with herpes B virus or other adventitious agents, or lesions of any kind attributable to the bulk suspension.

If the presence of herpes B virus is demonstrated, the measures concerning vaccine production described in section E.1.2.2, should be taken.

A test for the presence of Marburg virus may be carried out in guinea-pigs.

E.1.4.2 Monovalent bulk (after filtration) – tests for retroviruses

Test samples from the filtered monovalent bulk should be examined for the presence of retroviruses by an assay for reverse transcriptase that has been approved by the NRA (36).

Authors and acknowledgements

The first draft of these Recommendations was prepared by Dr M. Ferguson, England; Dr P. Minor, National Institute for Biological Standards and Control, England; Dr K. Chumakov, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr M. Baca-Estrada, Health Canada, Canada; and Ms V. Pithon, Agence Française de Sécurité Sanitaire des Produits de Santé, France; with support from the World Health Organization Secretariat including Dr J. Fournier-Caruana, Dr I. Knezevic, Dr D.J. Wood, Dr T.Q. Zhou (all from the Department of Immunization, Vaccines and Biologicals), and Dr R. Sutter (from the Global Polio Eradication Initiative), Switzerland.

The first draft took into consideration the discussions held at a working group meeting - Technical specifications for manufacturing and evaluating the WHO Recommendations for OPV: TRS Nos. 904 and 910 - held in Geneva. Switzerland 20-22 July 2010 and attended by Ms I.S. Budiharto and Mr A. Azhari, BioFarma, Indonesia (representing the Developing Countries Vaccine Manufacturers Network); Dr E. Coppens, Sanofi Pasteur, France (representing the International Federation of Pharmaceutical Manufacturers and Associations); Dr E. Dragunsky and Dr K. Chumakov, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr I. Ernest and Dr B. Descambe, GlaxoSmithKline Biologicals, Belgium (representing the International Federation of Pharmaceutical Manufacturers and Associations); Dr L. Fiore and Dr A.L. Salvati, Istituto Superiore di Sanità, Italy; Dr L. Herawati and Dr D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Mr D. Mattii and Mr T. Pasquali, Novartis Vaccines and Diagnostics, Italy (representing the International Federation of Pharmaceutical Manufacturers and Associations); Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, France; Dr P. Minor, Dr J. Martin, Dr A. MacAdam, Dr G. Dunn and Dr A. Heath, National Institute for Biological Standards and Control, England; Dr N. Nathanson, University of Pennsylvania, USA; Professor A. Nomoto, Microbial Chemistry Research Foundation, Japan; Ms V. Pithon, Agence

Française de Sécurité Sanitaire de Produits de Santé, France; Dr R.C. Rosales and Dr J.B. González, Birmex, Mexico (representing the Developing Countries Vaccine Manufacturers Network); Dr G. Waeterloos, Scientific Institute of Public Health, Belgium; Dr E. Wimmer, State University of New York, USA; Dr L.Y. Yuan, National Institute for the Control of Pharmaceutical and Biological Products, China; with support from the World Health Organization Secretariat including Dr M. Baca-Estrada, Dr C. Conrad, Dr J. Fournier-Caruana, Dr I. Knezevic, Dr D.J. Wood and Dr T.Q. Zhou (all from the Department of Immunization, Vaccines and Biologicals), and Dr R. Sutter (from the Global Polio Eradication Initiative), Switzerland.

A second draft was prepared following a meeting of the drafting group attended by Dr M. Ferguson, England; Dr P. Minor, National Institute for Biological Standards and Control, England; Dr K. Chumakov, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr M. Baca-Estrada, Health Canada, Canada; Ms V. Pithon, Agence Française de Sécurité Sanitaire des Produits de Santé, France; with support from the World Health Organization Secretariat including Dr J. Fournier-Caruana, Dr I. Knezevic, Dr D.J. Wood, Dr T.Q. Zhou (all from the Department of Immunization, Vaccines and Biologicals), Switzerland.

The following individuals provided comments on the second draft during a WHO consultation process that took place from 26 July to 15 September 2011: Dr W.A.M. Bakker and Dr M van Oijen, National Institute of Public Health and the Environment, the Netherlands: Ms I.S. Budiharto, BioFarma, Indonesia: Dr E. Coppens, Sanofi Pasteur, France; Dr L. Fiore and Dr A.L. Salvati, Istituto Superiore di Sanità, Italy; Dr J. Martin, National Institute for Biological Standards and Control, England; Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, France; Ms V. Pithon and Dr S. Morgeaux, Agence Française de Sécurité Sanitaire des Produits de Santé,⁷ France; Dr Y. Tano, Japan Poliomyelitis Research Institute, Japan; Dr M.J. Uribe Serralde, Dr A.M. Vionet and Dr J.B. Gonzalez, Birmex, Mexico; Dr G. Waeterloos, Scientific Institute of Public Health, Belgium; Dr H. Wang, Beijing Tiantan Biological Products Company, China; Dr L.Y. Yuan, National Institute for the Control of Pharmaceutical and Biological Products, China; with Dr L.A. Bigger and Ms O. Morin who coordinated and compiled comments from the Vaccines Committee of the International Federation of Pharmaceutical Manufacturers and Associations, Switzerland.

A third draft was then prepared by Dr M. Ferguson, England, and Dr T.Q. Zhou, Department of Immunization, Vaccines and Biologicals, World

On 1 May 2012 the tasks and duties of this agency were subsumed into the activities of the Agence Nationale de Sécurité du Médicament et des Produits de Santé.

Health Organization, Switzerland, with input from Dr J. Martin, Dr G. Cooper and Dr G. Dunn, National Institute for Biological Standards and Control, England; and the drafting group members, which included: Dr M. Baca-Estrada, Health Canada, Canada; Dr K. Chumakov, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr P. Minor, National Institute for Biological Standards and Control, England; and Ms V. Pithon, Agence Française de Sécurité Sanitaire des Produits de Santé, France; along with Dr J. Fournier-Caruana, Department of Immunization, Vaccines and Biologicals, and Dr R. Sutter, Global Polio Eradication Initiative, World Health Organization, Switzerland.

A fourth draft was prepared by Dr M. Ferguson, Norfolk, England, and Dr T.Q. Zhou, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland, following comments received during a WHOsponsored public consultation between November 2011 and February 2012. Comments were received from Mr A. Azhari, BioFarma, Indonesia; Dr L.A. Bigger, who coordinated and compiled comments from the Vaccines Committee of the International Federation of Pharmaceutical Manufacturers and Associations, Switzerland; Dr L. Fiore, Istituto Superiore di Sanità, Italy; Dr J. Fournier-Caruana, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland; Dr E. Leal, Fiocruz, on behalf of the National Institute of Quality Control in Health, Brazil; Dr A. Lopez, Laboratorios de Biológicos y Reactivos de México, Mexico; Dr S. Morgeaux, Agence Française de Sécurité Sanitaire des Produits de Santé, France; Dr S.R. Pakzad, Food and Drug Control Laboratory, Islamic Republic of Iran; Ms V. Pithon, Agence Française de Sécurité Sanitaire des Produits de Santé, France; Dr A.L. Salvati, Istituto Superiore di Sanità, Italy; Dr Y. Tano, Japan Poliomyelitis Research Institute, Japan; and Dr G. Waeterloos, Scientific Institute of Public Health, Belgium.

The fifth draft was prepared by Dr M. Ferguson, England and Dr T.Q. Zhou, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland, taking into consideration comments received on the fourth draft during a WHO-sponsored consultation held from 27–29 March 2012 and attended by Dr S. Abe, Japan Poliomyelitis Research Institute, Japan; Dr M. Baca-Estrada, Health Canada, Canada; Dr W.A.M. Bakker, National Institute for Public Health and the Environment, the Netherlands; Dr J. Fournier-Caruana, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland; Mr B.S. Chauhan, Bharat Biotech International, India; Dr K. Chumakov, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr E. Coppens, Sanofi Pasteur, France; Dr M. Duchêne, GlaxoSmithKline Biologicals, Belgium; Ms G. Dunn, National Institute for Biological Standards and Control, England; Dr D. Felnerova, Crucell, Switzerland; Dr M. Ferguson, England; Dr L. Fiore, Istituto Superiore di Sanità,

Italy; Mr J.B. González, Laboratorios de Biológicos y Reactivos de México, Mexico; Dr M.A. González, Federal Commission for the Protection from Sanitary Risks, Mexico; Professor V. Grachev, Russian Academy of Medical Sciences, Russian Federation; Mrs T. Jivapaisarnpong, Ministry of Public Health, Thailand; Dr I. Knezevic, Department of Immunization, Vaccines, and Biologicals, World Health Organization, Switzerland; Dr D. Kusmiaty, Directorate General of Drug and Food Control, Ministry of Health, Indonesia; Dr K. Katayama, National Institute of Infectious Diseases, Japan; Dr C.G. Li, National Institutes for Food and Drug Control, China; Dr J. Martin, National Institute for Biological Standards and Control, England; Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, France; Dr R. Modi, Cadila Pharmaceuticals, India; Ms E. Niogret, Sanofi Pasteur, France; Dr H. Okayasu, Research, Policy and Product Development, World Health Organization, Switzerland; Dr L.V. Phung, National Institute for Control of Vaccine and Biologicals, Viet Nam; Ms V. Pithon, Agence Française de Sécurité Sanitaire des Produits de Santé, France; Dr A. Sinyugina, Federal State Unitary Enterprise of Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russian Federation; Dr R. Sutter, Research, Policy and Product Development, World Health Organization, Switzerland; Mr D. Ugiyadi, BioFarma, Indonesia; Dr G. Waeterloos, Scientific Institute of Public Health, Belgium; Ms H. Wang, Tiantan Biological Products Company, China; Dr S. Yamazaki, Japan Poliomyelitis Research Institute, Japan; Mr L. Yi, Kunming Institute of Medical Biology, China; and Dr T.Q. Zhou, Immunization, Vaccines and Biologicals, World Health Organization, Switzerland.

The final draft document was prepared by Dr M. Ferguson, England and Dr T.Q. Zhou, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland following comments received on the fifth draft from Mr B.S. Chauhan, Bharat Biotech International, India; Dr K. Chumakov, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr E. Coppens, Sanofi Pasteur, France; Dr C. Dubeaux, GlaxoSmithKline Biologicals, Belgium; Dr J. Fournier-Caruana, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland; Dr M.A. González, Ministry of Health, Mexico; Dr C.G. Li, National Institutes for Food and Drug Control, China; Dr P. Minor, Dr A. Heath, Ms G. Dunn and Ms G. Cooper, National Institute for Biological Standards and Control, England; Dr V. Pithon, Agence Nationale de Sécurité du Médicament et des Produits de Santé, France; Dr D. Ugiyadi, BioFarma, Indonesia; Dr E. Vitkova, European Directorate for the Quality of Medicines & HealthCare, France; and Dr S.Yamazaki, Japan Poliomyelitis Research Institute, Japan.

Further changes were then made to document WHO/BS/2012.2185 by the Expert Committee on Biological Standardization, resulting in the present document.

The following individuals responded to a WHO survey on OPV seeds and quality-control information conducted during 2011–2012: Dr P. Amerlynck, GlaxoSmithKline Biologicals, Belgium; Mr A. Azhari, BioFarma, Indonesia; Dr W.A.M. Bakker and Dr M. van Oijen, National Institute of Public Health and the Environment, the Netherlands; Dr E. Coppens, Sanofi Pasteur, France; Professor V. Grachev, Dr A. Sinyugina and Dr A. Malkin, Federal State Unitary Enterprise of Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russian Federation; Professor N.D. Hien, Center for Research and Production of Vaccines and Biologicals, Viet Nam; Dr Q.H. Li, Kunming Institute of Medical Biology, China; Mr A. Mohammadi and Mr A. Zand, Razi Vaccine and Serum Research Institute, Islamic Republic of Iran; Dr M. Li, China National Biotec Group, China; Mr T. Pasquali, Novartis Vaccines and Diagnostics, Italy; Dr R.C. Rosales and Dr J.B. González, Birmex, Mexico; Dr S.V. Shankarwar, Haffkine Bio Pharmaceutical Corporation, India; Dr Y. Tano, Japan Poliomyelitis Research Institute, Japan; and Dr A. Vidmanić, Institute of Virology, Serbia.

References

- Requirements for Poliomyelitis Vaccine (oral) (Requirements for Biological Substances, No. 7).
 In: Requirements for Biological Substances. Report of a Study Group. Geneva, World Health Organization, 1962 (WHO Technical Report Series, No. 237), Annex.
- Requirements for poliomyelitis vaccine (oral) (Requirements for Biological Substances, No. 7): revised 1965. In: Requirements for Biological Substances. Manufacturing establishments and control laboratories – poliomyelitis vaccine (inactivated) – poliomyelitis vaccine (oral) – smallpox vaccine. Revised 1965. Report of a WHO Expert Group. Geneva, World Health Organization, 1966 (WHO Technical Report Series, No. 323), Annex 3.
- Requirements for poliomyelitis vaccine (oral). In: WHO Expert Committee on Biological Standardization. Twenty-fourth report. Geneva, World Health Organization, 1972 (WHO Technical Report Series, No. 486), Annex 1.
- Requirements for poliomyelitis vaccine (oral) (Revised 1982). In: WHO Expert Committee on Biological Standardization. Thirty-third report. Geneva, World Health Organization, 1983 (WHO Technical Report Series, No. 687), Annex 4.
- Requirements for poliomyelitis vaccine (oral) (revised 1989). In: WHO Expert Committee on Biological Standardization. Fortieth report. Geneva, World Health Organization, 1990 (WHO Technical Report Series, No. 800), Annex 1.
- 6. Requirements for continuous cell lines used for biologicals production. In: *WHO Expert Committee on Biological Standardization*. *Thirty-sixth report*. Geneva, World Health Organization, 1987 (WHO Technical Report Series, No. 745), Annex 3.
- 7. Acceptability of cell substrates for production of biologicals. Report of a WHO Study Group. Geneva, World Health Organization, 1987 (WHO Technical Report Series, No. 747) (apps.who.int/iris/bitstream/10665/38501/1/WHO_TRS_747.pdf, accessed 20 June 2013).
- 8. 1990 Requirements for poliomyelitis vaccine (oral) (Addendum 1998). In: *WHO Expert Committee on Biological Standardization. Forty-ninth report*. Geneva, World Health Organization, 2000 (WHO Technical Report Series, No. 897), Annex 2.

- 9. WHO global action plan to minimize poliovirus facility-associated risk after eradication of wild polioviruses and cessation of routine OPV use. Draft 2009. Geneva, World Health Organization, 2009 (http://www.polioeradication.org/Portals/0/Document/Resources/PostEradication/GAP3 2009.pdf, accessed 20 June 2013).
- Recommendations for the production and control of poliomyelitis vaccine (oral). In: WHO Expert Committee on Biological Standardization. Fiftieth report. Geneva, World Health Organization, 2002 (WHO Technical Report Series, No. 904), Annex 1.
- 11. Working group on transgenic mice as animal model for neurovirulence test of OPV, Geneva, 28–30 June 1999. Geneva, World Health Organization (unpublished document BS/99.1908; available on request from the Department of Quality, Safety and Biologicals, World Health Organization, 1211 Geneva 27, Switzerland).
- 12. Use of the MAPREC assay in the quality control of oral poliovirus vaccine. Report of a WHO working group. Geneva, 1–2 July 1999. Geneva, World Health Organization (unpublished document BS/99.1909; available on request from the Department of Quality, Safety and Biologicals, World Health Organization, 1211 Geneva 27, Switzerland).
- 13. Recommendations for the production and control of poliomyelitis vaccine (oral) (revised, Addendum 2000). In: *WHO Expert Committee on Biological Standardization. Fifty-first report*. Geneva, World Health Organization, 2002 (WHO Technical Report Series, No. 910), Annex 1.
- 14. WHO Working Group Meeting to Discuss the Revision of the WHO Recommendations for OPV: TRS No. 904 and 910. Geneva, Switzerland 20–22 July 2010. Geneva, World Health Organization, 2010 (http://www.who.int/biologicals/vaccines/OPV_Meeting_report_Final_Clean_13May2011.pdf, accessed 20 June 2013).
- 15. Polio vaccines and polio immunization in the pre-eradication era: WHO position paper. *Weekly Epidemiological Record*, 2010, 85:213–228.
- Sutter RW et al. Immunogenicity of bivalent types 1 and 3 oral poliovirus vaccine: a randomised, double-blind, controlled trial. *Lancet*, 2010, 376:1682–1688.
- 17. Risk assessment: frequency and burden of VAPP, cVDPV and iVDPV. In: *Report of the interim meeting of the Technical Consultative Group (TCG) on the Global Eradication of Poliomyelitis: Geneva, 13–14 November 2002*. Geneva, World Health Organization, 2002.
- 18. Kew OM et al. Vaccine-derived polioviruses and the endgame strategy for global polio eradication. *Annual Review of Microbiology*, 2005, 59:587–635.
- 19. Cockburn WC. The work of the WHO Consultative Group on Poliomyelitis Vaccines. *Bulletin of the World Health Organization*, 1988, 66:143–154.
- 20. Sabin AB, Boulger L. History of Sabin attenuated poliovirus oral live vaccine strains. *Journal of Biological Standardization*, 1973, 1:115–118.
- 21. Stones PB et al. *Preparation and properties of a derivative of Sabin's type 3 poliovirus strain Leon 12a,b*. 10th Symposium of the European Association against Poliomyelitis and Allied Diseases, 1964, 10:390–397.
- 22. Rezapkin GV et al. Reevaluation of nucleotide sequences of wild-type and attenuated polioviruses of type 3. *Virus Research*, 1999, 65:111–119.
- 23. Furesz J, Contreras G. Some aspects of the monkey neurovirulence test used for the assessment of oral poliovirus vaccines. *Developments in Biological Standardization*, 1993, 78:61–70.
- 24. Ren R et al. Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis. *Cell*, 1990, 63:353–362.
- 25. Koike S et al. Transgenic mice susceptible to poliovirus. *Proceedings of the National Academy of Sciences of the United States of America*, 1991, 88:951–955.

- 26. Dragunsky E et al. Transgenic mice as an alternative to monkeys for neurovirulence testing of live oral poliovirus vaccine: validation by a WHO collaborative study. *Bulletin of the World Health Organization*, 2003, 81:251–260.
- 27. Maintenance and distribution of transgenic mice susceptible to human viruses: memorandum from a WHO meeting. *Bulletin of the World Health Organization*, 1993, 71:493–502.
- Wood DJ, Macadam AJ. Laboratory tests for live attenuated poliovirus vaccines. *Biologicals*, 1997, 25:3–15.
- 29. Chumakov KM et al. Correlation between amount of virus with altered nucleotide sequence and the monkey test for acceptability of oral poliovirus vaccine. *Proceedings of the National Academy of Sciences of the United States of America*, 1991, 88:199–203.
- Chumakov K et al. Assessment of the viral RNA sequence heterogeneity for control of OPV neurovirulence. *Developments in Biological Standardization*, 1993, 78:79–89.
- Israel MA et al. Biological activity of polyoma viral DNA in mice and hamsters. *Journal of Virology*, 1979, 29:990–996.
- 32. Lebron JA et al. Adaptation of the WHO guideline for residual DNA in parenteral vaccines produced on continuous cell lines to a limit for oral vaccines. *Developments in Biologicals*, 2006, 123:35–44.
- Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture
 of biological medicinal products and for the characterization of cell banks. In: WHO Expert
 Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization,
 2013 (WHO Technical Report Series, No. 978), Annex 3.
- 34. WHO guidelines on nonclinical evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-fourth report. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.
- Guidelines on clinical evaluation of vaccines: regulatory expectations. In: WHO Expert Committee on Biological Standardization. Fifty-second report. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 924), Annex 1.
- 36. Good manufacturing practices: main principles for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report.* Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 3.
- 37. Good manufacturing practices for biological products. In: WHO Expert Committee on Biological Standardization. Forty-second report. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 1.
- Recommendations to assure the quality, safety and efficacy of BCG vaccines. Replacement of Annex 2 of WHO Technical Report Series, No. 745, and Amendment to Annex 12 of WHO Technical Report Series, No. 771. In: WHO Expert Committee on Biological Standardization. Sixty-second report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 979), Annex 3.
- 39. Requirements for the use of animal cells as in vitro substrates for the production of biologicals (Addendum 2003). In: *WHO Expert Committee on Biological Standardization. Fifty-fourth report*. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 4.
- 40. General requirements for the sterility of biological substances (Revised 1973). In: *WHO Expert Committee on Biological Standardization. Twenty-fifth report*. Geneva, World Health Organization, 1973 (WHO Technical Report Series, No. 530), Annex 4.
- 41. General requirements for the sterility of biological substances. (Requirements for Biological Substances No. 6, revised 1973, amendment 1995). In: *WHO Expert Committee on Biological Standardization. Forty-sixth report*. Geneva, World Health Organization, 1998 (WHO Technical Report Series, No. 872), Annex 3.

- 42. WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products. Geneva, World Health Organization, 2003 (WHO/BCT/QSD/03.01) (http://www.who.int/biologicals/publications/en/whotse2003.pdf, accessed 22 June 2013).
- 43. Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (revised 1992). In: WHO Expert Committee on Biological Standardization. Forty-third report. Geneva, World Health Organization, 1994 (WHO Technical Report Series, No. 840), Annex 2.
- 44. de Quadros CA et al. Eradication of poliomyelitis: progress in the Americas. *Pediatric Infectious Disease Journal*, 1991, 10:222–229.
- 45. Patriarca PA et al. Randomised trial of alternative formulations of oral poliovaccine in Brazil. *Lancet*, 1988, 331:429–443.
- 46. Expanded Programme on Immunization: Global Advisory Group. *Weekly Epidemiological Record*, 1991, 66:3–7 (Part I); and 9–12 (Part II).
- 47. Guidelines on stability evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-seventh report. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 962), Annex 3.
- 48. Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (jointly with the Expert Committee on Biological Standardization). In: WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 9.
- 49. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products. In: *The use of essential drugs. Sixth report of the WHO Expert Committee.* Geneva, World Health Organization, 1995 (WHO Technical Report Series, No. 850), Annex 3.
- 50. el-Sayed N et al. Monovalent type 1 oral poliovirus vaccine in newborns. *New England Journal of Medicine*, 2008, 359:1655–1665.
- 51. Plotkin SA, Vidor E. Poliovirus vaccine-inactivated. In: Plotkin SA, Orenstein WA, Offit PA, eds. *Vaccines*, 5th ed. London, Elsevier, 2008.
- 52. Cohen A et al. Seroresponse to trivalent oral poliovirus vaccine as a function of interval. *Pediatric Infectious Disease Journal*, 1995, 14:100–106.
- 53. *Manual for the virological investigation of polio*. Geneva, World Health Organization 1997 (http://whqlibdoc.who.int/hq/1997/WHO_EPI_GEN_97.01.pdf, accessed 20 June 2013).
- 54. Wood DJ, Heath AB. The second international standard for anti-poliovirus sera types 1, 2 and 3. *Biologicals*, 1992, 30:203–211.
- 55. The Immunological Basis for Immunization Series. Module 6: Poliomyelitis. Geneva, World Health Organization, 1993 (http://whqlibdoc.who.int/hq/1993/WHO_EPI_GEN_93.16_mod6.pdf, accessed 20 June 2013).
- 56. Guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report.* Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 2.
- 57. Guidelines for independent lot release of vaccines by regulatory authorities. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 2.
- 58. WHO manual for the establishment of national and other secondary standards for vaccines. Geneva, World Health Organization, 2011 (WHO/IVB/11.03) (http://whqlibdoc.who.int/hq/2011/WHO_IVB_11.03_eng.pdf, accessed 20 June 2013).

Overview of virus seeds used in OPV production

The history of the poliovirus strains used in the production of OPV is well documented (1-3). This appendix gives an overview of the virus seeds currently used in OPV production.

The flow diagrams shown in Figures 2.1 and 2.2 summarize the history of seed virus and reference materials used to manufacture OPV from Sabin 1 and Sabin 2 (Figure 2.1) and Sabin 3 (Figure 2.2) strains. Concentric circles indicate progressive virus passages made to prepare master seed stocks, working seed stocks and production lots of vaccine. Where relevant, submaster seed stocks are identified in the footnotes. Different seed viruses are identified as SO (Sabin original), SOM (Merck stock of SO), SOB (Behringwerke stock of SO), Pfizer (otherwise known as rederived SO, or RSO), SOJ (Japanese stock of SO) and SOR (Russian stock of SO).

These figures provide only a historical overview of the use of different seeds derived from the Sabin vaccine strain in OPV production (as of June 2012). They do not indicate any WHO "qualification" or "approval" of the strains or vaccines in the context of this document.

Figure 2.1
History of seed virus and reference materials used to produce type 1 and type 2 OPV from Sabin 1 and Sabin 2

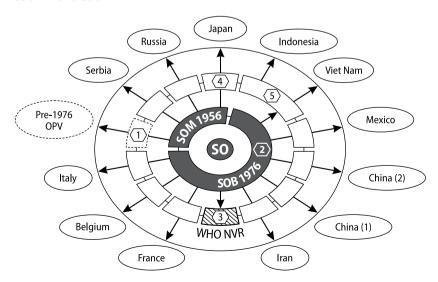
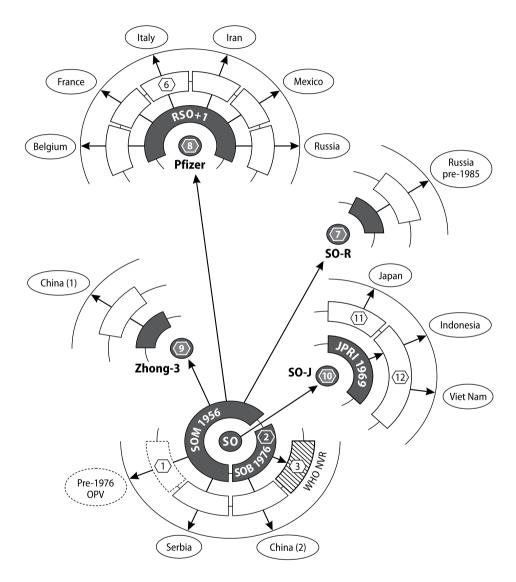


Figure 2.2 History of seed virus and reference materials used to produce type 3 OPV from Sabin 3



Manufacturers corresponding to the countries shown in Figure 2.1 and Figure 2.2

Belgium GlaxoSmithKline Biologicals

China (1) Institute of Medical Biology, Kunming

China (2) China National Biotec Group,

Beijing Tiantan Biological Products Company

France Sanofi Aventis Indonesia PT BioFarma

Islamic Republic of Iran Razi Vaccine and Serum Research Institute

Italy Novartis Vaccines

Japan Poliomyelitis Research Institute

Mexico Biologics and Reagents Laboratories of Mexico

Russian Federation Federal State Unitary Enterprise of Chumakov

Institute of Poliomyelitis and Viral Encephalitides

Serbia Torlak Institute of Virology, Vaccines and Serum

Viet Nam Center for Research and Production of Vaccines

and Biologicals

Notes 1–12 shown in Figure 2.1 and Figure 2.2

- 1. Working seeds were produced by different manufacturers before 1976.
- 2. WHO master seed stock.
- 3. WHO neurovirulence reference preparation.
- 4. Type-1 seed stock prepared at Japan Poliomyelitis Research Institute by four passages of SOM, including three terminal dilution passages (passage level SO+5).
- 5. Type-2 seed stock prepared at Japan Poliomyelitis Research Institute by one passage of SOM (SO+2). Seed stock prepared at Japan Poliomyelitis Research Institute by one passage of SOB (SO+2).
- 6. Novartis performed an additional passage to prepare submaster seed stock from which a working seed was produced.
- 7. In the Russian Federation, six plaques were selected, pooled, and grown to produce seed stock.

- 8. Pfizer (RSO): rederived Sabin original, produced by RNA plaque purification, passage.
- 9. Zhong-3: plaque purification, passage.
- 10. Produced by Japan Poliomyelitis Research Institute in 1969 from SO stock by one passage (SO+1).
- 11. Prepared from SOJ by passages in AGMK cells (SOJ+9), including two plaque purifications and three terminal passages (SO+10).
- 12. Prepared from SOJ by passages in AGMK cells (SOJ+6), including two plaque purifications (SO+7).

References

- Cockburn WC. The work of the WHO Consultative Group on Poliomyelitis Vaccines. Bulletin of the World Health Organization, 1988, 66:143–154.
- 2. Sabin AB, Boulger L. History of Sabin attenuated poliovirus oral live vaccine strains. *Journal of Biological Standardization*, 1973, 1:115–118.
- 3. Stones PB et al. *Preparation and properties of a derivative of Sabin's type 3 poliovirus strain Leon 12a,b.* 10th Symposium of the European Association against Poliomyelitis and Allied Diseases, 1964, 10:390–397.

In vivo tests for neurovirulence, and considerations in relation to assay choice

Live-attenuated vaccines were developed by Sabin in large part by using nonhuman primates, particularly Old World monkeys, to measure the level of residual neurovirulence. In the 1980s, tests of vaccine bulks and seeds were standardized, as a single dose of test material given by intraspinal inoculation and tested concurrently with a homologous reference. Vaccines derived from the Sabin strains that pass the MNVT have been shown to have an acceptable safety profile. However, in its current form, the MNVT is regarded as a test of consistency, and it is not known whether vaccines that fail the test are virulent in human recipients. Tests designed to replace the MNVT should be able to detect the same changes from batch to batch as the MNVT does, with similar sensitivity. The TgmNVT in mice expressing the human poliovirus receptor (TgPVR21 mice) has been developed as an alternative to the MNVT for all three poliovirus serotypes.

Summaries of the MNVT and TgmNVT are given below, along with the implementation process for the TgmNVT.

1. Summary of the MNVT

1.1 Key features

A detailed SOP for neurovirulence tests for types 1, 2 or 3 live-attenuated OPV in monkeys is available from WHO.¹ To perform the test, between 5.5 log₁₀ CCID₅₀ and 6.5 log₁₀ CCID₅₀ of monovalent virus is delivered in a single dose by intraspinal inoculation into the lumbar cord. A back titration of the inoculum should be carried out after the inoculation step has been completed. Residual paralysis, if any, occurring during the following 17–22 days should be noted. The animals are killed at the end of the test, or earlier on humanitarian grounds, and prepared for histological examination of the central nervous system. Damage to different regions is scored on a scale from 1 to 4, and a mean lesion score is calculated for each monkey and then for all the monkeys in the test. The clinical signs do not form part of the assessment or of the pass/fail criteria. The homologous WHO/SO+2 reference is tested in parallel. Laboratories that want to introduce the test should agree an implementation process with the NRA.

¹ Contact the Coordinator, Technologies, Standards and Norms, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).

1.2 Number of animals

The number of monkeys is chosen on statistical grounds, considering the variability of the test, so that a satisfactory vaccine will give the lesion score of a reference preparation only twice in 1% of tests, and therefore be incorrectly scored as a fail. Valid animals must show some sign of histological damage as evidence of correct placement of active virus. The number of "valid" monkeys required per virus preparation is 11 each for type 1 and type 2, and 18 for type 3. Because a reference must be tested at the same time, the total number of monkeys for type 1 and type 2 is at least 22 each; and for type 3 it is 36.

1.3 Sections examined

Sections are examined from defined regions of the spinal cord and brain, and scored histologically for virus activity on a scale of 1 (cellular infiltration only) to 4 (massive neuronal damage). At least 29 sections are examined per monkey, as specified in the WHO SOP for the MNVT. The readings are used to generate the mean lesion score for the animal, and the mean lesion scores for all animals are then used to generate the mean lesion score for the test as a whole.

1.4 Pass/fail criteria

The pass/fail criteria are based on variations occurring in the test from run to run, established from the scores obtained with the reference preparation, and are specific to each laboratory and operator. The within-test variance is used to calculate the statistical constants C1, C2 and C3. The vaccine is not acceptable if the mean lesion score of the test vaccine is greater than that of the concurrently tested reference by more than C1. If the test vaccine gives a higher score than the reference but the difference in scores lies between C1 and C2, the vaccine may be retested and the results pooled; if the difference for the pooled test results is greater than C3, the vaccine fails.

The values for C1, C2 and C3 are initially established on the basis of the data accumulated after four qualifying tests. These values should then be updated after every test until nine tests have been performed. After that, the C values are based on the 10 most recent tests. The C values must be established for each testing laboratory.

2. Summary of the TgmNVT

2.1 Key features

The detailed SOP for the TgmNVT is available from WHO for neurovirulence tests for type 1, 2 or 3 live-attenuated OPV.² The test for the neurovirulence of polio

Contact the Coordinator, Technologies, Standards and Norms, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).

vaccines in transgenic mice involves intraspinal inoculation of small volumes of test vaccine into a defined strain of transgenic mice carrying the human receptor for poliovirus. Two virus concentrations are used, and the outcome of the test is based on the clinical response to the dose. A reference preparation is tested at the same time. A clearly defined process has been established for laboratories that want to introduce the test.

2.2 Strain of transgenic mouse

Different transgenic mouse lines differ in their sensitivity to polio infection depending on their particular transgenic construct and genetic background, and only strains from a source approved by WHO should be used. As of 2012, the only approved transgenic mouse strain was TgPVR21, which was developed in Japan and can be sourced from the developers or from an approved subcontractor.

2.3 Titration of virus

Two doses of virus are inoculated in a volume of 5 μ l each: for type 1, 1.75 CCID₅₀ and 2.75 CCID₅₀; for type 2, 5.0 CCID₅₀ and 6.0 CCID₅₀; and for type 3, 3.5 CCID₅₀ and 4.5 CCID₅₀. The inocula must be prepared and titrated accurately to ensure that these doses are given; the precision of the determinations should be better than $\pm 0.3 \log_{10}$. A back titration of the inoculum should be performed after the inoculation step has been completed.

2.4 Inoculation and observation of animals

Animals procured at age 5–6 weeks are randomly allocated to cages, and allowed to recover for at least seven days. They are then appropriately anaesthetized and inoculated with $5\,\mu$ l of diluted test virus between the last thoracic vertebra and the first lumbar vertebra. Animals are observed for clinical signs once a day for the next 14 days, and ultimately scored either as normal throughout (slight weakness or no signs) or paralysed (paresis on two consecutive days, or paralysis on a single day). For the test to be valid, the lower doses and higher doses of the reference preparation should cause paralysis in more than 5% and less than 95% of animals, respectively. A test requires 128 mice for one vaccine plus the reference tested concurrently, or 192 mice for two vaccines and the reference. The reference is the same as that used in the monkey test; the use of other references may be acceptable but should be validated.

The vaccine passes if it is not significantly more virulent than the reference as defined in terms of the log of the odds ratio and the statistical constants L_1 and L_2 , which are based on the reproducibility of the test and define the pass/fail criteria as well as the grey zone in which a retest is required. The acceptance and rejection limits, L_1 and L_2 , have been selected so that a test vaccine that is equivalent to the reference will have a 0.95 probability of passing and a 0.01

probability of failing, respectively. The constants are regularly updated. The statistical evaluation of test validity includes assessments of linearity, and dose and sex effects.

3. Implementation process for the TgmNVT

If a manufacturer wishes to use the TgmNVT, relevant validation data should be available for the specific product to demonstrate the test's applicability. These data may include references to the collaborative studies by which the test was originally developed. A clear, stepwise process for implementing the TgmNVT has been established; it involves training staff in the inoculation technique by first injecting India ink, testing with vaccines, and testing using a blinded evaluation panel containing vaccines that pass, fail or marginally fail the test. Competence in clinical scoring is acquired by working through a standardized training procedure that involves scoring mice in parallel with an experienced scorer; there are also clear criteria for declaring a trainee competent.

Testing should be performed according to the procedures specified in the WHO SOP for the TgmNVT, using appropriate WHO reference materials unless modified procedures have been validated and shown to be suitable. The test chosen should be used to test virus seeds and bulks, as described in sections A.3.2.4.2 and A.4.4.7.2, respectively.

4. Considerations in relation to assay choice

The following specific issues suggest that care should be taken in the selection of the in vivo tests to be performed for neurovirulence, and that the selection should be justified. The report of the WHO working group meeting to discuss the revision of the WHO Recommendations for OPV: TRS Nos. 904 and 910 provides more detailed discussion (1).

4.1 Type-1 and type-2 Sabin vaccine viruses

The relative sensitivity of the TgmNVT and MNVT performed according to WHO procedures with respect to the presence of mutations in the 5' untranslated region in types 1 and 2 polioviruses appears to be comparable, but this sensitivity is significantly lower than that for type 3 (2, 3). It is unknown whether these two models are equally sensitive to other potentially neurovirulent mutations. Most manufacturers use essentially identical seeds of types 1 and 2, in contrast to the situation with type 3.

4.2 Type-3 Sabin vaccine virus

4.2.1 Molecular biology

Studies of the molecular biology of the Sabin polio vaccine virus strains have suggested that few mutations are involved in attenuation, and that for the type-3

strain, there may be only two: one base change in the 5′ noncoding region of the genome at base 472, and one coding change at base 2034 that introduces an amino acid change in the virus protein VP3. A third mutation at position 2493 has been described (4). Growth of Sabin 3 virus in cell culture or in vaccine recipients results in rapid accumulation of U instead of C at nucleotide 2493 (changing Thr to Ile at amino acid 6 of capsid protein VP1), and all Sabin 3 OPV batches contain variable amounts of these mutants. This mutation does not affect neurovirulence as determined by the MNVT but there is evidence that it influences the results obtained by the TgmNVT, which is described in the WHO SOP (5). Variations in the virulence of vaccine batches as measured in monkeys correlate well with variations in the base in the 5′ noncoding region as measured by the MAPREC assay. Changes in the amino acid in VP3, or changes at other positions that suppress its effect, are not thought to be generated in the course of well-controlled production runs, although this is possible in principle.

4.2.2 Current type-3 seed viruses

Seed viruses used for global vaccine production contain variable proportions of the bases found at position 2493 (C or U):

- The original WHO reference material for neurovirulence testing (passage level SO+2) contained about an equal mixture of both forms (2493 C or U).
- Batches prepared from RSO, the seeds most commonly used in production in Europe, typically contain about 5% or fewer of 2493-U (mutant).
- Seed viruses used in production by some manufacturers (i.e. a plaque purified from SO) result in batches containing 100% of the mutant form (2493-U) (6).

All OPVs in use are believed to have an acceptable safety profile.

5. Experience using the MNVT and TgmNVT with type-3 seeds and vaccines

There is evidence that the TgmNVT described in the WHO SOP is sensitive to the presence of 2493-U, whereas the monkey test is not sensitive to this mutation. Thus, batches produced from RSO seed will pass both the MNVT and TgmNVT tests, whereas batches produced from alternative seeds that contain 100% 2493-U will pass the MNVT but may fail the TgmNVT, although they still have an acceptable safety profile for clinical use.

The WHO SOP for the TgmNVT specifies the doses and the WHO reference material to be used, and includes the proportion of mice that need to

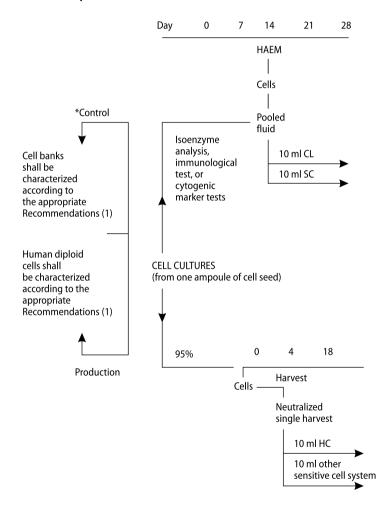
be affected at the two doses of virus for the test to be considered valid. The WHO reference material for the TgmNVT is the same as that used in the MNVT, and has approximately 50% 2493-C; it was validated primarily against vaccines made from SO or RSO seeds. However, if this reference material is used to test vaccines derived from seed that contains 2493-U, the seed may fail even if it contains little 472-C and would pass the MNVT. The TgmNVT could be adapted for testing bulks containing 2493-U – e.g. by changing the reference material, the doses or the validity criteria, or a combination of these. Manufacturers may wish to adapt the TgmNVT to make it applicable to their product. Any modified test should be validated and approved by the appropriate NRA.

6. References

- WHO Working Group Meeting to Discuss the Revision of the WHO Recommendations for OPV: TRS No. 904 and 910. Geneva, Switzerland 20–22 July 2010. Geneva, World Health Organization, 2010 (http://www.who.int/biologicals/vaccines/OPV_Meeting_report_Final_Clean_13May2011.pdf, accessed 20 June 2013).
- 2. Gennady V et al. Genetic stability of Sabin 1 strain of poliovirus: implications for quality control of oral poliovirus vaccine. *Virology*, 1998, 245:183–187.
- 3. Taffs RE et al. Genetic stability and mutant selection in Sabin 2 strain of oral poliovirus vaccine grown under different cell culture conditions. *Virology*, 1995, 209:66–73.
- 4. Tatem JM et al. A mutation present in the amino terminus of Sabin 3 poliovirus vp1 protein is attenuating. *Journal of Virology*, 1992, 66:3194–3197.
- 5. Chumakov KM et al. RNA sequence variants in live poliovirus vaccine and their relation to neurovirulence. *Journal of Virology*, 1992, 66:966–970.
- Rezapkin GV et al. Reevaluation of nucleotide sequences of wild-type and attenuated polioviruses of type 3. Virus Research, 1999, 65:111–119.

Preparation of poliomyelitis vaccines (oral, live, attenuated) using cell banks – example of a flowsheet

Figure 2.3 Flowsheet example



^a Control cells should be 5% of the total or 500 ml of cell suspension or 100 000 000 cells.

HAEM = test for haemadsorbing viruses; CL = cell line used for production but not the same batch of cells as used for production of the virus; SC = when a human diploid cell line is used for production, a simian kidney cell line should be used as the second indicator cell line.

Figure 2.3 continued

When a simian kidney cell line is used for production, a human diploid cell line should be used as the second indicator cell line; ¹ HC = human cells.

Note: this example includes all tests, whether obligatory or not. Since the requirements applicable in a particular place are those authorized by the NRA, this flowsheet should not be considered to be an integral part of the requirements; it has been included solely for guidance. Manufacturers should prepare their own flowsheet to clarify the procedures used.

Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. In: WHO Expert Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 3.

Cell-culture techniques for determining the virus content of poliomyelitis vaccines (oral, live, attenuated)

This appendix describes a method for determining the virus content of liveattenuated OPV in cell cultures. It is an example that is provided only for guidance.

The preparation to be assayed and the reference preparation are diluted in an appropriate medium. It is convenient to make 10-fold dilution steps of the virus suspensions initially, but for dilutions that are to be inoculated into HEp2 (Cincinnati) cell cultures, the dilutions should be prepared in $1.0 \log_{10}$ or smaller steps. A preliminary assay may be required to ensure that in the test the dilution range selected encompasses at least three dilutions that will infect between 0% and 100% of the cultures inoculated.

Titrate the vaccine for infectious virus using no fewer than three separate containers of vaccine following the method described below. Titrate one container of an appropriate virus reference preparation in triplicate to validate each assay. The virus titre of the reference preparation is monitored using a control chart, and a titre is established using historical data at each laboratory.

If the vaccine contains more than one type of poliovirus, titration of the individual serotypes is undertaken separately, using mixtures of appropriate type-specific antiserum (or preferably a monoclonal antibody) to neutralize each of the other types present.

To titrate individual serotypes, inoculate a suitable number of wells (ideally 8–10) in a flat-bottomed microtitre plate with equal volumes of the selected dilutions of virus and the appropriate antiserum mixture. Total virus content is determined, without any prior incubation, by directly diluting the vaccine in the assay medium. The assay is then incubated for 1–3 hours at 34–36 °C; this is followed by the addition of an appropriate volume of a suitable cell. The plates are further incubated at 34–36 °C, and examined between day 5 and day 9 for the presence of viral cytopathic effect.

The cytopathic effect can be observed by direct reading or after appropriate staining (vital or fixed staining). The individual virus concentration for each polio serotype and reference preparation is then calculated using an appropriate method.

The assay is considered valid if:

• the estimated virus concentration for the reference preparation is $\pm 0.5 \log_{10} \text{CCID}_{50}$ of the established value for this preparation;

• the confidence interval (P = 0.95) of the estimated virus concentration of the three replicates of the reference preparation is not greater than $\pm 0.3 \log_{10} \text{CCID}_{50}$.

The assay is repeated and results are averaged if:

• the confidence interval (P = 0.95) of the combined virus concentration of the vaccine is greater than $\pm 0.3 \log_{10} \text{CCID}_{50}$.

Model protocol for the manufacturing and control of poliomyelitis vaccines (oral, live, attenuated)

The following protocol is intended for guidance. It indicates the information that should be provided as a minimum by a manufacturer to the NRA. Information and tests may be added or omitted as necessary, with the authorization of the NRA.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO Guidelines for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that will accompany the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot-release certificate from the NRA or from the NCL in the country where the vaccine was produced or released stating that the product meets national requirements as well as the recommendations in Part A of this annex.

1. Summary information on finished product (final lot)

International name:
Trade name/commercial name:
Product licence (marketing authorization) number:
Country:
Name and address of manufacturer:
Name and address of licence
holder, if different:
Virus strain:
Origin and short history:
2. Summary information on manufacture

Batch number:	
Final bulk:	
Type of container:	
Number of doses per container:	
Number of filled containers in this final lot:	

Bulk numbers of monovalent bulk suspensions blended in monovalent/	Type 1	Type 2	Type 3
bivalent/trivalent vaccine:			
Site of manufacture of each monovalent	bulk.		
Date of manufacture of each monovalent			
Date of manufacture of final bulk (blend			
Date of manufacture (filling) of finished			
Date on which last determination of viru			
was started, or start date of period of			
Shelf-life approved (months):	•		
Expiry date:			
Storage conditions:			
Volume of human dose (in drops and/or			
Virus titre per single human dose:	III)		
Type 1:			
Type 2:			
Type 3:			
Nature and concentration of stabilizer:			
Nature of any antibiotics present in vacci			
and amount per human dose:			
Release date:			
Starting materials			
The information requested below is to be p	resented for ea	ch submission	ı. Full details
on master seed and working seed lots shou	•		
or whenever a change has been introduced	-	, 1	
C			
The following sections are intended for red	•		-
during the production of the vaccine, so t			
evidence of consistency in production. If			
indicated. Any abnormal result must be i		-	
If any cell lot or virus harvest int	-	•	
the control testing, this should also be re	ecorded, either	in the follow	ving sections
or on a separate sheet.			
3. Control of source materials (sect	ion A 3)		
•	1011 71.5)		
Cell banks (every submission)			
Information on cell banking system:			
Name and identification of substrate:			
Origin and short history:			

A4b:4b44
Authority that approved the cell bank:
Master cell bank (MCB) and working cell bank (WCB)
lot numbers and date of preparation:
Date the MCB and WCB were established:
Date of approval by NRA:
Total number of ampoules stored:
Passage level (or number of population
doublings) of cell bank:
Maximum number of passages approved:
Storage conditions:
Method of preparation of cell bank in terms of number of
freezes and efforts made to ensure that a homogeneous
population is dispersed into the ampoules:
Identity tests on MCB and WCB (first submission only) (section A.3.1.2)
Percentage of total cell-bank ampoules tested:
Identification of cell substrate:
Method:
Specification:
Date of test:
Result:
Growth characteristics:
Morphological characteristics:
Immunological marker:
Cytogenetic data:
Biochemical data:
Results of other identity tests:
Tests for adventitious agents
Method used:
Number of vials tested:
Volume of inoculum per vial:
Date of start of test:
Date of end of test:
Result:
Tests for bacteria, fungi and mycoplasmas
Tests for bacteria and fungi
Method used:
Number of vials tested:
Volume of inoculum per vial:

Volum Observ	e of mediu ation peri	ım per vial: _ od (specificat	tion):			
Incubation	Media us	ed Inoculun	n Da	te of start of test	Date of en of test	d Results
20–25 °C						
30−36 °C						
Negative control						
Volume Media Temper Observ	e tested: _ used: rature of in ration peri	od (specificat	tion):			
		Date of start of	of test	Date of e	nd of test	Results
Subcultures	at day 3					
Subcultures	at day 7					
Subcultures	at day 14					
Subcultures	at day 21					
Cell sul Inoculu Date of Passage Negative Positive Date of Results	bstrate use um: f test: e number: ve controls: f staining:					
Virus seeds (e	very suhm	nission) (sectio	n A 3 2)		
Vaccine viru	ıs strain(s)	and serotype	e(s): _			

Origin and short history of virus seeds:
Virus strains: information and seed lot preparation (every submission) (section A.3.2.1)
Virus master seed (VMS), virus submaster seed and virus working seed (VWS)
Source of VMS:
VMS and VWS lot numbers:
Name and address of manufacturer:
VWS passage level from VMS:
Dates of inoculation:
Dates of harvest:
Number of containers:
Conditions of storage:
Dates of preparation:
Maximum passage levels authorized:
Tests on VMS, virus submaster seed, and VWS (first submission only)
Tests for adventitious agents
Date(s) of satisfactory test(s) for freedom
from adventitious agent:
Volume of virus seed samples for
neutralization and testing:
Batch number of antiserum used for
neutralization of virus seed:
Method used:
Date of start of test:
Date of end of test:
Result:
Identity test
Method used:
Date of start of test:
Date of end of test:
Result:
Absence of SV40
Method used:
Date of start of test:
Date of end of test:
Results:

In vitro tests: MAPREC or rct/40 marker test
MAPREC test
Date of test:
Type 1
Ratio of % of the sum of both mutations 480-A
and 525-C in bulk sample to the International Standard
or level of mutations:
Result of test of consistency of production:
Result of test of comparison with the
International Standard:
Type 2
Ratio of % of 481-G in bulk sample to the International
Standard or level of mutations:
Result of test of consistency of production:
Result of test of comparison with the
International Standard:
Type 3
Ratio of % of 472-C in bulk sample to the International
Standard or level of mutations:
Result of test of consistency of production:
Result of test of comparison with the
International Standard:
In vitro rct/40 marker test
Date of test:
Reduction of titre in bulk sample:
Reduction of titre in negative reference:
Reduction of titre in positive reference:
Result:
Result of test of consistency of production:
In vive tests for a superincleuse
In vivo tests for neurovirulence Neurovirulence test in monkeys (MNVT)
Result of blood serum test in monkeys prior to inoculation:
Number and species of monkeys inoculated:
Quantity (CCID ₅₀) inoculated into each
test monkey:
test indiret,

Number of "valid" monkeys inoculated
with test sample:
Number of positive monkeys observed that were
inoculated with test sample or with reference:
Reference preparation:
Number of "valid" monkeys inoculated
with reference:
Number of positive monkeys observed:
Mean lesion score of test sample:
Mean lesion score of reference: (see also attached
forms giving details of histological observations
and assessment)
C ₁ constant value:
Neurovirulence test in transgenic mice (TgmNVT)
Strain of mice inoculated:
For each dose of the seed sample:
Number of mice inoculated:
Number of mice excluded from evaluation:
Number of mice paralysed:
Results of validity tests for each dose of the
reference virus:
Number of mice inoculated:
Number of mice excluded from evaluation:
Number of mice paralysed:
Virus assay results for each dose inoculated
(residual inocula):
Paralysis rates for test vaccine at each dose:
Paralysis rates for reference virus at each dose:
Results:
Log of the odds ratio:
L ₁ and L ₂ values:
Pass/fail decision:
Freedom from bacteria, fungi and mycoplasmas
Tests for bacteria and fungi
Method used:
Number of vials tested:
Volume of inoculum per vial:
Volume of medium per vial:
Observation period (specification):

Incubation	Media used	Inoculum	Date of start of test	Date of end of test	Results
20−25 °C					
30−36 °C					
Negative control					

Test for mycoplasmas

Method use	ed:		
Volume tes	ted:		
Media used	:		
Temperatui	re of incubation:		
Observation	n period (specificati	on):	
Positive cor	ntrols (list of species	used and results):	
	Date of start of test	Date of end of test	Results
Subcultures at day 3			
Subcultures at day 7			
Subcultures at day 14			
Subcultures at day 21			
Cell substra			
Inoculum:			
Date of test	:		
C			
Results:			
Virus titration			

Genotype characterization
Method used:
Date of test:
Result:
Test for mycobacteria
Method used:
Date of start of test:
Date of end of test:
Result:
4. Control of vaccine production (section A.4)
Control cell cultures (section A.4.1)
Lot number of MCB:
Lot number of WCB:
Date of thawing of ampoule of WCB:
Passage number of production cells:
Date of preparation of control cell cultures:
Results of microscopic observation:
results of interoscopic observation.
Tests of control cell cultures
Ratio of control cell cultures to production
cell cultures:
Incubation conditions:
Period of observation of cultures:
Dates observation started and ended:
Proportion of cultures discarded for
nonspecific reasons:
Results of observation:
Date supernatant fluid collected:
That for heavy adaptive views
Tests for haemadsorbing viruses Quantity of cells tested:
Method used:
Date of start of test:
Date of end of test:
Results:
Tests for adventitious agents in supernatant culture fluids
Method used:
Date of start of test

Date of end of test:
Result:
Identity test
Method used:
Date of start of test:
Date of end of test:
Result:
Control of single harvests (section A.4.3)
Volume harvested:
Date of sampling:
Identity test
Method used:
Date of start of test:
Date of end of test:
Result:
Virus titration
Date of test:
Reference batch number:
Date of test:
Result:
Tests of neutralized single harvests for adventitious agents
Method used:
Date of start of test:
Date of end of test:
Result:
Freedom from bacteria, fungi and mycoplasmas
Tests for bacteria and fungi
Method used:Number of vials tested:
Volume of modium por vial:
Volume of medium per vial:
Observation period (specification):

Incubation	Media used	Inoculum	Date of start of test	Date of end of test	Results
20−25 °C					
30−36 °C					
Negative control					

Test for mycoplas	smas		
Method use	ed:		
Volume tes	ted:		
		on):	
Positive con	ntrols (list of species	used and results):	
	· •	·	
	Date of start of test	Date of end of test	Results
Subcultures at day 3			
Subcultures at day 7			
Subcultures at day 14			
Subcultures at day 21			
		l l	
Indicator cell-cul	ture method (if appli	icable)	
U			
U			
	8.		
icourts.			
Test for mycobacteria			
Method used: _			
Date of start of t	est:		
Date of end of te	est:		
Result:			

Control of mo	novalent bulk	(section A.4	.4)		
Date of filtra	tion of bulk:				
Porosity of f	ilters used:				
Date of samp	oling:				
Identity test	1 1				
			s:		
Virus titratio	on				
Date of	test:				
Result:					
Method					
			on):		
Observ	ation period ((specificatio	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		
Incubation	Media used	Inoculum	Date of start of test	Date of end of test	Results
20−25 °C					
30−36 °C					
Negative control					
Test for myco	d used:				
	start of test:				
Result.					
Tests for cons	sistency of viri	us character	istics		
	rct/40 marke				
D	ate of test:				

Reference used:
Reduction of titre of negative reference:
Reduction of titre of positive reference:
Result:
Teodic
MAPREC test
Date of test:
2 4.00 02 00001
Type 1
Ratio of % of the sum of both mutations 480-A
and 525-C in bulk sample to the International Standard
or level of mutations:
Result of test of consistency of production:
Result of test of comparison with the
International Standard:
Type 2
Ratio of % of 481-G in bulk sample to the International
Standard or level of mutations:
Result of test of consistency of production:
Result of test of comparison with the
International Standard:
Type 3 Ratio of % of 472-C in bulk sample to the International
Standard or level of mutations:
Result of test of consistency of production:Result of test of comparison with the
International Standard:
international standard:
Neurovirulence tests in monkeys (MNVT)
Result of blood serum test in monkeys prior
to inoculation:
Date of inoculation of monovalent bulk:
Number and species of monkeys inoculated:
• '
·
± ,
<u>-</u>
• •
·
Quantity (CCID ₅₀) inoculated into each test monkey: Number of "valid" monkeys inoculated with test sample: Number of positive monkeys observed that were inoculated with test sample or with reference: Reference preparation: Number of "valid" monkeys inoculated with reference: Number of positive monkeys observed:

Mean lesion score of test san	nple:		
Mean lesion score of referen			
forms giving details of hi	`		
and assessment)	•		
C ₁ constant value:			
-1			
Neurovirulence test in transgenic r	nice (TømNVT)		
Strain of mice inoculated: _			
For each dose of the bulk sar			
Number of mice inoculated:			
Number of mice excluded fr			
Number of mice paralysed:			
Results of validity tests for ea			
reference virus:			
Number of mice inoculated:			
Number of mice excluded fr			
Number of mice paralysed:			
Virus assay results for each of			
(residual inocula):			
Paralysis rates for test vaccir	e at each dose:		
Paralysis rates for reference			
Results:			
Log of the odds ratio:			
L ₁ and L ₂ values:			
Pass/fail decision:			
Final bulk (section A.4.5)			
Preparation of bulk (types as	Type 1	Type 2	Type 3
appropriate)	71	71	71
Monovalent bulks in blend			
Volume in blend			
Nature and volume of stabilizer			
Nature and volume of diluent			
Total volume of blend:			
Tests for bacteria and fungi			
Method used:			
Number of vials tested:			
Volume of inoculum per vial:			
Volume of medium per vial:			
Observation period (specification			

Inoculum

Date of start

of test

Date of end

of test

Results

Media used

Incubation

20-25 °C					
30-36 °C					
Negative					
control					
5. Filling a	nd contai	ners (sect	ion A.5)		
•					
Number of	vials after ir	spection:			
Number of	vials filled:				
			t (section <i>F</i>	1.6)	
Inspection	of final cont	ainers			
Results:					
Extractable	volume				
Extractable	volume (m	l):			
			proved drop		
				ners:	
рН					
•	-				
icouit.					
Identity tes	t				
Method use	ed:				
Lot number	of reference	e reagents:			
Tests for ba	cteria and f	ungi			

Method used: _____

Number of vials tested:

Volume of in	noculum per v	vial:			
Observation	period (spec	ification): $_{-}$			
Incubation	Media used	Inoculum	Date of start of test	Date of end of test	Results
20–25 °C					
30−36 °C					
Negative control					
Virus titratio	n				
			test:		
Result		Vaccine		Refe	rence
Type 1					
Type 2 Type 3					
Type 3					
Thermal stal	oility				
Date of test:					
Batch numb	ers of antiseru	ım used in 1	test:		
Results		cine at 37 °C	C Vacc	ine	Difference
Total v	irus				
Residual ant	ibiotics (if ap _l	plicable)			
Date of start	of test:				
	of test:				
Results:					
Level of stab	oilizer (if appli	cable)			
Date of start	of test:				
Date of end	of test:				
Results:					

Production in primary monkey kidney-cell cultures	i cuitures
Control of vaccine production:	
Control of monkeys:	
Monkey species used for production:	
Quarantine batch number:	
Percentage of monkeys surviving quarantine period:	
Nature and concentration of antibiotics or selecting	
agent(s) used in the production cell culture's	
maintenance medium:	
Tests for antibodies to simian immunodeficiency virus, SV40,	
foamy viruses and herpes B virus	
Methods used:	
Date of start of test:	
Date of end of test:	
Results:	
Production details	
Production monkey number:	
Date of trypsinizing:	
Number of cultures prepared:	
Cell cultures for vaccine production	
Virus-seed lot number:	
Virus titre/cell ratio:	
Number of cultures inoculated:	
Date of inoculation:	
Date of harvest:	
Temperature of incubation:	
Period of incubation:	
Number of cultures harvested:	
Tests on pooled supernatant fluids	
Date of sampling from production cell cultures:	
Tests for adventitious agents:	
Volume tested and cell culture type:	
Observation period:	
Date of completion of tests:	
Results	

Date of sampling from cell cultures inoculated with the pooled fluid
Tests for adventitious agents:
Volume tested and cell culture type:
Date of completion of tests:
Results:
Total Sound his history and profession
Tests in rabbit kidney-cell cultures
Volume tested:
Date of completion of tests:
Results:
Control of cell cultures
Ratio of control cell cultures to production cell cultures, or control
cell cultures as a proportion of production cell cultures:
Period of observation of cultures:
Proportion of cultures discarded for nonspecific reasons:
Results:
Tests for he are adopting a views of
Tests for haemadsorbing viruses
Methods:
Date of test:
Results:
Tests for other adventitious agents
Methods:
Date of test:
Results:
Control of single harvests
Volume harvested:
Date of sampling:
Tests for bacteria, fungi and mycoplasmas:
Results:
Tests on neutralized single harvests in monkey kidney-cell and human-cell cultures
Batch number of antiserum used:
Volume tested:
Date primary cell-culture tests started:
Period of observation:
Date cell-culture fluids sampled:

WHO Expert Committee on Biological Standardization	Sixt	y-third i	report
--	------	-----------	--------

Period of observation:	
Date of completion of tests:	
Results:	
Control of monovalent bulk	
Tests in rabbits	
Number and weight range of animals:	
Date of inoculation:	
Quantity of monovalent bulk injected: _	
Results (survival numbers and other rele	vant
observations):	
Date of filtration of bulk:	
Porosity of filters used:	
Date of sampling:	
Tests for retroviruses	
Methods:	
Methods:	
Date:	
Date:	
Date: Results: 7. Certification by the manufacturer	
Date: Results: 7. Certification by the manufacturer Name of the manufacturer	laboratory of the manufacturing
Date:	laboratory of the manufacturing and control of the vaccine coliomyelitis vaccine (oral), whose her, meets all national requirements mendations to assure the quality,
Date:	laboratory of the manufacturing and control of the vaccine voliomyelitis vaccine (oral), whose there, meets all national requirements mendations to assure the quality, ral, live, attenuated) (2014). ²
Date:	laboratory of the manufacturing and control of the vaccine voliomyelitis vaccine (oral), whose there, meets all national requirements mendations to assure the quality, ral, live, attenuated) (2014). ²

² WHO Technical Report Series, No. 980, Annex 2.

8. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 6), a label from a final container and an instruction leaflet for users.

Appendix 6

Model certificate for the release of poliomyelitis vaccines (oral, live, attenuated) by NRAs

Lot release certificate
Certificate no
The following lot(s) of poliomyelitis vaccine (oral, live, attenuated) produced by
The release decision is based on

The certificate may include the following information:

- name and address of manufacturer
- site(s) of manufacturing
- trade name and common name of product
- marketing authorization number
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)

¹ Name of manufacturer.

² Country of origin.

³ If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.

With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

⁵ WHO Technical Report Series, No. 980, Annex 2.

⁶ WHO Technical Report Series, No. 961, Annex 3.

⁷ WHO Technical Report Series, No. 822, Annex 1.

⁸ WHO Technical Report Series, No. 978, Annex 2.

⁹ Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.

- type of container used
- number of doses per container
- number of containers or lot size
- date of start of period of validity (e.g. manufacturing date) and/or expiry date
- storage conditions
- signature and function of the person authorized to issue the certificate
- date of issue of certificate
- certificate number.

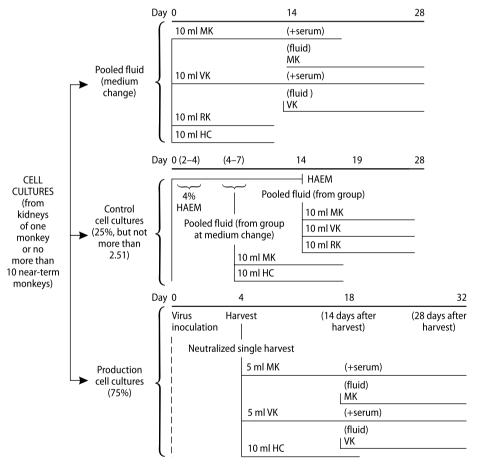
The Director of the NRA	(or other	appropriate a	uthority)
-------------------------	-----------	---------------	-----------

Name (typed)	
Signature	
Date	
Date	

Appendix 7

Preparation of poliomyelitis vaccines (oral, live, attenuated) using primary monkey kidney cells – example of a flowsheet

Figure 2.4 Flowsheet example



HAEM = test for haemadsorbing viruses; MK = monkey kidney cells from species (but not the same animal) used for production; VK = kidney cells from vervet monkey or one sensitive to SV40; RK = rabbit kidney cells; HC = human cells sensitive to measles.

Note: this example includes all tests, whether obligatory or not. Since the requirements applicable in a particular place are those authorized by the NRA, this flowsheet should not be considered to be an integral part of the requirements; it has been included solely for guidance. Manufacturers should prepare their own flowsheet to clarify the procedures used.

Annex 3

Guidelines on the quality, safety and efficacy of recombinant malaria vaccines targeting the pre-erythrocytic and blood stages of *Plasmodium falciparum*

Introdu	ction and scope	143
Backgro	ound	144
Part A.	Guidelines on manufacturing and control	15 1
A.1	Recombinant malaria antigens under evaluation	152
A.2	Terminology	153
A.3	General manufacturing guidelines	154
A.4	Control of source materials	154
A.5	Fermentation	155
A.6	Single harvests	156
A.7	Control of purified antigen bulk	156
A.8	Final bulk	159
A.9	Filling and containers	160
A.10	Control tests on final lot	160
A.1	Records, retained samples, labelling, distribution and transport	164
A.12	2 Stability testing, storage and expiry date	164
Part B. I	Nonclinical evaluation of malaria vaccines	165
B.1	Introduction	165
B.2	Product development and characterization	166
B.3	Pharmacodynamic studies	168
B.4	Toxicity	170
Part C. 0	Clinical evaluation of malaria vaccines	17 1
C.1	Introduction	17 1
C.2	Clinical studies	172
C.3	Post-licensure investigations	185
Part D. (Guidelines for NRAs	187
D.1	General	187
D.2	Release and certification by the NRA	187
Authors	•	188
Acknow	rledgements	191
Referen	ces	191

Appendix i	
Controlled human malaria infection trials (human-challenge studies)	195
Appendix 2	
Methodological considerations: quantification of human immunoglobulin G directed against the repeated region (NANP) of the circumsporozoite protein of the parasite <i>P. falciparum</i> (anti-CS ELISA)	197
Appendix 3 Model protocol for the manufacturing and control of recombinant malaria vaccines	199
Appendix 4 Model certificate for the release of recombinant malaria vaccines by NRAs	208

This document provides information and guidance on the development, production, quality control and evaluation of the safety and efficacy of candidate malaria vaccines produced using recombinant DNA technology. Since there is at present no licensed malaria vaccine, this document is written in the form of WHO Guidelines instead of Recommendations, and is intended to facilitate progress towards the eventual licensure of such a vaccine. Guidelines allow greater flexibility than Recommendations with respect to future developments in the field. The parts printed in small type in Part A are comments or examples that are intended to provide additional guidance on the currently most-advanced candidate vaccine. To facilitate the international distribution of vaccines produced in accordance with these Guidelines, a summary protocol for recording test results has been provided in Appendix 3.

Introduction and scope

These Guidelines are intended to provide guidance to national regulatory authorities (NRAs) and vaccine manufacturers on the quality (including the production, quality control, characterization and stability), and nonclinical and clinical aspects of recombinant malaria vaccines targeting the pre-erythrocytic and blood stages of *Plasmodium falciparum*.

Various approaches to the development of malaria vaccines are being investigated that employ different production platforms, and target different stages of the life-cycle of malaria parasites. As of 2012, only one candidate vaccine – RTS,S/AS01, a recombinant *P. falciparum* malaria vaccine produced in yeast that targets the pre-erythrocytic stage of the parasite – was under evaluation in phase III clinical trials (1). In early clinical trials, this vaccine demonstrated some degree of efficacy in reducing all episodes of clinical malaria (2–4).

These Guidelines consist of three technical sections – Part A: Guidelines on manufacturing and control; Part B: Nonclinical evaluation; and Part C: Clinical evaluation. These three sections differ somewhat in their scope to reflect the different stages of vaccine development, and the diversity of production platforms and vaccine targets.

Part A of this document focuses on the manufacturing process and the quality-control issues relevant to recombinant antigens. Specific information regarding RTS,S/AS01 vaccine, the vaccine currently under phase III evaluation, is provided in small print. Part B (nonclinical evaluation) and Part C (clinical evaluation) are written to have a wider scope, and may be applicable to the evaluation of other recombinant subunit malaria vaccines that target the pre-erythrocytic or blood-stage of *P. falciparum*. Additionally, the issues discussed in Part C may apply to whole-organism malaria vaccines; however, specific issues for this type of vaccine are discussed in more detail elsewhere (5). The appendices are provided as examples to illustrate the thinking and assay methods in place at the time these Guidelines were adopted. Therefore, they should not be considered as final procedures but as evolving approaches.

Additional and specific considerations for clinical development programmes are necessary for transmission-blocking malaria vaccines because these are intended to reduce malaria transmission by blocking or interfering with the sexual stage of the parasite's life-cycle, and are not expected to prevent malaria disease directly in vaccinated individuals (6).

At present, there is no vaccine licensed for malaria. Some methodological considerations are provided in appendices as examples based on the protocols used by the manufacturer of the most advanced candidate vaccine. These are provided for information only and should not be considered as endorsements of any candidate vaccines. When a malaria vaccine is licensed, the principles

detailed in these Guidelines may also apply to the evaluation of vaccines for which significant changes to the marketing authorization have been submitted.

This document should be read in conjunction with other relevant WHO Guidelines, including those on the nonclinical (7) and clinical (8) evaluation of vaccines.

It is desirable to apply the 3R principles (reduction, replacement, refinement) to minimize the use of animals for ethical reasons (9). Both manufacturers and the staff of NRAs and national control laboratories (NCLs) are encouraged to further develop and use in vitro assays, and to accumulate more data on their application to the quality control of vaccines. However, the type of testing chosen should be driven by the scientific need for valid and relevant data (10).

This document is a new set of Guidelines, and does not replace a specific earlier version; however, WHO has previously issued documents that have provided guidance on vaccine development. In 1997, WHO developed Guidelines that provided a theoretical and epidemiological framework for malaria-vaccine evaluation (11). In 2002, WHO established a Malaria Vaccine Committee (MALVAC). Under the guidance of MALVAC, and building on the 1997 document, WHO has facilitated a series of consultations on the clinical evaluation of malaria vaccine efficacy. A WHO Study Group on Measures of Malaria Vaccine Efficacy met in 2006 in Montreux, Switzerland (12). There was further elaboration of the methods of analysis for malaria vaccine in field-efficacy studies during the 2008 WHO MALVAC scientific forum (13). The outcomes of these consultations form the basis of the clinical section (Part C) of these Guidelines.

Background

Disease burden of malaria

Six identified species of the Plasmodium protozoan parasite can infect humans (*P. falciparum*, *P. vivax*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. malariae* and *P. knowlesi*). *P. falciparum* accounts for more than 90% of all malaria-attributable deaths. Vaccine development efforts have focused on *P. falciparum* and to a lesser extent on *P. vivax* (14). Morbidity and mortality from malaria is a consequence of the replication of parasites in red blood cells. Although it is beyond the scope of these Guidelines to discuss in detail the pathophysiological mechanisms by which morbidity and mortality are induced, there is evidence that all of the following can contribute to malaria-related disease: sequestration of infected red blood cells, severe anaemia due to red blood cell lysis, inflammation-related brain pathology, lactic acidosis, and a general shock-like syndrome with hypotension, hypoglycaemia and poor tissue perfusion. The blood stage is established following the injection of the sporozoite form of the parasite by female anopheline

mosquitoes; subsequent development occurs through the liver stage, which is followed by progression into the blood.

The disease burden of malaria is traditionally assessed by estimating the annual number of deaths due to malaria, the number of acute new cases of the disease per year, and the economic costs resulting from the deaths, illnesses and related treatment as well as the loss of productivity. The databases from which these estimates are made vary in accuracy from country to country. However, these annual data afford a global perspective on the malaria-disease burden. As of 2010, WHO estimated that approximately 655 000 deaths per year were attributable to malaria, with the vast majority of these deaths occurring in sub-Saharan Africa, and the majority of the remaining cases occurring in South-East Asia, the Indian subcontinent and South America. Most of the deaths in Africa occur in children who are younger than 5 years, and in primigravid females. The annual number of new cases of clinical episodes of malaria was estimated in 2010 to be 216 000 000 (15). Young children in malaria-endemic countries typically experience several clinical episodes of malaria before they develop immunity, which protects against the more severe forms of the disease. The economic costs of malaria are difficult to estimate, but in some heavily affected countries these costs run to the equivalent of billions of US dollars per year, and negatively impact the country's gross domestic product by several percentage points (16).

In many African countries substantial malaria-control efforts have been implemented, including the widespread deployment of long-lasting insecticide-treated nets (LLIN) and the use of indoor residual spraying. Studies have indicated reductions in the annual incidence rates of new malaria cases and in deaths due to malaria of \geq 50% in some settings (15, 17). There is a cost burden associated with maintaining vector-control measures and, in the past, control programmes have been underfunded and interrupted, leading to a corresponding resurgence of malaria transmission and the associated morbidity and mortality.

It is widely believed that substantial reductions in the disease burden of malaria will result from a combination of implementing vector-control measures, using selective chemoprophylaxis, strengthening diagnostic testing, effectively treating people with malaria and, potentially, preventing the disease through immunization. Although immunization may make a contribution, malaria-control efforts are unlikely to rely primarily on vaccination. Therefore, it is within this context of a significant disease burden, highly concentrated among children younger than 5 years of age, and multipronged approaches to malaria-disease control, that consideration of the evaluation of recombinant, stage-specific malaria vaccines will take place.

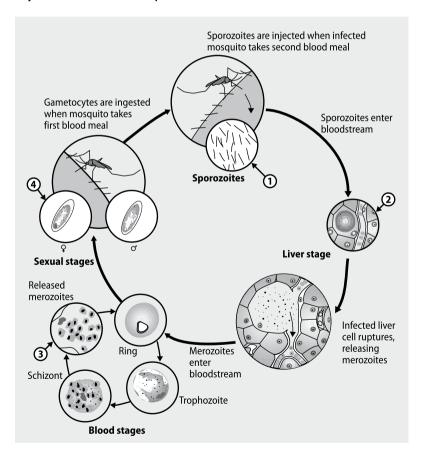
Life-cycle, vaccine targets and potential vaccine effects

Figure 3.1 illustrates the four distinct stages of the life-cycle of malaria parasites – with each stage providing potential vaccine-antigen targets (18). The pre-

erythrocytic stages (stages 1 and 2 in Figure 3.1) encompass the injection of the sporozoite stage of the parasite by the bite of an infected female anopheline mosquito, and the rapid homing of the sporozoite into the liver cells within a matter of minutes to a few hours. Antigens present on the surface of the sporozoite, such as circumsporozoite protein (CSP), or deployed to the surface of the infected hepatocyte, have been used as pre-erythrocytic-stage candidate vaccines. Immune responses directed at either the sporozoite stage or at the infected hepatocyte could, in theory, prevent the blood-stage infection from developing. Since it is during the blood stage of the infection that all morbidity and mortality occurs, a highly effective pre-erythrocytic vaccine could prevent infection or significantly reduce the disease burden associated with malaria.

Figure 3.1

The life-cycle of *Plasmodium falciparum*¹



¹ Source: Moorthy et al. (19). Used with permission from Elsevier.

After repeated rounds of replication in the hepatocyte, an intrahepatocytic stage develops, termed a hepatic schizont, which then ruptures, releasing thousands of small, round, merozoite forms of the parasite into the venous circulation. These merozoites rapidly invade human erythrocytes (see stage 3 in Figure 3.1). Numerous antigens that are unique to either the merozoite (e.g. the merozoite surface antigens) or to the infected erythrocyte (erythrocyte-associated surface antigens, such as RESA) are potential erythrocytic-stage vaccine antigens, and such vaccines would either prevent the invasion of the erythrocyte by the merozoite, or would target the infected erythrocyte for destruction by the host's immune system. The net effect of such erythrocyte-stage immune responses could be to limit or ameliorate the blood-stage manifestations of the malaria infection.

Small subsets of infected erythrocytes undergo a developmental switch into the sexual stage of the organisms, termed gametocytes (see stage 4 in Figure 3.1). Although most gametocytes remain within the host erythrocyte until they are taken up during a blood meal ingested by a female anopheline mosquito, some of the infected erythrocytes rupture in the host's reticuloendothelial system and present gametocyte-specific antigens to the host's immune system. Vaccines targeting gametocyte stages of the parasite, or targeting the fertilized gamete stage, which is found only in the mosquito midgut after fertilization occurs, may provide transmission-blocking immune responses that could interrupt transmission of the parasite from an infected person to an uninfected person by preventing development of a mature sporozoite in the mosquito.

Combination vaccines containing antigens expressed at different stages of the parasite's life-cycle may induce an immune response with a broad biological effect. To date, the most successful approaches to inducing protective antimalarial effects have used whole parasites that have been subjected to irradiation while still in the mosquito's salivary gland and subsequent inoculation of sporozoites by the direct bite of these irradiated mosquitoes. In these experiments it was demonstrated that highly effective infection-preventing immunity could be induced in malaria-naive volunteers (20). Such whole-organism approaches to malaria immunization continue to be explored using various methods, including genetic attenuation of sporozoites and irradiation of sporozoites with subsequent injection by needle and syringe rather than by bite of mosquitoes. However, the results of such efforts have been inconclusive. Additionally, the use of wholeorganism vaccines raises safety and standardization issues that are beyond the scope of this document. The focus of these Guidelines is therefore on firstgeneration recombinant malaria vaccines that have been developed and tested in humans primarily as single-stage and single-antigen constructs.

Naturally acquired antimalarial immunity

After repeated natural exposure to *P. falciparum* malaria infections, individuals develop a significantly reduced risk of developing serious illness or dying from

subsequent malaria infections. This acquisition of immunity through natural exposure is sometimes referred to as premunition. In areas of moderate-to-high transmission in malaria-endemic countries, premunition usually develops by the age of 5–7 years, depending on the intensity of malaria transmission. The development of premunition at these ages helps to explain why the preponderance of deaths due to P. falciparum malaria is found in children who are younger than 5 years. The mechanisms underlying premunition are not fully understood; however, there are two leading hypotheses. One is that the gradual acquisition of strain-specific immunity occurs; the other is that repeated antigenic exposure, perhaps in conjunction with an age-related immune maturation, is necessary for the development of premunition. This immunity does not prevent future malaria infections, and robust infection-blocking immunity is not thought to occur. Additionally, the immunity acquired during childhood does not protect primigravid women, thus accounting for a spike in malaria-attributable deaths in these women. Premunition (or partial immunity) is also known to wane to a significant degree if an individual migrates out of a malaria-endemic region and ceases to have regular exposure to malaria infection for a number of years. Severe malaria illness and death can occur in people who have migrated out of, and then have returned to, a malaria-endemic area (21), which suggests that premunition requires some level of ongoing re-exposure to critical malaria antigens in order to be maintained at effective, disease-ameliorating levels.

It is beyond the scope of these Guidelines to describe the effector mechanisms involved in naturally acquired antimalarial immunity. Significant roles for both humoral and cell-mediated effectors have been demonstrated in animal models, and both humoral and cell-mediated immune responses have been induced in humans after natural malaria infection and exposure to experimental malaria vaccine. No clear correlates of protection have been established for vaccines, although an accumulating body of evidence indicates that antibodies to CSP show some correlation to pre-erythrocytic protection (22), and antibodies to Pfs25, a sexual-stage protein, correlate in animal models with significant transmission-blocking effects (23).

The development of protection against severe disease after natural malaria infection, and the possible role of identifiable and quantifiable effector mechanisms of protection, both lend a positive perspective to the development of effective malaria vaccines. However, the complexity of the parasite and the strong suggestion that naturally induced protection wanes if malaria exposure ceases, pose significant challenges. It is a daunting task to expect a malaria vaccine to produce a better protective response than natural exposure, yet that must be the long-term goal if vaccination is to be the path to achieving sustained control of this disease. Nevertheless, even a partially protective vaccine could have an important role in reducing the burden of malaria disease if it is combined with existing preventive and treatment measures.

Variability in transmission intensity, and effects on clinical disease and acquired immunity

Malaria transmission may be seasonal and generally varies as a function of vector survival and longevity which, in turn, are functions of environmental factors, such as altitude, rainfall and humidity. It has long been recognized that certain areas of the world have intense malaria transmission all year round, whereas other areas have a seasonal pattern of transmission. In areas with a seasonal pattern, transmission may be intense during the transmission season. Although areas can be identified as having a certain level of transmission intensity, malariologists have noted for many years that the actual transmission rates observed tend to be highly local and focal, meaning that malaria transmission and the number of malaria infections identified in a particular area may vary substantially over a rather limited geographical range. The entomological inoculation rate (EIR) is defined as the number of times that an individual is bitten by an infective mosquito in a year. This can be as high as 1000 or more infective bites per year in some areas, but in other malaria-endemic areas the average EIR may be less than 1.

It has been noted that certain clinical manifestations of malaria, such as cerebral malaria, occur more frequently in settings in which transmission is episodic or seasonal, whereas severe and life-threatening anaemia following infection occurs more often in regions where malaria is a year-round threat. If transmission is highly intermittent, a situation may arise in which an entire segment of the population of an area lives through a time when transmission is quite low. Then, perhaps due to changing weather or environmental conditions, transmission may suddenly increase and result in many more-severe cases and deaths than would be expected if transmission had followed a more stable and regular pattern.

The performance of a malaria vaccine may vary according to the seasonal pattern and intensity of transmission. Interpretation of the results of a vaccine trial requires a comprehensive set of baseline data for a given trial location, as well as an understanding of the dynamics of malaria transmission, including the seasonal nature of the disease.

Clinical presentations of malarial disease vary by age, and influence the design of malaria-vaccine trials

The case definitions of an episode of clinical malaria and the methods of diagnosing malaria infection in the context of vaccine trials are addressed in detail later in these Guidelines as well as in related WHO documents (12). Episodes of clinical malaria may present with quite different clinical features, depending on the age of the individual, the intensity of malaria transmission and the clinical stage of the infection. Morbidity due to infection with *P. falciparum*

can range from a mild febrile illness, which is quite difficult to distinguish from many other similar illnesses, to fulminant and life-threatening disease with severe central nervous system stupor and coma, or to a full-blown shock syndrome requiring immediate blood volume support and ancillary supportive measures, or a combination of these. Furthermore, the clinical picture can change within 24 hours, from an illness that appears to be relatively mild to a life-threatening disease. The availability of rapid diagnostic tests can be of great help when expert clinical microscopy is not available. Well designed and carefully executed malaria-vaccine trials must clearly specify how an episode of clinical malaria is to be diagnosed and treated in order to ensure the safety of the subjects, and the validity of the efficacy results.

Antimalarial vaccines used in conjunction with other control measures

As noted above, there has been a resurgence of funding for malaria treatment and vector-control programmes, and these programmes show significant promise for decreasing the burden of malaria. Given the modest vaccine efficacy demonstrated to date, malaria-control efforts will have to combine vector-control strategies with immunization strategies once a licensed malaria vaccine becomes available. Phase III study designs will need to document carefully any control measures, such as the use of LLINs, indoor residual spraying or selective chemoprophylaxis programmes, so that the context in which the vaccine's efficacy was measured can be established; phase III studies also will need to document the comparability of the trial arms, with respect to these factors.

Information is insufficient to predict the public-health benefit of immunizations against malaria that are used in conjunction with the maintenance of other malaria-control measures. Well designed clinical trials can establish the clinical efficacy and confidence intervals for chosen end-points for a malaria vaccine, and can also control for confounding effects from vector-control efforts and programmes aimed at promoting prompt diagnosis and treatment. The longer-term public-health consequences of the simultaneous use of a malaria vaccine and other control measures can be assessed only through post-licensure studies (see section C.3).

Current phase III candidate malaria vaccines

The most advanced candidate is the vaccine against *P. falciparum* infection known as RTS,S/AS01. In phase II studies, RTS,S/AS01 has demonstrated efficacy against clinical malaria when given to children aged 5–17 months at first immunization (1, 2, 24). This vaccine, which is based on the *P. falciparum* sporozoite antigen CSP, was developed after a series of clinical trials demonstrated that simpler CSP-based vaccines provided inadequate clinical

efficacy. Furthermore, in addition to using a novel delivery system based on the hepatitis B-malaria antigen fusion protein (see section A.1.1), novel adjuvants have been utilized because RTS,S formulated on aluminium-containing adjuvants alone afforded no protection in human-challenge studies (25). Various RTS,S/adjuvant formulations have been compared in human-challenge studies (see section C.2.2), and the formulation designated as RTS,S/AS01 appeared to provide the greatest protection (26).

Currently, it is envisaged that RTS,S/AS01 may be considered for deployment to those parts of sub-Saharan Africa where *P. falciparum* is the main species of malaria parasite. This document does not consider evaluation of *P. falciparum* vaccines in areas coendemic for *P. vivax* and *P. falciparum*, such as parts of south-east Asia or South America. It is anticipated that trials in coendemic areas would be performed prior to consideration of deployment of a *P. falciparum* vaccine in such areas.

More than 30 *P. falciparum* malaria-vaccine projects are at either advanced preclinical or clinical stages of evaluation (*27*). As of 2012, RTS,S/AS01 was the only candidate vaccine in pivotal phase III evaluation.

Approaches that utilize recombinant protein antigens and target blood stages are being developed but only pre-erythrocytic vaccine approaches have entered pivotal phase III evaluation.

Part A. Guidelines on manufacturing and control

Part A of this document focuses on the relevant issues relating to the manufacturing process and quality control of recombinant subunit vaccines. The primary goal of this section is to outline the general principles that may apply to a number of recombinant malaria antigens. However, given that the pre-erythrocytic P. falciparum malaria vaccine (RTS,S/AS01) developed by GlaxoSmithKline Biologicals in conjunction with the Malaria Vaccine Initiative is the most advanced candidate (1), a description of this specific vaccine is provided in section A.1 and, where appropriate, additional specific information on the RTS,S/AS01 vaccine is provided in small print. Information on the general aspects of adjuvant formulations is also included in Part A, and some specific details of the proprietary adjuvant AS01 are indicated in small print. Selected details regarding the production and testing of RTS,S/AS01 vaccine are provided as additional guidance for NRAs that may be asked to review this vaccine in the future; these details illustrate the nature of the manufacturing and testing information that should be provided for other vaccines that are under development.

Quality control during the manufacturing process relies on the implementation of quality systems, such as those known as good manufacturing practice (GMP), to ensure the production of consistent vaccine lots with

characteristics similar to those of lots previously shown to be safe and effective. Throughout the process, a number of in-process control tests should be established (with acceptable limits) to allow quality to be monitored for each lot from the beginning to the end of production. It is important to note that most release specifications are product-specific, and therefore will be approved by the NRA as part of the marketing authorization.

A.1 Recombinant malaria antigens under evaluation

As of 2012, there were no licensed malaria vaccines. However, many candidate vaccines using recombinant subunit antigens were under development.

Recombinant subunit *P. falciparum* malaria candidate vaccines under preclinical or clinical evaluation include antigens expressed at different stages of the parasite's life-cycle (28). These antigens include the circumsporozoite (CS) antigen, thrombospondin-related adhesion protein (TRAP), merozoite surface protein type 1 (MSP1) and type 3 (MSP3), apical membrane antigen type 1 (AMA-1), and P. falciparum mosquito stage antigen (Pfs25). Modifications of these and other antigens have also been explored in attempts to overcome the complexities of obtaining correct folding and secondary structure, or to induce better immune responses (e.g. RTS,S). Combination vaccines containing two or more antigens are also under development.

The development of RTS,S/AS01 vaccine was initiated in the 1980s, and is based on a purified recombinant RTS,S antigen expressed in yeast (*Saccharomyces cerevisiae*) and formulated in a novel adjuvant system named AS01 (29).

The CSP gene from the P. falciparum NF54 strain was obtained as a DNA fragment encoding specific portions of the full-length CS (repeat region containing B-cell epitopes and a region containing the T-cell epitopes), and cloned into a suitable vector to produce a fusion protein with the hepatitis B surface antigen (HBsAg) (S antigen). The resulting fusion protein has been designated RTS. The combination of a portion of the CS gene with S antigen gene was selected due to the well known property of the S protein to form particles, and to improve antigen presentation to the immune system (30, 31). To stabilize the recombinant viral-like particles, genes encoding both the RTS fusion protein and the nonfused S antigen are inserted into yeast cells by means of appropriate expression vectors. Thus, the yeast cells produce both the RTS and S proteins, and these spontaneously coassemble into mixed particles that, when purified, comprise the final vaccine antigen (called RTS,S). In summary, the final vaccine antigen is a particle that includes, in defined proportions, the nonfused S hepatitis B antigen and a fusion protein (RTS) that combines the RT portion of the CSP with the S hepatitis B surface antigen.

In addition, the purified RTS,S antigen contains lipid as an integral component of the particle.

The vaccine currently under phase III efficacy evaluation includes the proprietary adjuvant called AS01. This novel adjuvant system contains monophosphoryl lipid A (MPL) and a saponin derived from the bark of the plant *Quillaja saponaria* (QS21) as immunostimulants formulated together with liposomes. Studies have demonstrated that adjuvants capable of enhancing antibody and cellular immunity are required to achieve protection against malaria in human-challenge models (26, 32).

The liquid adjuvant system AS01 is used to reconstitute the lyophilized RTS,S antigen prior to administration. The RTS,S antigen does not adsorb to the adjuvant when the antigen is reconstituted with AS01; thus antigen adsorption is not included among the control tests for this product. The RTS,S/AS01 vaccine is for prophylactic use.

A.2 **Terminology**

The definitions given below apply to the terms as used in these Guidelines. They may have different meanings in other contexts.

Adjuvant: a substance or a composition of substances that potentiates and/or modulates the immune response to an antigen towards the desired effect.

Adventitious agents: contaminating microorganisms of the cell substrate, or materials used in their culture; these may include bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses that have been unintentionally introduced.

Cell bank: a collection of ampoules containing aliquots of a suspension of cells from a single pool of cells of uniform composition, which are stored frozen under defined conditions.

Final bulk: the formulated antigen bulk, prepared from one or more batches of purified bulk, present in the container from which the final containers are filled prior to lyophilization.

Final lot: a collection of sealed final containers of lyophilized vaccine that is homogeneous with respect to the risk of contamination during the filling and lyophilization process. Therefore, a final lot must have been filled from a single vessel of final bulk during one working session.

Master cell bank (MCB): a collection of containers holding aliquots of a suspension of cells from a single pool of cells of uniform composition, which are stored frozen under defined conditions. The MCB is used to derive all working cell banks for the anticipated lifetime of vaccine production following licensure.

Production cell culture: a cell culture derived from one or more containers of the working cell bank and used for the production of vaccines.

Purified antigen bulk: the purified antigen prior to the addition of any substances, such as diluents and stabilizers.

Single harvest: the biological material prepared from a single fermentation run prior to the downstream (purification) process.

Working cell bank (WCB): a collection of containers holding aliquots of a suspension of cells from a single pool of cells of uniform composition, which have been derived from the MCB, and that are stored frozen under defined conditions (typically below –60 °C for yeast). One or more aliquots of the WCB are used for routine production of the vaccine. Multiple WCBs are made and used during the lifetime of the vaccine product.

A.3 General manufacturing guidelines

The general manufacturing recommendations contained in WHO good manufacturing practices for pharmaceutical products: main principles (33) and Good manufacturing practices for biological products (34) apply to the establishment of manufacturing facilities.

A.4 Control of source materials

A.4.1 Yeast cell substrates for antigen production

The use of any cell substrate should be based on a cell bank system. Only cells that have been approved and registered with the NRA should be used for production. The NRA should be responsible for approving the cell bank. An appropriate history should be provided for the cell bank.

A.4.1.1 Yeast cells

The characteristics of the recombinant production strain (host cell in combination with the expression vector system) should be fully described, and information should be given about the absence of adventitious agents (35, 36) and gene homogeneity for the MCBs and WCBs. A full description of the biological characteristics of the host cell and expression vectors should be given. The physiological measures used to promote and control the expression of the cloned gene in the host cell should be described in detail. These measures should include assessments of the genetic markers of the host cell; the construction, genetics and structure of the expression vector; and the origin and identification of the gene that is being cloned.

The nucleotide sequence of the gene insert and of adjacent segments of the vector, and restriction-enzyme mapping of the vector containing the gene insert, should be provided if required by the NRA. Thorough characterization of the gene product should be done during product development, and documentation should be provided in support of licensure (see Part B, Nonclinical evaluation).

Both the MCB and the WCB must be maintained in a frozen state that allows recovery of viable cells without alteration of the genotype.

If necessary, the cells should be recovered from the frozen state in selective media such that the genotype and phenotype consistent with the unmodified host and unmodified recombinant DNA vector are maintained and clearly identifiable. Cell banks should be identified and fully characterized by means of appropriate tests. The MCB and WCB should be tested for the absence of adventitious agents according to Part A of WHO General requirements for the sterility of biological substances no. 6 (1973) (35) or by a method approved by the NRA.

Data that demonstrate the stability of the expression system during storage of the recombinant WCB up to or beyond the passage level used for production should be provided to and approved by the NRA. Any instability of the expression system occurring in the seed culture or after a production-scale run should be documented.

A.4.2 Fermentation medium

Production fermentation should be performed in a defined culture medium that has been shown to be suitable for the production of relevant antigens with consistent yields. The acceptability of the source(s) of any components used of bovine, porcine, sheep or goat origin should be approved by the NRA. Components should comply with WHO guidance relating to animal-transmissible spongiform encephalopathies (37–39). The NRA should approve any change in the media used.

A.5 Fermentation

A.5.1 Production of cell cultures

Only cell cultures derived from the WCB should be used for production. All processing of cells should be done in a designated facility in which no cells or organisms are handled other than those directly required for the process.

A.5.1.1 Control of antigen production up to single harvest

Microbial purity in each fermentation vessel should be monitored at the end of the production run by methods approved by the NRA. Any agent added to the fermenter or bioreactor to feed cells or to induce or increase cell density should be approved by the NRA.

A.5.2 Genetic characterization and stability

Where the plasmid is integrated into the host-cell genome, the presence of the integrated antigen sequences should be confirmed. The DNA sequence of the cloned gene should normally be confirmed from the cell bank stage up to and beyond the usual level of population doubling for full-scale fermentation. Southern blot analysis of total cellular DNA or sequence analysis of the messenger

RNA (mRNA), may be helpful. The copy number, physical state and stability of the vector inside the host cell should be documented (40, 41).

For the RTS,S/AS01 vaccine, documentation of the characterization and stability of the genes encoding for the RT and S fusion protein and the nonfused S protein should be provided in support of licensure.

A.6 Single harvests

A.6.1 Storage and intermediate hold times

During the purification process, all intermediates should be maintained under conditions shown by the manufacturer to ensure they retain the desired biological activity. Hold times should be based on validation studies, and approved by the NRA.

A.6.2 Tests on single harvests

A.6.2.1 **Sampling**

Samples required for the testing of single harvests should be taken immediately on harvesting, prior to further processing.

A.6.2.2 Test for contamination by bacteria and fungi

Microbial and fungal contamination in the fermentation vessels should be monitored at the end of production, and should be tested for according to Part A, section 5.2 of General requirements for the sterility of biological substances no. 6 (1973) (35) or by methods approved by the NRA.

A.6.3 Consistency of yield

Data on the consistency of yield between runs and during individual production runs should be provided, and the NRA should approve the criteria for determining what constitutes an acceptable production run.

A.7 Control of purified antigen bulk

The purification procedure can be applied to a single harvest or to a pool of single harvests. When applicable, the maximum number of single harvests that may be pooled should be defined on the basis of validation studies, and should be approved by the NRA. Adequate purification may require several purification steps based on different principles (e.g. size, charge or hydrophobicity). The use of procedures that rely on different physicochemical properties of the molecules will minimize the possibility of copurification of extraneous cellular materials. The methods used for the purification of the vaccine antigen should be appropriately validated, and then approved by the NRA. Any agent added to the purification

process should be documented, and its removal should be adequately validated and tested for as appropriate (see section A.7.1.8).

The purified antigen bulk can be stored under conditions shown by the manufacturer to retain the desired biological activity. Intermediate hold times should be approved by the NRA.

A.7.1 Tests on the purified antigen bulk

The purified antigen bulk should be tested using the tests listed below. All quality-control release tests, and specifications for purified antigen bulk, should be validated and shown to be suitable for the intended purpose. Additional tests on intermediates during the purification process may be used to monitor the consistency and yield.

Purity A.7.1.1

The degree of purity of each purified antigen bulk should be assessed using suitable methods. Examples of suitable methods for analysing the proportion of degradation products and potential contaminating proteins in the total protein of the preparation are polyacrylamide gel electrophoresis (PAGE), optionally followed by densitometric analysis, and high-performance liquid chromatography (HPLC). Specifications should be established based on the formulation that provided acceptable data on safety and efficacy. Specifications should be set during the processes of product development and validation, and established by agreement with the NRA.

For the RTS,S/AS01 vaccine, the RTS and S proteins should be not less than 95% of the total protein in the purified antigen bulk.

Protein content A.7.1.2

The protein content should be determined by using a suitable method, such as the micro-Kjeldahl method, the Lowry technique or another method.

A.7.1.3 Antigen content

The antigen content of the purified antigen bulk should be determined by an appropriate immunochemical method that measures antigenic activity. An appropriate antigen reference material - of known purity, antigenic activity and protein content - should be included in these assays. The assays should be designed so that the consistency of production can be monitored. This reference material should either be a representative bulk or a highly purified preparation stored in single-use aliquots.

For the RTS,S/AS01 vaccine, the antigenic activity of the RTS,S antigen should be determined by means of an immunological assay, such as the enzyme-linked immunosorbent assay (ELISA), and a protein-content assay. The antigenic activity is defined as the ratio of the result from the immunological assay to the result from the protein-content test. The test method and acceptable limits should be established in agreement with the NRA.

For the RTS,S/AS01 vaccine, the ratio between the two polypeptides S and RTS should be determined using an appropriate analytical method. The S to RTS ratio should be within a range defined by those lots and shown to have acceptable performance in clinical trials. The limits should be established in agreement with the NRA.

A.7.1.4 **Identity**

Tests used for assessing other properties of the antigen, such as antigen content or purity, will generally be suitable for assessing the identity of the protein in the bulk. For instance, immunoblots of PAGE separations using antigen-specific antibodies could be used to confirm the molecular identity of the product. The identity testing approach should be defined during the processes of product development and validation, and should be approved by the NRA.

A.7.1.5 Lipids

The lipid content of each purified antigen bulk should be determined using an appropriate method. The methods used and the permitted concentrations of lipid should be approved by the NRA. This test may be omitted after the consistency of the purification process has been demonstrated to the satisfaction of the NRA.

A.7.1.6 Carbohydrates

The carbohydrate content of each purified antigen bulk should be determined using an appropriate method. The methods used and the permitted concentrations of carbohydrates should be approved by the NRA. This test may be omitted after the consistency of the purification process has been demonstrated to the satisfaction of the NRA.

A.7.1.7 Sterility tests for bacteria and fungi

Each purified antigen bulk should be tested for freedom from bacteria and fungi following WHO General requirements for the sterility of biological substances no. 6 (1973) (35) or by using a method approved by the NRA.

A.7.1.8 Tests for agents used during purification or other phases of manufacture

The purified antigen bulk should be tested for the presence of any potentially hazardous agents used during manufacture. The method and the concentration

limits used should be approved by the NRA. These tests may be omitted for routine lot release after it has been demonstrated that the validated purification process consistently eliminates the agents from the purified bulks.

A.7.1.9 Tests for residuals derived from the antigen expression system

The amount of residuals derived from the antigen expression system (e.g. DNA or host-cell proteins) should be determined in each antigen purified bulk using acceptable methods. These tests may be omitted for routine lot release after it has been demonstrated that the validated purification process consistently eliminates the residual components from the bulks to the satisfaction of the NRA.

A 7 1 10 Bacterial endotoxins

Each final purified antigen bulk should be tested for bacterial endotoxins. The method and the concentration limits used should be approved by the NRA. At the concentration of the final formulation of the vaccine, the total amount of residual endotoxins should not exceed that found in vaccine lots shown to be safe in clinical trials or in data from other lots used to support licensing.

A.8 Final bulk

The final bulk may be prepared from one or more purified antigen bulks. Only bulks that have satisfied the requirements outlined in previous sections should be included in the final bulk. The antigen concentration in the final formulation should be sufficient to ensure that the dose is consistent with that shown to be safe and effective in human clinical trials. Formulation is generally based on protein content, but antigen content may be used.

The operations necessary for preparing the final bulk should be conducted in such a manner as to avoid contaminating the product. In preparing the final bulk, any substances that have been added to the product, such as adjuvants, diluents and stabilizers, should have been shown to the satisfaction of the NRA not to impair the safety and efficacy of the vaccine in the concentration used. Until the bulk is filled into containers to prepare the final vaccine or the lyophilized antigen, the final bulk suspension should be stored under conditions that, according to the manufacturer, will ensure that the desired biological activity is retained during the time-limit for holding approved by the NRA.

A.8.1 Tests on the final bulk

Depending on the production process or the characteristics of the vaccine, some tests may be performed on the final bulk rather than on the final product if the NRA agrees.

A.8.1.1 Sterility tests

Each final antigen bulk should be tested for bacterial and fungal sterility according to WHO General requirements for the sterility of biological substances no. 6 (1973) (35) or by using a method approved by the NRA.

A.9 Filling and containers

The general requirements concerning filling and containers given in Good manufacturing practices for biological products (34) apply to vaccine filled in the final form.

Care should be taken to ensure that the materials from which the container and, if applicable, the closure are made do not adversely affect the immunogenicity of the vaccine under the recommended storage conditions.

A.10 Control tests on final lot

Samples should be taken from each final vaccine lot to be tested; these samples must fulfil the requirements of this section. All of the tests and specifications, including the methods used and the permissible limits for different parameters described in this section, unless otherwise specified, should be approved by the NRA. The specifications should be defined based on the results of tests on lots that have been shown to have acceptable performance in clinical studies.

The requirements concerning filling and containers given in WHO Good manufacturing practices for biological products (34) must be met, or an NRA-approved method applied.

The RTS,S vaccine is a two-component vaccine consisting of the RTS,S antigen (lyophilized) and the AS01 adjuvant (liquid). Immediately prior to administration, the full contents of the AS01 vial are withdrawn and added to the antigen vial to reconstitute the RTS,S. During product development and process validation, relevant testing, including evaluation of potential interactions between the antigen and adjuvant, should be conducted on batches of final-container RTS,S antigen lot that have been reconstituted using the final adjuvant system lot. This should be done until consistency has been demonstrated to the satisfaction of the NRA. Routine release testing of only the individual vaccine components (i.e. antigen final container and adjuvant system final container) may be justified when the consistency and compatibility of the antigen and adjuvant have been demonstrated.

Care should be taken to ensure that the materials comprising the container and, if applicable, transference devices and closure systems, do not adversely affect the quality of the vaccine. The manufacturer should provide the NRA with adequate data to prove that the product remains stable under appropriate conditions of storage and shipping.

A.10.1 Inspection of containers

Each container of each the final vaccine-antigen lot should be inspected visually or mechanically, and those containers showing abnormalities should be discarded.

A.10.2 Appearance

Visual inspection of the appearance of the vaccine should be described with respect to the form and colour.

The appearance of each of the individual components of the RTS,S/ AS01 vaccine should be examined (i.e. the RTS,S final-container lot and the AS01 final-container lot) as should the final reconstituted vaccine. Visual inspection of the reconstituted vaccine may be discontinued when consistency has been demonstrated to the satisfaction of the NRA.

A.10.3 **Identity**

An identity test should be performed on at least one labelled container from each final lot using methods approved by the NRA. The test used for determining the antigen content will generally be suitable for assessing identity. Alternatively, immunoblots using antigen-specific antibodies could also be used to confirm the molecular identity of the product.

A.10.4 Sterility tests

Each final vaccine lot should be tested for bacterial and fungal sterility according to the requirements outlined in WHO General requirements for the sterility of biological substances no. 6 (1973) (35) or by using an acceptable method that has been approved by the NRA.

For RTS,S/AS01 vaccine, vials from both the lyophilized final lot and the liquid adjuvant AS01 should be tested for bacterial and fungal sterility.

A.10.5 General safety test

Each final lot should be tested in mice or guinea-pigs to confirm the absence of abnormal toxicity using a test approved by the NRA. This test may not be required for routine lot release after the consistency of production has been established to the satisfaction of the NRA.

Each final lot of RTS,S antigen should be tested to confirm the absence of abnormal toxicity upon reconstitution with the proprietary adjuvant system AS01.

A.10.6 **pH**

The pH value of each final batch of containers should be tested. Lyophilized products should be reconstituted prior to testing using the diluent approved by the NRA

A.10.7 Pyrogen and endotoxin content

The vaccine in the final container should be tested for pyrogenic activity through intravenous injection into rabbits. A Limulus amoebocyte lysate (LAL) test may be used in lieu of the rabbit pyrogen test if it has been validated. Similarly, a suitably validated monocyte activation test may be considered as an alternative to the pyrogen test. The endotoxin content or pyrogenic activity should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and approved by the NRA. The test may not be required after the consistency of production has been demonstrated to the satisfaction of the NRA. Lyophilized products should be reconstituted prior to testing using the diluent approved by the NRA.

When testing is performed on RTS,S reconstituted with the adjuvant system, the rabbit test for pyrogens should be performed because the adjuvant may interfere with the LAL test.

A.10.8 Protein content

The protein content should be determined using a method approved by the NRA. For some products, the protein content may be calculated using an intermediate from an earlier process if this can be adequately justified and has been approved by the NRA. Specifications should be within the limits for vaccine preparations shown to have acceptable performance in clinical studies.

For the RTS,S/AS01 vaccine, the protein content should be measured in the lyophilized RTS,S antigen.

A.10.9 Moisture content

When the antigen is lyophilized, the average moisture content should be determined using methods that have been accepted by the NRA. Values should be within the limits for preparations shown to be adequately stable in stability studies of the vaccine.

For RTS,S/AS01, the moisture content of the vaccine should be measured in the lyophilized RTS,S antigen.

A.10.10 Potency test

An appropriate quantitative test for potency should be performed on samples representative of the final vaccine lot. The method used and the data analysis

should be approved by the NRA. The vaccine's potency should be compared with that of an approved reference preparation. Establishment of the specifications for each product should be based on the response observed in tests on vaccine clinical lots and data from other lots used to support licensing. The specifications for each antigen claimed to contribute to efficacy should be approved by the NRA.

Because no International Standard for the RTS,S antigen is available, the manufacturer should establish a product-specific reference preparation that can be traced to a vaccine lot that has had demonstrated efficacy in clinical trials. Methodological considerations regarding in vivo and in vitro potency assays for RTS,S/AS01 are available on the WHO Biologicals web site at: http://who.int/biologicals/vaccines/malaria/en/index.html and will be updated when necessary.

A.10.11 Control tests on the adjuvant

The quality control tests for adjuvants and source materials are specific to the components (e.g. MPL or lipids) and the characteristics of the adjuvant formulation (e.g. water–in-oil emulsions). All of the tests and specifications, including the methods used and permitted concentrations, should be approved by the NRA.

The tests for the proprietary adjuvant system AS01 should be carried out on the final containers of the AS01 adjuvant.

A.10.11.1 Identity and content of adjuvant system components

Components of the final adjuvant system should be identified and quantified using appropriate methods.

A.10.11.2 Adjuvant system quality attributes

Specifications for the relevant quality attributes of the adjuvant system should be set and approved by the NRA. Each component of the adjuvant system should be shown to meet defined purity characteristics.

- For the RTS,S/AS01 vaccine, each final lot of the AS01 adjuvant system should be assayed for average liposome size and size distribution using suitable analytical methods. The methods and limits used should be approved by the NRA.
- The pH of each final lot of the AS01 adjuvant system should be tested.
- Sterility tests should be performed on each final lot of the AS01 adjuvant system to comply with WHO Guidelines (35, 36).

■ The AS01 adjuvant system in the final container should be tested for pyrogenic activity by intravenous injection into rabbits. The test for pyrogenic activity may not be required after the consistency of production has been demonstrated to the satisfaction of the NRA.

Each final lot of AS01 should be assayed for immunostimulants (i.e. QS21 and MPL) and the content of liposome components. The methods used and the concentrations permitted should be approved by the NRA.

A.11 Records, retained samples, labelling, distribution and transport

The requirements given in WHO Good manufacturing practices for biological products (34) apply. In addition, the label on the carton, the container or the leaflet accompanying the container should state:

- the nature of the cells used to produce the antigen;
- the nature and amount of adjuvant present in the vaccine;
- the volume of one recommended human dose, the immunization schedule, and the recommended routes of administration (this information should be given for neonates, children and immunosuppressed individuals, and should be the same for a given vaccine in all parts of the world);
- the amount of active substance contained in one recommended human dose.

Efforts should be made to ensure that shipping conditions are such as to maintain the vaccine in an appropriate environment. Temperature indicators should be packaged with each vaccine shipment to monitor fluctuations in temperature during transportation. Further guidance is provided in WHO Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (42).

A.12 Stability testing, storage and expiry date

A.12.1 Stability testing

Adequate stability studies form an essential part of vaccine development. Guidance on evaluating vaccine stability is provided in WHO Guidelines on stability evaluation of vaccines (43). In addition to testing the final product, stability testing should include testing at intermediate stages of production.

For the RTS,S/AS01 vaccine, the stability of the final vaccine antigen lot (lyophilized RTS,S antigen) and the adjuvant system (liquid AS01 adjuvant) should be demonstrated at the recommended storage temperatures to the satisfaction of the NRA. The formulation of RTS,S

antigen and the adjuvant system must be stable throughout the claimed conditions for use (e.g. up to 6 hours after reconstitution) when stored according to instructions. Acceptable limits for stability should be agreed with the NRA.

A.12.2 Storage conditions

Storage conditions should be fully validated and approved by the NRA. The vaccine (including the antigen and adjuvant system) should have been shown to maintain its potency for a period equal to the duration from the date of release to the expiry date.

A.12.3 Expiry date

The expiry date should be fixed with NRA approval, and should take into account the experimental data on the stability of the vaccine (including both the antigen and the adjuvant system).

Part B. Nonclinical evaluation of malaria vaccines

B.1 Introduction

The nonclinical evaluation of malaria vaccines should be based on WHO guidelines on nonclinical evaluation of vaccines (7). Prior to the clinical testing of any new or modified malaria vaccine in humans, there should be extensive product characterization, immunogenicity and safety testing, and proof-of-concept studies in animals. The nonclinical testing of vaccines is a prerequisite for the initiation of clinical studies in humans. There is no laboratory test or series of tests that will unequivocally ensure that a newly developed malaria vaccine will be adequately safe and effective. In view of this limitation, manufacturers are expected to provide information describing the approach taken to the collection of supporting evidence, beginning with a comprehensive programme of nonclinical testing, and followed by a progression of clinical evaluations. The extent to which nonclinical studies will be required depends on the type of antigen and the complexity of the formulation, particularly when novel adjuvant systems are employed.

The following sections describe the types of nonclinical information that should be submitted to the NRA. The purpose of the submissions will vary during the product-development process. In some cases, these nonclinical data will be submitted to support the initiation of a specific clinical study; in other cases, the nonclinical data will be included in a marketing-authorization application.

These Guidelines should be read in conjunction with WHO guidelines on nonclinical evaluation of vaccines (7). They are specifically aimed at the nonclinical evaluation of malaria vaccines that are based on recombinant antigens in the context of the development of a new vaccine, or when significant changes to the manufacturing process require re-evaluation and re-characterization of a vaccine.

The goal of preclinical testing, defined as the nonclinical testing carried out prior to initiation of any clinical investigations, is to develop a package of supporting data and product information that justifies the move to clinical studies. These data should provide evidence that:

- the vaccine antigens and final product are well defined and thoroughly characterized;
- the vaccine administered to humans is likely to be well tolerated by the target population;
- the vaccine is reasonably likely, on the basis of animal immunogenicity and, when applicable, protection data, to provide protection from clinical malaria.

These issues are discussed in detail below.

Vaccine lots used in nonclinical studies should be adequately representative of those intended for clinical investigation and, ideally, should be the same lots as those used in clinical studies. If this is not feasible, then the lots used in nonclinical studies should be comparable to clinical lots with respect to formulation, physicochemical data, quality characteristics and the stability profile. Details on the design, conduct, analysis and evaluation of nonclinical studies are available in WHO guidelines on nonclinical evaluation of vaccines (7).

Similar considerations should be given to vaccines based on alternative technologies (e.g. viral vectors, DNA or whole organisms). However, it is beyond the scope of this document to provide testing requirements for these vaccine platforms.

Product development and characterization

The general principles of vaccine production, testing and stability are described in WHO guidelines on nonclinical evaluation of vaccines (7). The nonclinical and preclinical testing should include extensive product characterization; however, the nature and extent of the characterization studies may vary according to the stage of development. The testing information obtained during development provides guidance on the product's characteristics, as well as on the tests and evaluation criteria that are appropriate for quality control, as defined in Part A of this document.

B.2.1 Strategy for cloning and expressing the gene product

A full description of the biological characteristics of the host cell and expression vectors used in production should be given. This should include details of: (i) the

construction, genetics and structure of the expression vector; and (ii) the origin and identification of the gene that is being cloned. The physiological measures used to promote and control the expression of the cloned gene in the host cell should be described in detail.

Data should be provided to demonstrate the stability of the expression system during storage of the WCB, and beyond the passage level used for production. Any instability of the expression system occurring in the seed culture or after a production-scale run – such as rearrangements, deletions or insertions of nucleotides – must be documented. The NRA should approve the system used.

B.2.2 Characterization of the vaccine antigen

The molecular size and integrity of the expressed protein and its composition should be established by techniques such as sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining under reducing and nonreducing conditions, size-exclusion chromatography, and appropriate analyses of any carbohydrate and lipid components or modifications.

The identity of the protein should be established by peptide mapping or terminal amino acid sequence analysis, or both. Following SDS-PAGE, the protein bands should be identified in immunoblots using specific antibodies (e.g. monoclonal antibodies) to confirm the presence of the expected gene product(s). The primary structure of the protein should be further characterized by suitable methods, such as partial amino acid sequence analysis and peptide mapping. Mass spectrometry may be used to confirm the average molecular mass and the presence of the protein in the preparation.

Rigorous identification and characterization of recombinant DNAderived antigens are required as part of the marketing-authorization application. If applicable and relevant, the ways in which the recombinant antigens differ chemically, structurally, biologically or immunologically from naturally occurring antigens must be fully documented. Such differences could arise during processing at the genetic or post-translational level, or during purification.

Characterization of vaccine formulations B.2.3

Justification for the selection of the antigen(s) and the adjuvant system, in particular supporting evidence of the adjuvant's mode of action, should be given. It is important to note that adjuvants are not licensed in their own right but only as a component of a particular vaccine. Evaluations of the vaccine formulation should include proof-of-concept studies that evaluate the enhancement of the desired immune response. Additionally, antigen/adjuvant formulations will need to be fully characterized, and have undergone adequate pharmacotoxicological studies, before clinical evaluation, as discussed in section B.4. Advances in the understanding of the mechanisms that protect against malaria suggest that both

humoral and cell-mediated immunity (22) are important. This, together with the generally poor immunogenicity of recombinant subunit antigens, has led to the development and assessment of various adjuvant systems that are capable of stimulating both adaptive and innate immunity, including a broad range of antibody and cellular immune responses.

Further guidance on the general principles of the nonclinical assessment of adjuvants can be found in the WHO guidelines on nonclinical evaluation of vaccines (7) and in the European Medicines Agency's Guideline on adjuvants in vaccines for human use (44).

B.3 Pharmacodynamic studies

Immunogenicity studies in animal models can provide important information with respect to the optimization of adjuvant formulations and the evaluation of immunological characteristics of the antigen including, for some antigens, the ability to induce functional antibodies. Animal models of malaria have provided insights into the mechanisms of both pathogenesis and immune protection, and these studies have led to the identification of potential vaccine approaches. However, experience has shown that extrapolating data from animal models to human disease should be done with caution.

B.3.1 Rodent models

Rodent models have frequently provided initial evidence of the immunogenicity or efficacy, or both, of potential malaria vaccines despite the significant limitation that these models are unnatural hosts for human parasites. In contrast to the chronic infection that develops in the natural host and parasite combination, rodent models develop acute, and often lethal, infections. A major potential limitation of these models is that rodents' immune responses to malarial antigens may be not relevant to natural human infections. Additionally, the lack of standardization in the route of administration, method of challenge and endpoints of the numerous rodent models makes comparisons and comprehensive evaluations difficult. Nevertheless, the ability to utilize these models provides mechanisms for the scientific examination of immunological relationships, and facilitates understanding of potential clinical issues. Immunization-challenge models utilizing sporozoites or erythrocytes parasitized with P. yoelii, P. berghei and P. chabaudi are often used in conjunction with vaccine constructs based on these orthologue Plasmodium species. When interpreting data from these studies, the caveats of the evolutionary distance between rodent biology and human biology, as well as between the rodent and human species of Plasmodium, must be considered. Lack of protection in such models is taken as an indication not to progress a given candidate vaccine; and while demonstration of protection does not necessarily predict clinical protection, it provides some

rationale and justification for the clinical development of candidate antigens or vaccine platforms.

In the protection against blood-stage challenge with sporozoites or parasitized red blood cells, the usual end-points measured with lethal and nonlethal strains of parasites are the reduction in peak parasitaemia, prolongation of the prepatent period and protection from mortality.

B.3.2 Nonhuman primate models

The genetic and morphological similarity between humans and nonhuman primates makes nonhuman primates potentially useful models for the evaluation of candidate vaccines. The susceptibility of these primates to human malaria, albeit in modified forms, is their chief advantage over other animal models; however, the nonhuman primate model should not necessarily be given preference over other available models.

The Aotus and Saimiri species of New World monkeys and the Macaca mulatta species (i.e. rhesus macaque) of Old World monkeys are commonly used to examine the immunogenicity and potential efficacy of candidate malaria vaccines. New World monkeys are most useful for their receptivity to P. falciparum, P. vivax and P. malariae. The malaria model in the Aotus monkey is useful for investigations of blood-stage infections of these strains of malaria. In addition, Aotus species have been used for the study of mosquito transmission and for susceptibility studies of sporozoite-induced infections and liver-stage studies. The Saimiri monkey model provides useful information for investigating P. vivax infections and P. falciparum blood-stage infection. When compared with humans, New World monkey models often demonstrate a more rapid acquisition of effective immunity, and the development of life-threatening anaemia. Additionally, these nonhuman primates may demonstrate variable parasitological parameters and a tolerance for high parasitaemia. There is a limited set of P. falciparum and P. vivax isolates and antigenic types adapted for these models; the limitation being that the 3D7 isolate is chloroquine-sensitive.

The rhesus monkey possesses a relatively high degree of homology to humans; however, the rhesus monkey is refractory to most human species of malaria parasites. These monkeys are susceptible to sporozoite or blood-stage-induced infections with *P. knowlesi* (a species for which human infections have been reported) and, when infected with appropriate parasite species such as *P. simiovale* or *P. cynomolgi*, these nonhuman primates can develop chronic infections, semi-immune states, frequent recrudescence and patterns of relapsing infections.

Although nonhuman primates are particularly useful for assessing potential efficacy because of their similarity to humans, the availability, cost and ethical considerations surrounding their acquisition, housing, care and disposition present practical limitations.

B 3.3 Immunological end-points

In the immunization-challenge models, the end-points used to define protection are important and vary among different models. The absence of blood-stage infection or delay in patency is used as a measure of pre-erythrocytic protection in the sporozoite-challenge model. In the same model, liver-stage parasite burden can be measured by the reduction in the number of late liver-stage parasites following challenge.

Immunogenicity should be measured as humoral, cellular or functional immune responses in the various models. Parasite-specific assays – such as immunofluorescence assays against sporozoites, liver-stage or blood-stage parasites, or a combination of these – or antigen-specific assays – such as ELISA, that measure the quantity and subclass of antibody to recombinant proteins or synthetic peptides – should be used to characterize humoral responses. Cellular responses should examine CD8+ and CD4+ T cells, and Th1 and Th2 responses using assays such as the enzyme-linked immunosorbent spot (ELISPOT), intracellular cytokine staining or multiparameter flow cytometry. Functional immune-response activity may be measured in vitro using methods such as the growth inhibition assay (45) and antibody-dependent cellular inhibition assay. More extensive analyses of the functional activity of immune responses may include the kinetics and duration of CD8+ and CD4+ cells and antibody responses, as well as assessing the quality or fine specificity of the antibody response.

B.4 Toxicity

Toxicology studies should be undertaken on the final vaccine formulation in accordance with WHO guidelines on nonclinical evaluation of vaccines (7). If the vaccine is to be used in adults, additional studies may be needed (e.g. to assess the risk of using a particular adjuvant/antigen combination during pregnancy).

If the vaccine is formulated with a novel adjuvant, nonclinical toxicology studies should be conducted, as appropriate, for the final vaccine (these should include the antigens and the adjuvant). A repeated-dose toxicity study may be used to compare the safety profile of the novel adjuvant with the safety profile of an established vaccine formulation, taking into account existing guidelines (7, 44). If no toxicological data exist for a new adjuvant, toxicity studies of the adjuvant alone may, in some situations, provide useful information; however, consultation with the NRA is encouraged.

If a novel cell substrate (i.e. a substrate that has not been previously licensed or used in humans) is used for the production of the recombinant antigen (46), safety aspects, such as potential immune responses elicited by residual host-cell proteins, should be investigated in a suitable animal model.

Part C. Clinical evaluation of malaria vaccines

C.1 Introduction

Clinical trials should adhere to the principles described in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (8) and in WHO Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (47), as well as other guidance (48). All clinical trials should be approved by the relevant NRA. This document adheres to the definition of phases of clinical trials as defined in Guidelines on clinical evaluation of vaccines: regulatory expectations (8). Details specific to malaria vaccines, particularly those related to the definitions of phase IIa and phase IIb trials, are included in section C.1.1 below.

The guidance regarding clinical development programmes provided in this section should be viewed in the light of data on the safety, immunogenicity, efficacy and effectiveness of malaria vaccines that may become available in the future. For example, the existence of a licensed malaria vaccine in the future may influence the design of clinical trials of new malaria vaccines in some settings.

C.1.1 Outline of the clinical development programme

No licensed malaria vaccines are currently available, and no immunological correlates of protection have been established that could be used to predict the protection afforded to individuals by vaccination against clinically apparent malaria. Therefore, the protective efficacy of candidate malaria vaccines has to be demonstrated in prelicensure studies of an appropriate design in which subjects in the control groups do not receive the test product.

Before proceeding to large-scale phase III efficacy studies, the evidence regarding safety, immunogenicity and efficacy obtained from nonclinical studies and phase I and phase II clinical studies should support an expectation that a clinically useful degree of efficacy may be achievable without unacceptable adverse effects, when the vaccine is administered using the planned dosing regimen.

As described in the sections that follow, a typical clinical development programme for a candidate malaria vaccine could include the following, using a logical progression between phases:

- extensive nonclinical studies that provide data to support human use (as described in Part B);
- initial safety and immunogenicity studies in healthy adults i.e.
 phase I studies (since it is envisaged that malaria vaccines would
 ultimately be intended for use from infancy, these studies should
 include adults who are naive to malaria as well as non-naive adults);
- human-challenge studies in naive adult subjects i.e. phase IIa studies (see Appendix 1);

- safety and immunogenicity data from subjects aged < 18 years who are resident in endemic areas; with progression to the target age group;
- one or more larger clinical studies in endemic areas that evaluate safety and immunogenicity and are of a sufficient size to provide preliminary evidence of efficacy – i.e. phase IIb studies (some of these studies should be conducted in, or at least include some data from, the target age group);
- one or more concomitant immunization trials in the target population that evaluate potential interactions with other vaccines administered on the same schedule;
- one or more randomized controlled studies of sufficient size to provide definitive evidence of protection in at least one pivotal study of efficacy – i.e. a phase III study;
- post-licensure studies of safety and effectiveness i.e. phase IV studies.

Clinical studies

C.2.1 Immunogenicity

The assessment of the immunogenicity of candidate malaria vaccines is an essential part of the clinical-development programme (see Appendix 2 for an example). This is needed to underpin:

- the selection of the dose of antigen;
- the inclusion of an adjuvant (if this is proposed);
- the selection of a primary vaccine regimen to be evaluated for efficacy in the target population, including the route of administration and the immunization schedule;
- the potential for boosting immune responses by revaccination should the efficacy studies or effectiveness data, or both, indicate a waning of protection over time;
- the possible need to adjust the dose regimen for subgroups that may have lower immune responses – such as HIV-positive people with low CD4 cell counts or people who are severely malnourished (immune-response data could be obtained from such subgroups in separate studies or during protective efficacy studies as part of an immunogenicity substudy).

Identifying the parameters that may be most sensitive for demonstrating differences in immune responses (e.g. between vaccine formulations and

populations) is also necessary to ensure that clinical studies are well designed and will support:

- changes in manufacture, which may occur during or after licensure, or both, that are considered to have some potential to affect immune responses;
- assessment of the effects of co-administration with other vaccines (i.e. assessment of immune-interference phenomena);
- comparisons between populations (e.g. in different geographical locations) with varied transmission intensities and varied malaria strains in circulation, and with various host factors that could affect immune responses.

As part of the overall evaluation of immunogenicity, it is recommended that sufficient blood samples be obtained during the phase III efficacy studies to enable both a comprehensive exploration of the immune response to malaria vaccines and an evaluation of any correlation there may be between immune responses and protection against clinical malaria.

C.2.1.1 Measurement of immune responses to vaccination

The mechanism(s) of naturally acquired immunity to *Plasmodium* species, including *P. falciparum*, are not fully understood. It is thought that humoral immunity is directed against antigens expressed during the asexual blood stages, but seroprevalence studies in residents of endemic areas have indicated low-to-moderate levels of immunoglobulin G (IgG) directed against the *P. falciparum* circumsporozoite protein (anti-CS), which is the leading pre-erythrocytic vaccine antigen.

Given the many unknowns and uncertainties regarding the immune mechanisms of naturally acquired protection against clinical malaria, it is preferable that a wide range of immunological parameters are assessed when evaluating vaccine immunogenicity. This approach may also facilitate attempts to detect possible correlations between the immune response to vaccination and protection against clinical malaria (49, 50).

There is no animal model or in vitro assay of functional immune responses to malaria vaccines known to correlate with efficacy. Most experience in evaluating the immune response to malaria vaccines has been gained using ELISA to measure antigen-specific IgG. It is expected that, at a minimum, a validated IgG ELISA for determining antibody concentrations to the relevant antigen will be applied as an indication of vaccine immunogenicity. Sponsors are encouraged to explore alternative assays, including the possibility of measuring functional antibody. The selection of assays used to evaluate the human immune response to a vaccine should be justified by the manufacturer. The use of validated

quantitative assays is critical, and the validation report should include a detailed description of the calibration of any in-house references, and of the processing and storage of samples, reference standards and reagents. Data on assay validation should be reviewed and approved by the NRA.

It is recommended that sponsors also conduct an explorative assessment of cell-mediated immunity (CMI). Both ELISPOT (e.g. for interferon gamma) and intracellular cytokine staining have been used to assess CMI, and efforts to standardize these assays are under way in the context of assessing responses to malaria vaccines (51, 52).

Data on immune responses should be obtained throughout the clinical-development programme (see sections C.2.2.1 and C.2.2.2 on phase I–III studies). The blood volume and the number of samples that can be collected from paediatric subjects may limit the number and type of assays that are possible, especially for infants. It may be necessary to prioritize the assays applied to each specimen or use randomized subsets of samples for each assay, or both.

C.2.1.2 Interpretation of immune responses to vaccination

In order to attempt to identify immunological correlates of protection and in the absence of knowledge regarding which immune parameter is most closely associated with protection against clinical malaria, there should be plans in place to utilize the data obtained from prelicensure and post-licensure clinical studies in which immunogenicity and efficacy have been documented.

The basis for assessing responses to different vaccine doses and for comparing antibody levels between vaccination and control groups should take into account derived measures such as:

- seroconversion rates (using an appropriate definition)
- geometric mean concentrations or geometric mean titres
- reverse cumulative distributions (53).

C.2.2 Efficacy

2.2.2.1 Phase I–II studies

The initial (phase I) studies should be sufficient to provide an early indication of whether severe local or systemic adverse events, or both, occur commonly after vaccination. The data on immune responses from such studies should assist in the identification of candidate malaria vaccines suitable for further investigation.

The first studies will most likely enrol healthy adults who are naive to malaria (as assessed using their residence history, medical history and, possibly, serological testing). It is preferable that the initial evaluation includes subjects who reside in non-endemic areas so that they are not at risk of natural infection

with any species of malaria during the study period because this could complicate the assessments of both safety and immunogenicity.

An initial exploration of safety and immunogenicity may also be conducted in healthy adult residents of an endemic area (i.e. including subjects with evidence of pre-existing immunity to malaria). Such a study could provide further reassurance about the safety and immunogenicity of the vaccine before studies progress to younger age groups and larger numbers of subjects who live in endemic areas.

The preliminary data on safety and immunogenicity should support the selection of one or more vaccine formulations (i.e. in terms of antigen doses, and the need for, and amount of, any adjuvant) and regimens to be used in studies that assess efficacy in the target population. To provide a sound basis for future studies, the immunogenicity data should include measurement of responses after sequential doses and, ideally, an exploration of different dose intervals. After a vaccine has been approved, the most practical way to deploy such vaccines may be by incorporating them into the schedule for the Expanded Programme on Immunization (EPI), in which case consideration should be given to the possibility that three doses of the malaria vaccine may not be required to obtain protection. For example, studies could be conducted to compare regimens with different numbers of doses administered at different time points within the EPI schedule.

Human-challenge studies, which are often referred to as phase IIa studies (see Appendix 3), have been instrumental in identifying pre-erythrocytic candidate malaria vaccine formulations for further evaluation. If performed, such challenge studies should be conducted only in highly specialized units that have appropriate expertise and facilities, and only after approval by local authorities, which should include a review of ethical and technical considerations.

The initial evidence of efficacy may be obtained from phase IIb field-efficacy studies. These should generally follow the design principles of phase III efficacy studies, as described below in section C.2.2.2. Phase IIb studies are intended to provide an estimate of protective efficacy that can inform the design of phase III studies. Since phase IIb studies require fewer subjects than phase III studies, there is usually less geographical spread of study sites and less population diversity.

C.2.2.2 Phase III studies

C.2.2.2.1 Overview

Candidate malaria vaccines should be evaluated in randomized double-blind studies in which the safety and efficacy of the vaccine in the vaccinated group are compared with a control group that does not receive the vaccine. If the results of earlier studies have not provided definitive evidence to support the selection of a single formulation or regimen for the vaccine, then the study design may need to include more than one group assigned to receive the test product. If so, this has implications for the size of the study and the plan for analysis.

The recommendations made in this section may require reconsideration after at least one malaria vaccine has been approved.

Efficacy studies of pre-erythrocytic vaccines should be designed to allow assessment of protection against blood-stage infection and against disease. Studies designed to assess protection against incident infection require pretreatment with antimalarials, and regular cross-sectional surveys to detect new asymptomatic infections.

Phase III studies should include a sufficient number of subjects to ensure that there is adequate power to allow statistically robust conclusions to be drawn from the predefined primary analysis. A double-blind study design should be maintained at least until all data have been collected for the planned primary analysis or a decision has been taken to terminate the study on the basis of predefined stopping rules or safety concerns. In order to avoid the use of placebo injections (at least for some, if not all, visits) the control group may receive an ethically appropriate licensed comparator vaccine that is not expected to have an impact on the risk of malaria.

The use of an independent monitoring committee (consisting of persons independent of the sponsor and the investigators), with an adequately constituted charter, is strongly recommended. This committee should review the safety data that emerge during the study. The committee may also be charged with implementing stopping rules for reasons of unexpectedly low or high efficacy, if this is in accordance with the study protocol (48).

The following factors should be borne in mind when planning the phase III programme.

- All episodes of malaria that meet the case definition described below), and not just the first episode of malaria, should be captured for the duration of the study since many children will experience several clinical episodes of malaria.
- It is most likely that the ultimate target population for primary vaccination will be (or will at least include) infants. Thus, delivery of malaria vaccine may be incorporated into the EPI schedule with or without a need for one or more booster doses. In addition, the target population may include toddlers or older children, and the vaccine may be incorporated into catch-up programmes. Whatever the immunization schedule, the prelicensure and post-licensure studies should evaluate whether efficacy against clinical malaria persists throughout the age range in which the bulk of malaria-related morbidity and mortality occurs. This is important in order to assess

- the possibility that vaccination could result in an upward shift in the age at which severe and potentially life-threatening malaria occurs.
- Vaccine efficacy may differ according to transmission intensity, and genetic factors in humans and parasites. Studies should be conducted in settings of both seasonal and year-round transmission, and in settings with a range of transmission intensities. It is desirable that data on efficacy should be obtained from all, or from a representative selection of, areas in which the vaccine may ultimately be deployed. The sponsor may choose to perform separate studies in different geographical areas, or to conduct one large study that includes study sites considered likely to provide representative data. If the latter approach is adopted, a predefined stratification of enrolment by area could be used to support secondary analyses of efficacy by area or by transmission category or type.
- The concomitant use of other malaria interventions should be documented.

C.2.2.2.2 Design and analysis

The following section discusses essential features of study design that have the potential to affect estimates of efficacy and to influence the extrapolation of the results to non-study populations. These features include:

- the study population
- adjunctive measures
- case-ascertainment methods
- case definitions that have pre-specified:
 - clinical criteria (including severity of illness)
 - laboratory criteria (including sensitivity and specificity)
- approaches to analyses.

C.2.2.2.3 Study population

The protocol-defined selection criteria should aim to enrol a study population that is as representative as possible of the target population in which the vaccine is expected to be used. It may be appropriate to exclude persons with severe concomitant disease, including severe malnutrition, but subjects who are only mildly malnourished should not be excluded. A risk assessment should be made of the suitability of a given vaccine construct for administration to those with varying degrees of immunodeficiency. It is recommended that asymptomatic HIV-positive subjects should not be excluded unless this is deemed necessary due to the nature of the vaccine construct.

C.2.2.2.4 Adjunctive measures

In general, treating study subjects with antimalarial agents immediately prior to vaccination is not encouraged since this would not reflect the expected mode of deployment of a malaria vaccine. If pretreatment is used, the pharmacology of the antimalarial agents administered must be well understood, and the plasma half-lives should be sufficiently short to ensure that no impact on the estimate of vaccine efficacy would be expected.

Sponsors and investigators have an obligation to ensure that effective antimalarial chemotherapy is available to treat any subjects who develop clinical malaria during the study. Throughout the study, it is essential to document, as far as is possible, all antimalarial therapy administered to subjects, including the use of intermittent preventive treatment. It is recommended that study sites should not include sites where a significant number of people with malaria might be treated without contact with investigators (e.g. by the purchasing of antimalarial medicines).

The distribution of bednets to study subjects and the indoor residual spraying of their homes should take place in accordance with the policy of the national malaria-control programme. Sponsors and investigators should liaise with the national malaria-control programme to determine whether LLIN should be supplied as part of the study, or whether the programme will ensure distribution through local channels. Sponsors should endeavour to ensure that access to and use of LLIN are maximized throughout the study, given that the use of LLIN is known to reduce malaria morbidity and mortality in endemic settings (54).

To the extent that it is possible, it is desirable to document use of LLIN during the study both for individual subjects and at the community level in order to allow an assessment of the value of the malaria vaccine in the context of LLIN. Information on other issues (e.g. any entomological control measures) that may have an impact on the rates of clinical cases of malaria should be recorded at each study site.

C.2.2.2.5 Case definition

The following criteria apply to the evaluation of pre-erythrocytic vaccines. Immune responses to blood-stage vaccines are intended to reduce asexual parasite density, and it is therefore possible that the relationship between fever and parasite density could be altered in those receiving blood-stage vaccines. Thus the following criteria are not acceptable for the evaluation of blood-stage vaccines, and research is continuing to try to develop appropriate case definitions for blood-stage vaccines.

Clear definitions of clinical malaria and severe malaria are critical. However, the definition of clinical malaria is not straightforward because in malaria-endemic settings a child will often have parasitaemia and a coincidental fever that is not caused by parasites in the blood.

A case of clinical malaria should satisfy the clinical diagnostic criteria and should fulfil the relevant parasitological criteria for defining clinical malaria or severe malaria as follows.

C.2.2.2.6 Clinical diagnostic criteria

The recommended definition of a case of clinical malaria is the presence of fever, defined as an axillary temperature of \geq 37.5 °C, in a child presenting to a health-care facility with an illness consistent with malaria. This definition has been used in many field-efficacy studies of malaria vaccines. It should be noted that it is not the Brighton Collaboration's definition of fever (55), which is applicable to post-immunization events.

Cases of severe malaria represent a subset of all cases of clinical malaria and are characterized by the presence of at least one of the following:

- prostration
- respiratory distress
- Blantyre coma score ≤ 2
- seizures (two or more)
- hypoglycaemia (blood glucose < 2.2 mmol/L)
- anaemia (haemoglobin < 5g/dl)
- acidosis (base excess $\leq -10.0 \text{ mmol/L}$)
- lactate \geq 5.0 mmol/L

without any of the following:

- pneumonia (based on clinical assessment and culture)
- meningitis (based on examination of cerebrospinal fluid)
- bacteraemia (based on blood culture)
- gastroenteritis (based on clinical assessment).

All cases of severe malaria should be included with the clinical malaria cases. This case definition of severe malaria is intended to apply to infants and young children. The low incidence of severe disease in adults would most likely preclude measurement of efficacy against severe disease in adults.

C.2.2.2.7 Parasitological criteria for case definitions

If the vaccine is expected to prevent only *P. falciparum* malaria then the case definition should incorporate this restriction. If the vaccine has the potential to

prevent malaria due to other species of Plasmodium, the definition should be adjusted accordingly.

Parasitological diagnosis is based on a parasite density threshold quantified by microscopy. In high-transmission settings the threshold for defining a case should be derived by using recent historical data from each study area that is appropriate to the age group and case-detection system. Within each study, a single threshold should be selected that is considered to be sufficiently specific to all study areas. For a study conducted in low-transmission settings, it may be that the presence of a specific number of parasites defines a case.

Various methods are applied to calculate parasite density from microscopic observations (56). The acceptable method(s) should be predefined in the protocol, and a quality assurance scheme and laboratory accreditation system should be in place to ensure standardization among sites and consistency of data.

C.2.2.2.8 Case ascertainment

The case-detection system has an important bearing on the interpretation of vaccine efficacy. Either active case detection (ACD) or passive case detection (PCD) may be used.

In phase IIb efficacy studies with a relatively modest number of study subjects, the use of ACD that includes regular home visits by study staff may be appropriate and, depending on the study setting, PCD may also be appropriate. ACD may identify higher numbers of cases of malaria than PCD.

In phase III efficacy studies in which, on suspicion of malaria, study subjects present to designated health-care facilities (i.e. children are taken by caregivers) for diagnosis and treatment, PCD systems may be acceptable.

In both instances it is important that clinical episodes of malaria are captured from the time of the first vaccination onwards, and that the time of onset of clinical malaria is carefully documented to allow for determination of the time elapsed since the last dose of vaccine.

C.2.2.2.9 End-points and analyses

For each study, the statistical analysis plan (SAP), defining the primary, secondary and any exploratory analyses that are envisaged, should be finalized before unblinding the treatment assignment; ideally, the SAP should be submitted with the protocol. The SAP submission date should allow sufficient time for regulatory review, as well as provide some assurance that finalization of the SAP was not influenced by interim analyses, whether scheduled or unscheduled. If analyses of specific subsets of the study population are warranted, they should be prespecified in the SAP along with methods of analysis. Similarly, if any ad hoc analyses are added, the addition should be justified.

C.2.2.2.10 Primary analysis of efficacy

It is recommended that the primary analysis compares the rates of all episodes of clinical malaria between vaccinated and unvaccinated groups because this approach best reflects the impact of the vaccine on a community's burden of disease

Alternatively, sponsors may choose to predefine the rates of all episodes and the rates of the first (or only) episodes per participant as coprimary end-points.

The protocol and SAP should specify and define all study cohorts to be evaluated, and should justify the set that will be used for each type of analysis (efficacy, immunogenicity and safety). In vaccine efficacy studies, a common approach is to predefine the per protocol population as primary, and to base the primary analysis on the number of episodes meeting the case definition as counted from a minimum number of days after the final dose of the course is given. Immunogenicity data, including the kinetics of the antibody response, should be taken into account when defining the minimum time elapsed since the last dose. Such an analysis would be expected to provide an estimate of the maximum vaccine efficacy achievable in the study setting. However, it is essential that adequate sensitivity analyses of efficacy are performed.

The timing of the primary analysis should be carefully chosen to provide a robust comparison between vaccination and control groups that is appropriate to support a decision on licensure. When planning the study, investigators should consider whether the sponsor's staff, investigators and subjects will or will not be unblinded with respect to vaccination group at the time of unblinding of the study for the purposes of regulatory submission. The study protocol and SAP must clarify exactly who will be unblinded, and when they will be unblinded, taking into account the possibility that it may not be tenable in some settings to continue to withhold vaccine from unvaccinated persons if the results indicate very high efficacy. These issues need to be carefully considered at the study-planning stage.

As a minimum, the primary analysis should not take place until all subjects have completed at least 12 months of follow up after the last dose of vaccine. The minimum period of post-vaccination follow-up before the primary analysis is performed should also take into account the seasonality of malaria at the study sites since it may be desirable to follow up for more than one transmission season. Prior to study initiation, the sponsors should reach agreement with the relevant NRAs with respect to the duration of follow-up that will be completed by all subjects before the primary analysis is performed that is intended to support initial licensure. Further follow-up should then continue (see section on Study duration) regardless of whether there has been unblinding at the participant level. If an interim analysis is planned, the protocol and SAP should define who will carry out the analysis, describe the analysis schedule, and provide the details of the analysis along with any statistical adjustments considered.

An alternative approach is to plan to conduct the primary analysis when a predefined number of cases of malaria have occurred. However, it would also be necessary to prespecify the minimum duration of follow-up before the primary analysis is performed. The relative merits of these approaches need to be considered early in the protocol-planning phase.

Potential covariate factors for efficacy should be identified in the study protocol so that, where possible, the information required to evaluate their effect will be collected during the study. The SAP should specify the covariates that are to be explored, and the methods to be applied.

C.2.2.2.11 Secondary analyses

Some of the data required for secondary analyses will become available only after the study has been unblinded and the primary analysis has been conducted to support initial licensure. The range of secondary efficacy analyses should include at least the following:

- efficacy against all cases of malaria counting from the time of the first dose;
- efficacy against clinical malaria cases based on higher and lower thresholds of parasite density;
- efficacy against cases of severe malaria;
- efficacy as calculated from predefined data pools by study site(s) and area(s) (e.g. sites or areas with comparable transmission rates, with and without seasonality);
- vaccine efficacy against all episodes of malaria by time since the last vaccine dose.

With regard to the last point on vaccine efficacy against all episodes of malaria by time since the last vaccine dose, the precise period will depend on individual trial designs and the total duration of the trial, although periods of 6–12 months will usually be appropriate. An illustrative example is provided in Table 3.1. This analysis addresses the important public-health issue of whether the relative risk of disease varies in vaccinated and unvaccinated individuals according to the time since vaccination. There may be several reasons for such variation, including waning vaccine efficacy and the future risk of disease being influenced – through acquired partial immunity – by prior disease experience. However, the analysis addresses the important issue of the extent to which, at a given time after vaccination, a vaccinated person is at a differential risk of disease when compared with an unvaccinated person. The initial protocol-defined duration of follow-up may require amendment if there is evidence of waning efficacy and a possible need to assess the effect of one or more booster doses.

In addition to the efficacy analyses, investigators should prespecify the planned analyses of immune responses, including comparisons between vaccination and control groups at various intervals.

Table 3.1 Reporting of all malaria episodes with time since vaccination

Time period ^a	Vaccine group			Control group		
	Malaria episodes	Person- years at risk	Rate ^b	Malaria episodes	Person- years at risk	Rate
(0-X months)						
(X–Y months)						
(Y–Z months)						
(0–Z months)						

^a Z months is the total duration of the trial; X and Y are intermediate points during the trial. The number of such subdivisions used may be more or less than shown in this table.

C.2.2.2.12 Exploratory analyses

Exploratory analyses may include:

- analyses of associations between antibody concentrations and efficacy
- analyses of associations between cell-mediated immune responses and efficacy
- malaria-related admissions to hospital
- all episodes of hospitalization from any cause
- mortality attributed to malaria
- all-cause mortality
- anaemia
- changes in the prevalence of malaria infections.

An exploration of any possible correlation between immune responses and protection against clinical malaria requires a specific plan to collect and analyse a sufficient number of serum samples from subjects in the vaccination and control groups. There are several possible methods that may be applied to these types of exploratory analyses, and it is recommended that advice should be obtained from appropriate experts in this field when developing the protocol.

^b The rate is the number of malaria episodes per person-year at risk.

C.2.2.2.13 Study duration

Whatever the timing of the primary analysis discussed above in section C.2.2.2.10 and the decisions taken regarding unblinding, it is recommended that protocols should plan to follow up on subjects, or on randomly selected subsets of subjects, for a prespecified period of time. While the protocol should propose an initial period of follow-up that takes into account feasibility issues, this may require amendment depending on the early results of the study, possible evidence of waning protection, and the extent of the post-licensure programme to assess effectiveness (see section C.3). If it becomes necessary for subjects assigned to the unvaccinated group to receive the candidate malaria vaccine before the planned end of follow-up, the vaccinated cohort should still be followed for cases of malaria.

Where there is the possibility of waning efficacy over time, there are advantages in identifying a randomized subset of study subjects (from both the vaccination and control groups) to give prior consent to receive booster doses at timed intervals after completing the initial series. This would allow for evaluation of the safety and immunogenicity of booster doses in the previously vaccinated group relative to the first doses received by the control group. These data would be especially valuable if longer-term follow-up eventually indicates waning efficacy, since data would already be available on the safety and immunogenicity of a booster dose.

In addition to following up on cases of malaria, the study population (or, in some cases, predefined subsets of the study population) should be followed up for safety, including assessing the incidence of serious adverse events, and, depending on the vaccine composition, specific events of interest. For example, if the vaccine incorporates a novel adjuvant there may be a theoretical reason to document any possible cases of autoimmune disease occurring in the longer term.

A cohort should also be followed up for immunogenicity. This cohort will need to be identified at the time of randomization to obtain consent for additional blood samples to be taken.

C.2.2.2.14 Concomitant administration with other vaccines

Studies that evaluate co-administration of the candidate malaria vaccine with routine childhood immunizations that deliver antigens are encouraged; such immunizations include diphtheria-tetanus-whole-cell pertussis (DTwP) or diphtheria-tetanus-acellular pertussis (DTaP) vaccines, *Haemophilus influenzae* type b (Hib) vaccine, oral poliovirus vaccine (OPV) or inactivated poliomyelitis vaccine (IPV), and hepatitis B virus vaccine. Depending on the study site and the schedule for the candidate vaccine, there may be co-administration with other vaccines, including conjugated pneumococcal vaccine, conjugated meningococcal vaccine, oral rotavirus vaccine and measles vaccine.

It is desirable to obtain some information on the possible effects of co-administration on the safety and immunogenicity of the candidate malaria vaccine before commencing large-scale efficacy studies. The effect of malaria vaccination on the immune response to other vaccines is of interest, as is the effect of vaccination with other vaccines on the immune response to the malaria antigen. If necessary, further co-administration studies could be performed in parallel or following completion of phase III efficacy studies. The principles of the design of such studies are discussed in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (8).

C.2.3 Safety evaluation

The prelicensure assessment of vaccine safety is a critically important part of the clinical programme, and should be developed to meet the general principles described in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (8). The assessment of safety, with appropriately defined objectives, should be part of the studies described above. Such studies should include a comprehensive assessment of adverse events. Particular attention should be paid to the possible occurrence of specific adverse events that have been associated with vaccines of similar composition.

The minimum acceptable size of the safety database at the time of approval should take into account the vaccine's composition (including all antigens and any adjuvants), whether novel antigens are present, the platforms or recombinant vectors, and the severity of the infectious diseases being prevented.

In the field of blood-stage malaria vaccines, many of the immunological targets are ligand–receptor interactions that mediate adhesion to or invasion of erythrocytes, or both. Clinical evaluation of such vaccines should include haematological monitoring for adverse events, such as anaemia or haemolysis.

Additionally, the dossier should include consideration of safety evaluations in high-risk individuals who may benefit from vaccination. Safety in these groups is often assessed in post-marketing studies (see section C.3); however, a pre-specified plan for such studies is often required at the time of application for marketing authorization.

C.3 Post-licensure investigations

Many malaria vaccines either include novel adjuvants or are based on novel recombinant vector systems. As is the case for all vaccines, there must be adequate systems of pharmacovigilance in place at the time of initial licensure and subsequent to it. In the post-licensure period, longer-term follow-up of subjects enrolled into prelicensure studies will continue. For malaria vaccines intended primarily for use in populations in developing countries, this places a

major emphasis on the strengthening of pharmacovigilance systems in order to detect rare adverse events that may not have been detected during the prelicensure phase. For many vaccines, large pharmacovigilance databases generated in high-income countries may become available after licensure. This may not be the case for some malaria vaccines. In addition, there will be a need to conduct specific studies of effectiveness.

In particular, there will be a need to evaluate the vaccine's effectiveness during routine vaccination programmes as a result of the unknown validity of extrapolating an estimate of vaccine efficacy from a phase III study to other populations, areas and transmission settings. There are several possible factors that may have an impact on the level of protection (and, hence, the benefit) that can be achieved by a malaria vaccine, including:

- transmission intensity
- the use of other preventive measures such as LLIN and indoor residual spraying
- the health-care system, including the availability of access, diagnosis and treatment.

The degree to which the immunization of different age groups does or does not reduce malaria transmission is an additional piece of information that is important when assessing the benefit conferred by a malaria vaccine. Where the anticipated effects on transmission have not been demonstrated prelicensure, it may be appropriate to design specific post-licensure effectiveness studies to address this issue. Such studies would most probably be cluster-randomized (6).

In addition, if a malaria vaccine were to confer only a limited duration of protection, it is possible that widespread use in routine vaccination programmes could result in deferment of malaria-associated morbidity to an older age range compared with the pre-vaccine situation. Furthermore, it is unlikely that the possible need for booster doses can be fully addressed until several years after the vaccine has been in widespread use. Such decisions would not usually be based on waning immune responses alone, which would be an expected finding. Therefore, the overall plans for monitoring the effectiveness of the vaccine should be adequate for assessing shifts in the demographics and number of malaria cases over time.

The manufacturer has a responsibility to assess the vaccine's safety and effectiveness following initial approval of a new malaria vaccine. At the time of first licensure, there should be adequate plans in place regarding these activities and these should conform to applicable legislation. The geographical areas or countries in which such data can be collected will depend on where and when the vaccine is introduced into routine programmes. As far as possible, manufacturers should plan to collect data in specific areas and countries that have been

selected on the basis of their similarity and lack of similarity (in terms of the factors mentioned above and any others that seem important) to regions where the phase III studies were performed. Basic principles for the conduct of post-licensure studies (e.g. that they are intended to provide estimates of effectiveness) and safety surveillance are outlined in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (8).

The collection of reliable and comprehensive data on safety and effectiveness must involve close cooperation between manufacturers and publichealth authorities in the areas and countries selected. Preapproval and post-approval discussions between vaccine manufacturers responsible for placing the product on the market and national and international public-health bodies are essential for adequate planning. There may be a need to select areas and countries in which the strengthening of pharmacovigilance functions has occurred or is continuing.

Part D. Guidelines for NRAs

D1 General

The general recommendations for NRAs and NCLs given in Guidelines for national authorities on quality assurance for biological products (57) and in Guidelines for independent lot release of vaccines by regulatory authorities (10) apply. These Guidelines specify that no new biological substance should be released until consistency in manufacturing and quality have been established. The detailed production and control procedures, as well as any significant change in them that may affect the quality, safety or efficacy of the malaria vaccine, should be discussed with and approved by the NRA.

Consistency in production has been recognized as an essential component in the quality assurance of malaria vaccines. In particular, NRAs should carefully monitor production records and the results of quality-control tests on clinical lots as well as from a series of consecutive lots of the vaccine.

D.2 Release and certification by the NRA

A vaccine should be released only if it fulfils all national requirements or satisfies Part A of these Guidelines, or both (*10*).

A protocol based on the model given in Appendix 3, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for release of the vaccine for use.

A statement signed by the appropriate official of the NRA should be provided if requested by a manufacturing establishment, and should certify whether the lot of vaccine in question meets all national requirements as well as Part A of these Guidelines. The certificate should provide sufficient information

about the product. A model certificate is given in Appendix 4. The official national release certificate should be provided to importers of the vaccine. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

Authors

The first draft of these Guidelines was prepared by the following lead authors for the parts indicated: (1) Part A – Dr M. Baca-Estrada, Quality, Safety and Standards, World Health Organization, Switzerland; (2) Part B – Dr M. Baca-Estrada, Quality, Safety and Standards, World Health Organization, Switzerland; and (3) Part C – Dr V. Moorthy, Initiative for Vaccine Research, World Health Organization, Switzerland.

The first draft was discussed at a WHO working group meeting held on 4-5 October 2010 in Geneva, Switzerland attended by: Dr L. Chocarro, LC Plus Consulting, Canada; Dr P. Corran, honorary position at the London School of Hygiene and Tropical Medicine, England; Dr J.R. Daugherty, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Mr A. Mitangu Fimbo, Tanzania Food and Drugs Authority, United Republic of Tanzania; Professor B. Genton, Swiss Tropical and Public Health Institute, Switzerland; Dr I. Hansenne, Scientific Institute of Public Health, Belgium; Mr M.D. Jere, Pharmacy Medicines and Poisons Board, Malawi; Mr E. Karikari-Boateng, Food and Drugs Board Ghana, Ghana; Dr R. Leblanc, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr B.D. Meade, Meade Biologics, USA; Dr P. Milligan, London School of Hygiene and Tropical Medicine, England; Dr P. Neels, Federal Agency for Medicinal and Health Products, Belgium; Mr J. Pandit, Ministry of Medical Services, Kenya; Ms M.H. Pinheiro, European Medicines Agency, England; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Professor P. Smith, London School of Hygiene and Tropical Medicine, England; Professor T. Smith, Swiss Tropical and Public Health Institute, Switzerland; Dr J.W. van der Laan, National Institute for Public Health and the Environment, the Netherlands; Ms F.A. Kaltovich, Malaria Vaccine Initiative, USA (representing the Program for Appropriate Technology in Health); Dr D. Leboulleux, Malaria Vaccine Initiative, France (representing the Program for Appropriate Technology in Health); Mrs M-C. Uwamwezi, GlaxoSmithKline Biologicals, Belgium (representing the International Federation of Pharmaceutical Manufacturers and Associations); Dr P. Vandoolaeghe, GlaxoSmithKline Biologicals, Belgium (representing the International Federation of Pharmaceutical Manufacturers and Associations); Dr D. Wood, Quality, Safety and Standards, World Health Organization, Switzerland; Dr I. Knezevic, Quality, Safety and Standards, World Health Organization, Switzerland; Dr M. Baca-Estrada, Quality, Safety and Standards, World Health Organization, Switzerland; Dr C. Conrad, Quality, Safety and Standards, World Health Organization, Switzerland; Dr N. Dellepiane, Quality, Safety and Standards, World Health Organization, Switzerland; Dr V. Moorthy, Initiative for Vaccine Research, World Health Organization, Switzerland; Dr S. Nishioka, Quality, Safety and Standards, World Health Organization, Switzerland.

Taking into account comments received at the WHO working group meeting, a second draft was prepared by the following lead authors for the parts indicated: (1) Introduction – Dr C. Conrad, Quality, Safety and Standards, World Health Organization, Switzerland; (2) Background – Dr R. Leblanc, United States Food and Drug Administration, USA; Dr V. Moorthy, Initiative for Vaccine Research, World Health Organization, Switzerland; (3) Part A – Dr M. Baca-Estrada, Health Canada, Canada; (4) Part B – Dr M. Green, United States Food and Drug Administration, USA; and (5) Part C – Dr V. Moorthy, Initiative for Vaccine Research, World Health Organization, Switzerland; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England.

The second draft was further discussed and revised by the extended drafting group via a series of telephone conferences involving: Dr M. Baca-Estrada, Health Canada, Canada; Dr L. Chocarro, LC Plus Consulting, Canada; Dr M. Green, United States Food and Drug Administration, USA; Dr R. LeBlanc, United States Food and Drug Administration, USA; Dr B.D. Meade, Meade Biologics, USA; Dr P. Milligan, London School of Hygiene and Tropical Medicine, England; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Professor P. Smith, London School of Hygiene and Tropical Medicine, England; Dr C. Conrad, Quality, Safety and Standards, World Health Organization, Switzerland; Dr V. Moorthy, Initiative for Vaccine Research, World Health Organization, Switzerland.

The revised second draft was discussed at a WHO informal consultation on the development of guidelines to evaluate recombinant malaria vaccines, held on 21–22 November 2011 in Geneva, Switzerland, and attended by: Dr N.Y. Benno, National Agency for Food and Drug Administration and Control, Nigeria; Dr D. Bryan, National Institute for Biological Standards and Control, England; Dr L. Chocarro, LC Plus Consulting, Canada; Dr P. Corran, honorary position at the London School of Hygiene and Tropical Medicine, England; Dr R. Dobbelaer, Consultant, Belgium; Mr A. Mitangu Fimbo, Tanzania Food and Drugs Authority, United Republic of Tanzania; Professor B. Genton, Swiss Tropical and Public Health Institute, Switzerland; Dr M. Green, United States Food and Drug Administration, USA; Dr S. Spinosa Guzman, European Medicines Agency, England; Dr I. Hansenne, Scientific Institute of Public Health, Belgium; Mr M.D. Jere, Pharmacy Medicines and Poisons Board, Malawi; Mr E. Karikari-Boateng, Food and Drugs Board Ghana, Ghana; Dr R. LeBlanc, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr B.D. Meade, Meade Biologics, USA; Dr P. Milligan, London School of Hygiene

and Tropical Medicine, England; Dr P. Neels, Federal Agency for Medicinal and Health Products, Belgium; Mr J. Pandit, Ministry of Medical Services, Kenya; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Professor T. Smith, Swiss Tropical and Public Health Institute, Switzerland; Dr J.W. van der Laan, National Institute for Public Health and the Environment, the Netherlands; Ms F.A. Kaltovich, Malaria Vaccine Initiative, USA (representing the Program for Appropriate Technology in Health); Dr D. Leboulleux, Malaria Vaccine Initiative, France (representing the Program for Appropriate Technology in Health); Mrs M-C. Uwamwezi, GlaxoSmithKline Biologicals, Belgium (representing the International Federation of Pharmaceutical Manufacturers and Associations); Dr P. Vandoolaeghe, GlaxoSmithKline Biologicals, Belgium (representing the International Federation of Pharmaceutical Manufacturers and Associations); Dr J.M. Okwo-bele, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland; Dr D. Wood, Quality, Safety and Standards, World Health Organization, Switzerland; Dr I. Knezevic, Quality, Safety and Standards, World Health Organization, Switzerland; Dr C. Conrad, Quality, Safety and Standards, World Health Organization, Switzerland; Dr N. Dellepiane, Quality, Safety and Standards, World Health Organization, Switzerland; Dr V. Moorthy, Initiative for Vaccine Research, World Health Organization, Switzerland; Dr S. Nishioka, Quality, Safety and Standards, World Health Organization, Switzerland; Dr B.D. Akanmori, Regional Office for Africa, World Health Organization, Republic of the Congo; Professor P.M. Ndumbe, Regional Office for Africa, World Health Organization, Republic of the Congo; Dr G.A. Ki-Zerbo, Regional Office for Africa, World Health Organization, Republic of the Congo.

On the basis of the comments received at the WHO informal consultation, a third draft was prepared by a drafting group consisting of: Dr M. Green, United States Food and Drug Administration, USA; Dr R. LeBlanc, United States Food and Drug Administration, USA; Dr B.D. Meade, Meade Biologics, USA; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Dr C. Conrad, Quality, Safety and Standards, World Health Organization, Switzerland; Dr H.N. Kang, Quality, Safety and Standards, World Health Organization, Switzerland; Dr V. Moorthy, Initiative for Vaccine Research, World Health Organization, Switzerland.

The draft Guidelines were posted on the WHO web site for public consultation from 16 April to 15 May 2012.

The document WHO/BS/2012.2186 was then prepared by Dr B.D. Meade, Meade Biologics, USA, in coordination with: Dr C. Conrad, Paul-Ehrlich-Institut, Germany; Dr M. Green, United States Food and Drug Administration, USA; Dr H.N. Kang, Quality, Safety and Standards, World Health Organization, Switzerland; Dr R. LeBlanc, United States Food and Drug Administration, USA; Dr P. Milligan, London School of Hygiene and Tropical Medicine, England;

Dr V. Moorthy, Initiative for Vaccine Research, World Health Organization, Switzerland; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Professor P. Smith, London School of Hygiene and Tropical Medicine, England. The authors took into account comments received from the following reviewers: Dr M.F. Ahmed, National Organization for Drug Control and Research, Egypt; Ms L. Bigger on behalf of the Vaccines Committee's experts of the International Federation of Pharmaceutical Manufacturers and Associations, Switzerland; Dr D. Bryan, National Institute for Biological Standards and Control, England; Dr Y. Chen, LanZhou Institute of Biological Products, China; Dr M. Farag and Dr K. Goetz, Paul-Ehrlich-Institut, Germany; Dr S. Spinosa Guzman on behalf of the European Medicines Agency (reviewed by Dr K. Ho), Agence Nationale de Sécurité du Médicament et des Produits de Santé, France; Dr V. Irwin, Irish Medicines Board, Ireland; Dr B. Patel, Medicines and Healthcare Products Regulatory Agency, England; Dr M.M. Ho, National Institute for Biological Standards and Control, England; Dr T. Horii, Osaka University, Japan; Dr E. Babatunde Imoukhuede, European Vaccine Initiative, Germany; Dr S. Kumar, United States Food and Drug Administration, USA; Dr D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Dr G. Raychaudhuri, on behalf of the United States Food and Drug Administration Center for Biologics Evaluation and Research (reviewed by Dr R. Chattopadhyay, Dr J. Daugherty, Dr G. Ghebregiorgis, Dr D. Horne, and Dr D. Pratt), USA; Professor T. Smith, Swiss Tropical and Public Health Institute, Switzerland; Dr P. Volkers, Paul-Ehrlich-Institut, Germany; Dr J. Wittes, Statistics Collaborative, USA; Dr E. Woro on behalf of the National Agency of Drug and Food Control, Indonesia; Dr H. Xiaoxu, Chinese Pharmacopoeia, China; Dr M. Xu on behalf of the National Institutes for Food and Drug Control, China.

Further changes were made to document WHO/BS/2012.2186 by the WHO Expert Committee on Biological Standardization, resulting in the present document.

Acknowledgements

GlaxoSmithKline Biologicals is acknowledged for providing information on the manufacturing process and quality control of the RTS,S/AS01 vaccine.

References

- Agnandji ST et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. New England Journal of Medicine, 2011, 365:1863–1875.
- Asante KP et al. Safety and efficacy of the RTS,S/AS01E candidate malaria vaccine given with expanded-programme-on-immunisation vaccines: 19 month follow-up of a randomised, openlabel, phase 2 trial. Lancet Infectious Diseases, 2011, 11:741–749.

- 3. Bejon P et al. Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. *New England Journal of Medicine*, 2008, 359:2521–2532.
- Sacarlal J et al. Long-term safety and efficacy of the RTS,S/AS02A malaria vaccine in Mozambican children. *Journal of Infectious Diseases*, 2009, 200:329–336.
- 5. Pinder M et al. MALVAC 2009: Progress and challenges in development of whole organism malaria vaccines for endemic countries, 3–4 June 2009, Dakar, Senegal. *Vaccine*, 2010, 28:4695–4702.
- Pinder M et al. MALVAC 2010: Measures of Efficacy of Anti-malarial Interventions against Malaria Transmission, 15–16 November 2010, Geneva, Switzerland. Geneva, World Health Organization 2010 (http://www.who.int/vaccine_research/MALVAC_2010_meeting_report.pdf, accessed 22 June 2013).
- WHO guidelines on nonclinical evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-fourth report. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.
- Guidelines on clinical evaluation of vaccines: regulatory expectations. In: WHO Expert Committee on Biological Standardization. Fifty-second report. Geneva, World Health Organization, 2004 (WHO Technical Report Series No. 924), Annex 1.
- Shin J et al. International regulatory requirements for vaccine safety and potency testing: a WHO
 perspective. Procedia in Vaccinology, 2011, 5:164–170.
- Guidelines for independent lot release of vaccines by regulatory authorities. In: WHO Expert Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 2.
- 11. Guidelines for the evaluation of Plasmodium falciparum vaccines in populations exposed to natural infection. Special Programme for Research and Training in Tropical Diseases. Geneva, World Health Organization, 1997 (TDR/MAL/VAC/97).
- 12. Moorthy V et al. Measurement of malaria vaccine efficacy in phase III trials: report of a WHO consultation. *Vaccine*, 2007, 25:5115–5123.
- 13. Moorthy VS et al. MALVAC 2008: Measures of efficacy of malaria vaccines in phase 2b and phase 3 trials scientific, regulatory and public health perspectives. *Vaccine*, 2009, 27:624–628.
- Good MF. Our impasse in developing a malaria vaccine. Cellular and Molecular Life Sciences, 2011, 68:1105–1113.
- 15. World Malaria Report 2011. Geneva, World Health Organization, 2011 (http://www.who.int/malaria/world_malaria_report_2011/en/index.html, accessed 22 June 2013).
- 16. Sachs J, Malaney P. The economic and social burden of malaria. *Nature*, 2002, 415:680–685.
- 17. O'Meara WP et al. Changes in the burden of malaria in sub-Saharan Africa. *Lancet Infectious Diseases*, 2010, 10:545–555.
- 18. Greenwood BM et al. Malaria: progress, perils, and prospects for eradication. *Journal of Clinical Investigation*, 2008, 118:1266–1276.
- 19. Moorthy VS, Good MF, Hill AV. Malaria vaccine developments. *Lancet*, 2004, 363:150–156.
- 20. Hoffman SL et al. Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. *Journal of Infectious Diseases*, 2002, 185:1155–1164.
- Malaria. Geneva World Health Organization, 2012 (Fact sheet No. 94) (http://www.who.int/mediacentre/factsheets/fs094/en/, accessed 22 June 2013).
- Moorthy VS, Ballou WR. Immunological mechanisms underlying protection mediated by RTS,S: a review of the available data. Malaria Journal, 2009, 8:312.

- 23. Miura K et al. Transmission-blocking activity induced by malaria vaccine candidates Pfs25/Pvs25 is a direct and predictable function of antibody titer. *Malaria Journal*, 2007, 6:107.
- 24. Olotu A et al. Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anticircumsporozoite antibody titres and protection in children aged 5–17 months in Kenya and Tanzania: a randomised controlled trial. *Lancet Infectious Diseases*, 2011, 11:102–109.
- 25. Gordon DM et al. Safety, immunogenicity, and efficacy of a recombinantly produced Plasmodium falciparum circumsporozoite protein-hepatitis B surface antigen subunit vaccine. *Journal of Infectious Diseases*, 1995, 171:1576–1585.
- 26. Kester KE et al. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. *Journal of Infectious Diseases*, 2009, 200:337–346.
- 27. Tables of malaria vaccine projects globally. The Rainbow Tables. Initiative for Vaccine Research. Geneva, World Health Organization, 2012 (http://www.who.int/vaccine_research/links/Rainbow/en/index.html, accessed 22 June 2013).
- 28. Schwartz L et al. A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malaria Journal*, 2012, 11:12.
- 29. Casares S, Brumeanu TD, Richie TL. The RTS, S malaria vaccine. Vaccine, 2010, 28:4880–4894.
- 30. Rutgers T et al. Hepatitis B surface antigen as carrier matrix for the repetitive epitope of the circumsporozoite protein of Plasmodium falciparum. *Biotechnology*, 1988, 6:1065–1070.
- 31. Jacobs E et al. Simultaneous synthesis and assembly of various hepatitis B surface proteins in Saccharomyces cerevisiae. *Gene*, 1989, 80:279–291.
- 32. Stoute JA et al. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. *New England Journal of Medicine*, 1997, 336:86–91.
- 33. Good manufacturing practices: main principles for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report.* Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 3.
- 34. Good manufacturing practices for biological products. In: WHO Expert Committee on Biological Standardization. Forty-second report. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 1.
- 35. General requirements for the sterility of biological substances (Revised 1973). In: *WHO Expert Committee on Biological Standardization. Twenty-fifth report*. Geneva, World Health Organization, 1973 (WHO Technical Report Series, No. 530), Annex 4.
- General requirements for the sterility of biological substances. (Requirements for Biological Substances No. 6, revised 1973, amendment 1995). In: WHO Expert Committee on Biological Standardization. Forty-sixth report. Geneva, World Health Organization, 1998 (WHO Technical Report Series, No. 872), Annex 3.
- 37. WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products. Geneva, World Health Organization, 2003 (WHO/BCT/QSD/03.01) (http://www.who.int/biologicals/publications/en/whotse2003.pdf, accessed 22 June 2013).
- 38. WHO guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies. Geneva, World Health Organization, 2006 (http://www.who.int/bloodproducts/tse/WHO%20 TSE%20Guidelines%20FINAL-22%20JuneupdatedNL.pdf, accessed 22 June 2013).
- WHO tables on tissue infectivity distribution in transmissible spongiform encephalopathies. Geneva, World Health Organization, 2010 (WHO/EMP/QSM/2010.1) (http://www.who.int/bloodproducts/tablestissueinfectivity.pdf, accessed 22 June 2013).

- 40. Recombinant DNA technology, products of. In: *European Pharmacopoeia*, 6th ed. Strasbourg, Directorate for the Quality of Medicines of the Council of Europe (EDQM), 2008 (01/2008:0163).
- 41. Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology. In: WHO Expert Committee on Biological Standardization. Forty-first report. Geneva, World Health Organization, 1991 (WHO Technical Report Series, No. 814), Annex 3.
- 42. Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (jointly with the Expert Committee on Biological Standardization). In: WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 9.
- 43. Guidelines on stability evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-seventh report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 962), Annex 3.
- 44. Guideline on adjuvants in vaccines for human use. London, European Medicines Agency, 2005 (EMEA/CHMP/VEG/134716/2004).
- 45. Persson KE et al. Development and optimization of high-throughput methods to measure Plasmodium falciparum-specific growth inhibitory antibodies. *Journal of Clinical Microbiology*, 2006, 44:1665–1673.
- Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture
 of biological medicinal products and for the characterization of cell banks. In: WHO Expert
 Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization,
 2013 (WHO Technical Report Series, No. 978), Annex 3.
- 47. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products. In: *The use of essential drugs. Sixth report of the WHO Expert Committee.* Geneva, World Health Organization, 1995 (WHO Technical Report Series, No. 850), Annex 3.
- 48. Guideline for good clinical practice E6(R1). ICH harmonized tripartite guideline. Geneva, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 1996.
- 49. Madore DV et al. Utilization of serologic assays to support efficacy of vaccines in nonclinical and clinical trials: meeting at the crossroads. *Vaccine*, 2010, 28:4539–4547.
- 50. Plotkin SA. Vaccines: correlates of vaccine-induced immunity. *Clinical Infectious Diseases*, 2008, 47:401–409.
- 51. Cavanagh DR et al. Towards validated assays for key immunological outcomes in malaria vaccine development. *Vaccine*, 2011, 29:3093–3095.
- 52. *Initiative on Optimising Malaria Vaccine Lab Assays Evaluation*. Heidelberg, European Vaccine Initiative, (http://www.optimalvac.eu/, accessed 22 June 2013).
- 53. Reed GF, Meade BD, Steinhoff MC. The reverse cumulative distribution plot: a graphic method for exploratory analysis of antibody data. *Pediatrics*, 1995, 96(3 Pt 2):600–603.
- 54. Lengeler C. Insecticide-treated bednets and curtains for preventing malaria. *Cochrane Database of Systematic Reviews*, 2000, (2):CD000363.
- 55. Bonhoeffer J et al. The Brighton Collaboration: addressing the need for standardized case definitions of adverse events following immunization (AEFI). *Vaccine*, 2002, 21:298–302.
- 56. Moorthy VS, Reed Z, Smith PG. Clinical trials to estimate the efficacy of preventive interventions against malaria in paediatric populations: a methodological review. *Malaria Journal*, 2009, 8:23.
- 57. Guidelines for national authorities on quality assurance for biological products. In: WHO Expert Committee on Biological Standardization. Forty-second report. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 2.

Appendix 1

Controlled human malaria infection trials (human-challenge studies)

Human-challenge studies may be conducted to demonstrate a proof of concept or to gain preliminary information on the efficacy of pre-erythrocytic stage candidate vaccines prior to conducting further clinical studies of efficacy. These studies are considered to be phase IIa studies (see section C.2.2.1). Because human-challenge studies present unique considerations, the sponsor should discuss the study-development plan with the relevant local authorities, including committees that review ethical considerations, prior to initiating such studies for either proof of concept or vaccine efficacy.

Sponsors should provide a description of the human-challenge facility; they should identify any other malaria strains, nonmalaria microorganisms, and other mosquito species cultured and processed in the facility; and they should include details of changeover procedures used to prevent contamination among different strains, microorganisms and mosquito species. In addition, details should be provided regarding the controlled environmental conditions under which the parasites and infected mosquitoes are grown in the facility, and the procedures by which the escape of infected mosquitoes into the environment is monitored and prevented. Also, the sponsors should provide the local authorities with copies of the procedures used for parasite culture, mosquito infection, and challenge of human subjects with infected mosquitoes, as well as the results of tests for sensitivity to antimalarial medicines in the parasite strain(s) used for the challenge.

Administration of sporozoite-stage malaria parasites by mosquito bites has been used extensively to test pre-erythrocytic stage vaccines, and has been instrumental in selecting the most advanced candidate. In the most commonly used model, *Anopheles stephensi* mosquitoes feed on either the chloroquine-sensitive NF54 strain of *P. falciparum* or the 3D7 clone of NF54. Between 14 and 21 days after feeding, the mosquitoes are examined for infection by microscopic examination of their salivary glands to ensure a reliable and reproducible challenge. Subsequently, healthy human volunteers, including immunized subjects and nonimmunized controls, are allowed to be bitten by infected mosquitoes. Volunteers must be carefully screened for their suitability for such studies, and they must provide fully informed consent to indicate that they understand the risks and benefits of challenge studies. Following delivery of the malaria parasites, clinical signs and symptoms are monitored, and a thick blood smear is examined to diagnose blood-stage infection. Upon detection of parasites

microscopically, volunteers must be treated without delay with a rapidly curative antimalarial regimen. The validity of the challenge is verified by concurrently challenging the nonimmunized control group, who are expected to develop malaria infection after being bitten by malaria-infected mosquitoes.

Following sporozoite challenge, it is essential that volunteers be closely monitored in a medical facility or in local hotels. Alternatively, adequate monitoring of subjects at their homes during this phase of the study may be achieved using a combination of technology (e.g. mobile telephones, pagers) and frequent contact between the clinical investigator and the subjects. The monitoring protocol that is to be followed should be specified in the clinical protocol. This should include the criteria to be used to determine whether and when to transfer subjects to an emergency medical facility that has appropriate expertise in the management of malaria.

For the human-challenge study, the sponsor should propose screening and monitoring regimens for cardiac-related adverse events associated with the challenge of subjects with malaria sporozoites or with subsequent antimalarial treatment.

The informed consent form should include information on the previous acute coronary syndrome that occurred in temporal association with a malaria-challenge study in the Netherlands (1). In addition, subjects should be instructed that if such signs or symptoms develop when they are off-site, they should immediately call the clinical investigator or seek care at the nearest appropriate hospital.

Harmonized procedures for designing and conducting controlled humanmalaria infection studies are available. They show paramount consideration for safeguarding subjects' safety and maximizing the comparability of assessments among centres (2). As with all vaccine evaluation methods, new and potentially optimized methods may emerge; these may be considered for adoption in cases in which subjects' safety and the ability to compare assessments are maintained, and the bridging of results from previous methods to new methods has been demonstrated.

References

- Nieman AE et al. Cardiac complication after experimental human malaria infection: a case report. Malaria Journal, 2009, 8:277.
- Laurens MB et al. A consultation on the optimization of controlled human malaria infection by mosquito bite for evaluation of candidate malaria vaccines. *Vaccine*, 2012, 30:5302–5304.

Appendix 2

Methodological considerations: quantification of human immunoglobulin G directed against the repeated region (NANP) of the circumsporozoite protein of the parasite *P. falciparum* (anti-CS ELISA)

Background

This two-step enzyme-linked immunosorbent assay (ELISA) is based on the selective reaction between an antibody and its specific antigen, and allows determination of the titre of antigen-specific antibodies.

Based on information provided by the manufacturer, 96-well polystyrene plates are coated with the R32LR protein corresponding to the repeated region (NANP) of the *P. falciparum* circumsporozoite protein (CSP). Serial dilutions of serum samples are added directly to the plate. Antibodies to R32LR present in serum samples bind to the precoated R32LR.

Antihuman immunoglobulin G (IgG) horseradish peroxidase (HRP) conjugated antibodies are used as detection reagents; a chromogen substrate solution specific for HRP is used as a colorimetric detection system. The optical density is then obtained to quantify anti-CS IgG in serum samples.

The negative control is a pool of serum samples from nonimmunized individuals (without anti-CS IgG). A positive control and standard control (both containing well defined levels of anti-CS IgG) are run on each plate in order to assess the relative titre of each sample and to control the quality of each assay plate. A standard curve is generated by plotting the optical density of the serum standard against its assigned value (e.g. the titre). The optical density measured for a given sample allows extrapolation of its antibody titre.

Validation issues

The assessment of the immune response should be based on measuring the antibody concentration in serum using a validated and standardized assay. The validation studies should be designed to demonstrate that the assay is suitable for the clinical study. The validation report should include a detailed description of the calibration of any in-house references, and the processing and storage of samples, reference standards and reagents. The assay validation data should be reviewed and approved by the NRA.

Assay characteristics

The limit of detection for the assay is 0.2 endotoxin units per millilitre (EU/ml), and the limit of quantification is 0.3 EU/ml. The analytical range has been defined from 0.3 EU/ml to 190 EU/ml. However, if the value of a sample is above the upper limit of the analytical range, the sample is further diluted, and quantified again. Therefore, technically speaking, no upper limit is applied for the anti-CS ELISA. The cut-off of the ELISA is based on the upper limit of the 99.9% one-sided confidence interval of the anti-CS titres in a naive population originating from a non-endemic malaria region. This cut-off has been set at 0.5 EU/ml, and subjects with antibody titres \leq 0.5 EU/ml are considered to be seropositive.

On the basis of reproducibility experiments, the overall coefficient of variation of the assay has been estimated at 22.5%, which is in the range of coefficients of variation usually observed for reproducible ELISAs. The anti-CS ELISA has been shown to be linear in a range of titres from 1.1 EU/ml to 2440 EU/ml. Data from competition experiments have suggested that the anti-CS ELISA was specific for R32LR and did not cross-react with the AMA-1 antigen or hepatitis B antigen. Those who have used the assay concluded that the anti-CS IgG ELISA is a reproducible and robust method for quantifying anti-CS antibodies in human noninactivated serum.

Appendix 3

Model protocol for the manufacturing and control of recombinant malaria vaccines

The following protocol is intended for guidance. It indicates the information that should be provided as a minimum by a manufacturer to the NRA. Information and tests may be added or omitted as necessary, with the authorization of the NRA.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO Guidelines for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that will accompany the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot-release certificate from the NRA or from the NCL in the country where the vaccine was produced or released stating that the product meets national requirements as well as the recommendations in Part A of this annex.

1. Summary information on finished product (final lot)

International nonproprietary name

international nonproprietary name.
Commercial name:
Product licence (marketing authorization) number:
Country:
Name and address of manufacturer:
Name and address of product licence holder, if different:
Batch number(s):
Finished product (final lot):
Final bulk:
Type of container:
Number of filled containers in this final lot:
Number of doses per container:
Composition (antigen concentration)/volume of
single human dose:
Target group for immunization:
Expiry date:
Storage conditions:

A genealogy of the lot numbers of all components used in the formulation of the final product should be provided.

The following sections are intended for reporting the results of tests performed during the production of the vaccine, so that the complete document will provide evidence of the consistency of production. Thus, if any test has to be repeated, this must be indicated. Any abnormal results should be recorded on a separate sheet.

2. Control of source materials (section A.4)

The information requested below is to be presented for each submission. Full details on the master seed and working seed lots, and cell banks, are to be presented only upon first submission or whenever a change has been introduced.

Cell banks

Source of antigen (expression system):
Master cell bank (MCB) lot number and
preparation date:
Date of approval of protocols indicating compliance with the
requirements of the relevant monographs and with the
marketing authorization:
Manufacturer's working cell bank lot number and
preparation date:
Date of approval of protocols indicating compliance with the
requirements of the relevant monographs and with the
marketing authorization:
Production cell lot number:
Storage conditions:
Identification of cell substrate
Method:
Specification:
Date of test:
Result:
Nature and concentration of antibiotics or selecting agent(s)
used in the production cell culture's maintenance
medium:
Identification and source of starting materials used in preparing
production cells, including excipients and preservatives
(particularly any materials of human or animal origin -
e.g. albumin, serum):

3. Fermentation (section A.5)

Provide information on cells corresponding to each single harvest.

Yeast cells
Bacteria and fungi
Method:
Media used and temperature of incubation:
Volume inoculated:
Date of inoculation:
Date of end of observation:
Result:
4. Single harvests (section A.6)
Batch number(s):
Date of inoculation:
Date of harvesting:
Volume(s) of fermentation paste, storage temperature,
storage time and approved storage period:
Culture purity or sterility for bacteria and fungi
Method:
Media used and temperature of incubation:
Volume inoculated:
Date of start of test:
Date of end of test:
Result:
5. Control of purified antigen bulk (section A.7)
Batch number(s) of purified bulk:
Date(s) of purification(s):
Volume(s), storage temperature, storage time and
approved storage period:
Purity
Method:
Specification:
Date of test:
Result:

4	7	4	
ı	_	٧	
3	2		
(Ç	٠	2
(ŕ	١	١,
(٠	•
i	7		
3			
١	Ċ	ï	٦
	(
		3	
4	2		
	Ç		
	7	ζ	
	(d	ė
		۱	
ŧ	ď		
٦	_		
,	÷		
	3	5	
	(
	¢	f	
	į		۰
	_	d	
(Ξ		
•	-	٠,	
			٩
	í	٠	
	2	۰	۰
	į		
	(
ŀ	-	٠,	
١.			
(`
-			
3		5	
1 4 7			5

Protein content
Method:
Specification:
Date of test:
Result:
Antigen content/Identity
Method:
Specification:
Date of test:
Result:
Lipids
Method:
Specification:
Date of test:
Result:
Carbohydrates
Method:
Specification:
Date of test:
Result:
Sterility for bacteria and fungi
Method:
Media used and temperature of incubation:
Volume inoculated:
Date of start of test:
Date of end of test:
Result:
Potential hazards – e.g. residual chemical(s) (if relevant)
Method:
Specification:
Date of test:
Result:
Desidual DNA (if applicable)
Residual DNA (if applicable)
Method:Specification:
Specification:

Date of test:
Result:
Bacterial endotoxins
Method:
Specification:
Date of test:
Result:
6. Final bulk (section A.8)
Batch number(s) of final bulk:
Formulation date:
Batch number(s) of all components used
during formulation:
Volume, storage temperature, storage time and
approved storage period:
Sterility for bacteria and fungi
Method:
Media and temperature of incubation:
Volume inoculated:
Date of start of test:
Date of end of test:
Result:

7. Filling and containers (section A.9)
Lot number:
Date of filling:
Type of container:
Filling volume:
Number of containers filled:
Date of freeze-drying (if applicable):
Number of containers rejected during inspection:
Number of containers sampled:
Total number of containers:
Maximum period of storage approved:
Storage temperature and period:

	n the final lot (section A.10)
Inspection of contain	· · · · · · · · · · · · · · · · · · ·
Specification:	
Result:	
Appearance (A.10.2))
Method:	
Result:	
Identity (A.10.3)	
•	
Specification:	
Media used an Volume inocu	d temperature of incubation:lated:f test:
	test:
General safety test (unless omission authorized by NRA) (A.10.5)
Test in mice	,
Number	of mice tested:
Volume i	njected and route of injection:
	njection:
Date of e	nd of observation:
Specificat	cion:
Result: _	
Test in guinea-	pigs
	of guinea-pigs tested:
	njected and route of injection:
	ois ation.



Date of end of observation:
Specification:
Result:
pH (A.10.6)
Method:
Specification:
Date of test:
Result:
Pyrogen and endotoxin content (unless omission authorized by NRA) (A.10.7)
Method:
Specification:
Date of test:
Result:
Protein content (A.10.8)
Method:
Specification:
Date of test:
Result:
Moisture content (A.10.9)
Method:
Specification:
Date of test:
Result:
Potency test (A.10.10)
In vitro assay
Method:
Specification:
Date of test:
Result:
In vivo assay
Number of mice tested:
Species, strain, sex, ages and weight range:
Dates of immunization:
Date of test:

Vaccine doses (dilutions) and number of animals
responding at each dose:
ED ₅₀ of standard vaccine and test vaccine:
Potency of test vaccine versus standard vaccine,
with 95% confidence interval of the mean:
Validity criteria:
Date of start of period of validity:
Control tests on the adjuvant (A.10.11)
Identity (A.10.11.1)
Method:
Specification:
Date of test:
Result:
Content of component 1 (A 10.11.1)
Content of component 1 (A.10.11.1)
Method:
Specification:
Date of test:
Result:
Content of component 2 (A.10.11.1)
Method:
Specification:
Date of test:
Result:
A Jimont and an anality attailants and lite (A 10.11.2)
Adjuvant system quality attributes – quality (A.10.11.2)
Method:
Specification:
Date of test:
Result:
Adjuvant system quality attributes – purity (A.10.11.2)
Method:
Specification:
Date of test:
Result:
A limited and an experience of the state of
Adjuvant system quality attributes – sterility tests for bacteria and fungi (A.10.11.2) Method:
Media used and temperature of incubation:

Volume inoculated:	
Date of start of test:	
Date of end of test:	
Result:	
9. Certification by the manufacturer	
Name of the manufacturer	
Name of head of production (typed)	
Certification by the person from the cont company taking responsibility for the produc	
I certify that lot no whose number appears on the label of the requirements and/or satisfies Part A¹ of the Vand efficacy of recombinant malaria vaccinal blood stages of <i>Plasmodium falciparum</i> (20)	e final container, meets all national VHO Guidelines on the quality, safety es targeting the pre-erythrocytic and
Signature	
Name (typed)	
Date	

10. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 3), a label from a final container and an instruction leaflet for users.

¹ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

² WHO Technical Report Series, No. 980, Annex 3.

Appendix 4

Model certificate for the release of recombinant malaria vaccines by NRAs

Lot release certificate
Certificate no
The following lot(s) of recombinant malaria vaccine produced by
on the labels of the final containers, complies with the relevant national specifications and provisions for the release of biological products ³ and with Part A ⁴ of the WHO Guidelines on the quality, safety and efficacy of recombinant malaria vaccines targeting the pre-erythrocytic and blood stages of <i>Plasmodium falciparum</i> (2014), ⁵ and complies with WHO good manufacturing practices: main principles for pharmaceutical products; ⁶ Good manufacturing practices for biological products; ⁷ and Guidelines for independent lot release of vaccines by regulatory authorities. ⁸
The release decision is based on9

The certificate may include the following information:

- name and address of manufacturer
- site(s) of manufacturing
- trade name and common name of product
- marketing authorization number

¹ Name of manufacturer.

² Country of origin.

³ If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.

With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

⁵ WHO Technical Report Series, No. 980, Annex 3.

⁶ WHO Technical Report Series, No. 961, Annex 3.

⁷ WHO Technical Report Series, No. 822, Annex 1.

⁸ WHO Technical Report Series, No. 978, Annex 2.

⁹ Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.

- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)
- type of container used
- number of doses per container
- number of containers or lot size
- date of start of period of validity (e.g. manufacturing date) and/or expiry date
- storage conditions
- signature and function of the person authorized to issue the certificate
- date of issue of certificate
- certificate number.

Director of the NRA (or other appropriate author	ity	r)
--	-----	----

Name (typed)	
Signature	
Date	

Annex 4

Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed)

Replacement of Annex 2 of WHO Technical Report Series, No. 800, and Annex 5 of WHO Technical Report Series, No. 927

muou	uction	213
Scope	of the Recommendations	217
Genera	al considerations	218
Part A.	. Manufacturing recommendations	221
A. A. A. A. A.	1 Definitions 2 General manufacturing recommendations 3 Production, processing and control 4 Filling and containers 5 Control of final product 6 Records 7 Retained samples 8 Labelling 9 Distribution and transport 10 Stability, storage and expiry date	221 221 222 234 235 237 237 238 238
	Nonclinical evaluation of diphtheria vaccines	239
B.: B.:	Nonclinical testing and characterization of intermediates and in-process materials	239 240 241
Part C.	Clinical evaluation of diphtheria vaccines	244
C.:	 Introduction Assessment of immunogenicity in humans Safety evaluation Post-marketing studies and surveillance 	244 246 251 252
Part D	. Recommendations for NRAs	252
D. D.		252 253
Autho	rs and acknowledgements	253
Refere	nces	255

Appendix 1

Model protocol for the manufacturing and control of diphtheria vaccines (adsorbed) 258 **Appendix 2**

268

Model certificate for the release of diphtheria vaccines (adsorbed) by NRAs

Recommendations published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes recommendations for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments or examples intended to provide additional guidance to manufacturers and NRAs.

Introduction

Diphtheria toxoid, produced by the chemical detoxification of diphtheria toxin, was one of the earliest vaccines available for protection against a bacterial disease, and it remains the basis for diphtheria vaccines today. The widespread use of diphtheria vaccines in routine immunization programmes has significantly reduced the incidence of the disease and its related mortality both in developed and developing countries (1).

Diphtheria-tetanus-pertussis vaccine has been part of the WHO Expanded Programme on Immunization since the inception of the programme in 1974, and during 1980-2000, the reported number of diphtheria cases was reduced by more than 90% (2). However, diphtheria is still a significant health concern in countries with poor vaccination coverage. In addition, a large proportion of the adult population in countries with good vaccination coverage may be susceptible to diphtheria due to the waning of immunity and the absence of natural boosting. The potential for severe outbreaks of the disease is enhanced in populations where there are large numbers of susceptible adults and unimmunized children. This was evident during the epidemic affecting countries in the former Soviet Union during the 1990s; however, the epidemic also highlighted the protective efficacy of the diphtheria vaccines used to control it (3, 4). Such outbreaks highlight the need to maintain good coverage of childhood immunizations and appropriate booster immunizations, which are given beyond infancy and early school age, in order to provide sustained protective immunity against diphtheria.

Single-antigen diphtheria toxoid vaccine (i.e. containing only diphtheria toxoid) is rarely used for immunization, and the antigen is most commonly used in combination with tetanus toxoid alone, or with tetanus toxoid and whole-cell pertussis ("DTP"), or with tetanus toxoid and acellular pertussis antigens ("DTaP"). Diphtheria toxoid is also used in other combination vaccines that may contain, in addition to tetanus and pertussis antigens, inactivated poliovirus, hepatitis B surface antigen or *Haemophilus influenzae* type b capsular polysaccharide conjugates, or some combination of these. All diphtheria vaccines that are currently in use contain a mineral carrier, such as aluminium hydroxide or aluminium phosphate, as an adjuvant. Diphtheria vaccines intended for booster immunizations in older children, adolescents and adults are formulated with a lower amount of diphtheria toxoid when compared with vaccines intended for primary immunization; these lower doses for booster immunizations are designated with a lower-case "d", while those intended for primary immunization are designated with an upper-case "D".

New diphtheria vaccines are evaluated in populations with a low disease burden and, as such, are not evaluated according to their efficacy. Rather, evaluations assess their ability to induce levels of diphtheria antitoxin that are considered to offer protection against the disease. Further considerations surrounding the clinical evaluation of diphtheria vaccines are included in Part C of these Recommendations.

History of WHO Requirements and Recommendations, and standardization

The early developments leading to the formulation of the first Requirements for diphtheria toxoid are described in detail in Requirements for diphtheria toxoid and tetanus toxoid (Requirements for biological substances No. 10), published in 1964 (5). The development of diphtheria toxoid vaccines, and the publication of Requirements for their manufacture and for quality control, was helped considerably by the availability of international standards and international reference preparations for diphtheria toxoid and antitoxin. The International Standard for diphtheria antitoxin, equine (established in 1934) enabled toxoid to be assessed in terms of its ability to produce diphtheria antitoxin in humans and animals; results were expressed in IUs. In addition, the International Standard for Diphtheria Antitoxin for Flocculation Test (established in 1956) enabled antigen concentrations to be expressed in limit for flocculation units (Lf), and led to requirements for antigen content and purity being included in the 1964 publication (5). International standards for diphtheria toxoid, plain (established in 1951) and adsorbed (established in 1955), for use in biological potency assays, had been available for a number of years, but there was no general agreement on how they should be used in assaying different types of preparations, and they were not widely included in potency assays. Prior to 1964 the requirements for potency were specified by national regulatory authorities (NRAs) on the basis of the results of laboratory and field studies, and there was little uniformity among countries. As a result, the 1964 Requirements included a recommendation that the international standard for toxoid should be more widely used in biological assays in order to permit the formulation of more satisfactory potency tests based on the use of the international standard, which would be incorporated into future revisions of the Requirements (5).

The subsequent formulation of requirements for the assay of diphtheria vaccine potency was a significant milestone in the history of diphtheria vaccine production and quality control. The 1978 revision of the Requirements, which covered tetanus and pertussis vaccines as well as diphtheria, included a requirement for a potency assay that involved immunizing guineapigs, and following this with a challenge from a lethal dose of toxin (given subcutaneously) or a challenge with a series of toxin doses given intradermally (6). The requirement to compare immunizing potency against a reference material calibrated against the international standard was also included so that vaccine potency could be expressed in IUs. A minimum requirement

for diphtheria potency (for vaccines intended for the primary immunization of children) of 30 IU per single human dose (SHD) was also included in the revised Requirements, together with a requirement that sufficient animals should be used to achieve a 95% confidence interval that is less than 50-200% (6). It has been recognized that there are difficulties in providing evidence of a direct correlation between the estimated potency of a vaccine in a biological assay and the level and duration of protective immunity in humans. Despite this lack of direct evidence, the minimum requirement for diphtheria potency of 30 IU/SHD has helped to ensure the production and release of safe and effective diphtheria vaccines as assessed by the satisfactory performance of the vaccines in clinical studies and the low incidence of diphtheria in populations with good immunization coverage. The recommendation of 30 IU/SHD as a minimum requirement for diphtheria potency for primary immunization is therefore retained in this latest revision of the Recommendations. Following the publication in 1978 of revised Requirements, it became apparent that the large numbers of guinea-pigs required for the potency test made conformity difficult to achieve in many countries, and in 1986 an addendum to the Requirements specified that 95% confidence intervals greater than 50-200% were acceptable provided that the lower limit of the 95% confidence interval was still above the minimum potency required in each SHD (7).

Subsequent activities were undertaken aimed at providing greater flexibility in procedures, reducing the number of animals used and refining endpoints without prejudice to the principle of expressing vaccine potency in IUs to demonstrate whether the product being tested meets the minimum requirement for potency. In 1988, WHO held a scientific consultation in Geneva during which special emphasis was placed on methods of determining the potency of diphtheria (and tetanus) toxoid vaccines that would require fewer animals. It was acknowledged that measurement of toxoid antigen content by in vitro methods would not necessarily indicate whether a vaccine was of acceptable potency, and that immunogenicity tests in animals remained necessary for assessing potency (8). When the Requirements were revised again in 1989, they included the option to refine the end-point of the potency assay by using toxin neutralization tests (TNTs) in vivo or in vitro after bleeding instead of a toxin challenge, which would in turn allow mice (which are not sensitive to challenge with the toxin) to be used instead of guinea-pigs (8). In addition, although multiple-dilution assays were still recommended for the demonstration of production consistency, product stability and the calibration of reference materials, the option to perform the routine potency test using a single dilution of the test and reference vaccines was included, with the provision that consistency in production and quality control had been demonstrated previously for that product (8). Further extensive international consultation highlighted a need to clarify the recommendations on the use of simplified potency assays for routine lot release, and an amendment was added

to the Requirements to include a division of the section on potency testing to distinguish clearly the recommendations for licensing from the recommendations for routine lot release (9). This latest revision of the Recommendations includes a new section on the nonclinical evaluation of diphtheria vaccines (see Part B). As a result, the procedures for potency testing included in Part A refer to routine lot-release testing, while procedures for potency testing before licensure are included in Part B.

Developments in biological standardization continue to play a crucial part in the formulation of requirements and recommendations for the production and quality control of diphtheria vaccines. For potency testing of diphtheria vaccines, the approach taken by the European Pharmacopoeia (10), like that of WHO, relies on the use of a reference preparation calibrated against the international standard as well as the expression of vaccine potency in IUs. In some countries (including the United States), the potency test is based on the United States National Institutes of Health assay for diphtheria toxoid (11). In this test, the vaccine is assessed according to its ability to induce an antibody response in guinea-pigs that reaches a minimum threshold of 2 units per ml (as measured by an in vivo TNT against a standard antitoxin preparation) without comparison to a reference vaccine. Although data have demonstrated that vaccines meeting such requirements can induce significant levels of antitoxin response in humans, the use of quantitative assays is recommended by WHO, and the expression of diphtheria vaccine potency in IUs remains the approach recommended by WHO. Nevertheless, there are no universally accepted methods for potency testing for diphtheria vaccines, and the global harmonization of procedures and requirements remains a challenge. The lack of harmonization leads to problems with the international exchange of vaccines due to difficulties in the mutual recognition of the results of testing.

During the revision of these Recommendations, WHO held a scientific consultation in Beijing, China, in November 2011. At that meeting, the option of harmonizing the minimum potency requirements for diphtheria vaccine with those recommended in the European Pharmacopoeia was discussed. It was acknowledged that amending the WHO minimum requirement for potency could improve harmonization and the international exchange of vaccines. As a result, the minimum requirement for the potency of diphtheria vaccine, tested according to the methods described in these Recommendations, was amended so that the specification of 30 IU/SHD for vaccines intended for primary immunization now applies to the lower 95% confidence limit, thus demonstrating that the vaccine potency significantly exceeds 30 IU/SHD. Because the minimum potency requirement now applies to the lower limit of the 95% confidence interval, there is no requirement to achieve a 95% confidence interval narrower than 50–200%. However, the revised section on potency testing in Part A includes information on criteria that should be met in order for the potency estimate to

be statistically valid. This latest revision of the Recommendations also includes a recommendation that the use of product-specific minimum requirements for potency is acceptable, provided they are based on the results of clinical and laboratory studies, and have been approved by the NRA.

The main changes in this latest revision include:

- a change of title from Requirements to Recommendations;
- an update of the section on international standards and reference preparations, which has been moved to the General considerations section;
- an update of the section on general manufacturing recommendations and control tests;
- amendment of the minimum requirements for the potency of diphtheria vaccines to clarify the value that applies to the lower limit of the 95% confidence interval;
- inclusion of new sections to provide guidance on the clinical and nonclinical evaluations of diphtheria vaccines to assess safety, quality and efficacy.

In order to facilitate the release process of vaccines made in accordance with these Recommendations, a model protocol is provided in Appendix 1.

Scope of the Recommendations

These Recommendations apply to the production and quality control of adsorbed diphtheria vaccines, and have been updated from the 1989 revision of the Requirements for diphtheria, tetanus, pertussis and combined vaccines (8) and the amendments made in 2003 (9). These current Recommendations highlight advances in the production and testing of diphtheria vaccines and their related intermediates. The recommendations for the testing and quality control of diphtheria vaccines included in this document are based on currently licensed vaccines. Other products (such as those containing a new type of antigen or produced using novel technology) may require additional considerations. Other issues, such as guidelines for lot release (12), are covered in more detail by other documents.

Although these recommendations apply to the production and quality control of diphtheria vaccines, most diphtheria vaccines are presented in their final formulation with at least one other vaccine. Therefore, in addition to monovalent diphtheria vaccine, these recommendations also apply to diphtheria vaccines used in combination vaccines. The tests recommended for the final bulk or final fill also apply to combined vaccines where appropriate.

General considerations

The supply of effective diphtheria vaccines depends on the use of well characterized and standardized production processes, together with extensive in-process quality control tests and monitoring of the product and its related intermediates. A detailed, written description of clearly defined standard operating procedures used for the production and testing of diphtheria vaccines (or combined vaccines containing diphtheria vaccine), together with evidence of appropriate validation for each critical production step and relevant control tests, should be submitted by the vaccine manufacturer to the NRA for approval as part of the licensing application. Proposals for any variations to manufacturing or quality control methods should be submitted to the NRA for approval before implementation and should conform to national regulatory requirements.

For the production of diphtheria toxoid, the Park Williams 8 strain of *Corynebacterium diphtheriae* has been successfully used as the source of diphtheria toxin owing to its low infectivity and high capacity for toxin production in vitro, and this strain continues to be recommended for use. The approach adopted for diphtheria vaccine production is to obtain the greatest possible quantity of toxin during the growth phase of the microorganisms, and thereafter to convert the toxin into stable toxoid by the most effective method. Formaldehyde is most commonly used for detoxifying the toxin to produce toxoid.

The demonstration of safety and the confirmation of vaccine potency are fundamental requirements for the production of diphtheria toxoid vaccine. The requirement for the product to be purified (either before or after detoxification) is retained, since diphtheria toxoid in unpurified form is liable to cause severe vaccination reactions in humans. In view of the risk of reversion to toxicity, especially when a toxin is detoxified after purification, the present recommendations have been formulated to address this risk by retaining the recommended 6-week incubation period for diluted, purified toxoid stored at elevated temperatures during the irreversibility test. The assay to detect diphtheria toxin as part of in-process safety testing can be performed using guinea-pigs or using an in vitro cell culture system. The purpose of the potency test is to demonstrate, using a suitable animal model, the capacity of the product being tested to induce an immune response analogous to that of toxoid shown to be efficacious in humans. Although there is no direct correlation between the potency result obtained in a biological assay and the level and duration of immunity induced in humans after immunization, diphtheria vaccines that have been released based on the minimum requirement of 30 IU/ SHD, introduced in the 1978 revision to the previous Requirements, have been

shown to be clinically effective. Clinical studies should also be performed to support the licensure of a new diphtheria vaccine. Long-term studies to monitor antibody persistence and to determine the need for booster doses should also be considered, although these are not necessarily a prelicensure requirement. More information on clinical evaluation is included in Part C of these Recommendations.

Terminology

Definitions for some common terms used throughout this document are given below. They may have different meanings in other contexts.

Bulk purified toxoid: the processed, purified material that has been prepared from either a single harvest or a pool of single harvests. It is the parent material from which the final bulk is prepared.

Final bulk: the homogeneous final vaccine present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

Final lot: a collection of sealed final containers that is homogeneous in all respects. In principle, a final lot must have been filled from a single final bulk container and processed further (e.g. freeze-dried) in one continuous working session. Different final lots may be filled or processed from the same final bulk in different working sessions. These related final lots (or batches) are sometimes referred to as sub-batches, sublots, filling lots or freeze-drying lots, and should be identifiable by a distinctive final lot number.

Master seed lot: a quantity of bacterial suspension that has been derived from a single strain, has been processed as a single lot, and has a uniform composition. It is used to inoculate media for preparation of the working seed lot. The master seed lot should be stored as frozen stock in liquid glycerol (usually at or below $-80\,^{\circ}\text{C}$) or as lyophilized stock at a temperature known to ensure stability.

Seed lot: a quantity of bacterial suspension that has been derived from one strain, has been processed as a single lot, and has a uniform composition. It is used to prepare the inoculum for the production medium.

Single harvest: the toxic filtrate or toxoid obtained from one batch of cultures that have been inoculated, harvested and processed together.

Working seed lot: a bacterial culture consisting of a single substrain derived from the master seed lot. Working seed lots are stored in aliquots under the conditions described above for master seed lots. The working seed lot should be prepared from the master seed lot using as few cultural passages as possible; it should have the same characteristics as the master seed lot. It is used to inoculate media for the preparation of single harvests.

International reference materials

Subsequent sections of this document refer to WHO reference materials that may be used in laboratory or clinical evaluations. Key standards used in the control of diphtheria vaccines include the following.

- The Second International Standard of Diphtheria Toxoid for Flocculation Test this material (NIBSC code 02/176) was established in 2007 (13), with an assigned unitage of 1100 Lf/ampoule, replacing the First International Reference Reagent of Diphtheria Toxoid for Flocculation Test. This standard is intended for use in flocculation tests to determine the antigen content of diphtheria toxoid.
- The Fourth WHO International Standard for Diphtheria Toxoid Adsorbed this material (NIBSC code 07/216) was established in 2009 (14), and has an assigned potency of 213 IU/ampoule based on calibration against the Third WHO International Standard for Diphtheria Toxoid Adsorbed (NIBSC code 98/560) in guinea-pig challenge assays. This standard replaces the previous standard and is intended for use as a reference vaccine in diphtheria vaccine potency assays.
- The WHO International Standard Diphtheria Antitoxin Equine this dried hyperimmune equine serum was established in 1934. The material is used to prepare a liquid fill containing 10 IU/ml in 66% glycerol in normal saline approximately every two years. The current fill has the NIBSC code number 11/200, and is intended for use as a reference preparation in TNTs in vivo or in vitro to determine the potency of diphtheria antitoxin.
- The First WHO International Standard for Diphtheria Antitoxin Human – this material (NIBSC code 10/262) was established in 2012, and has an assigned unitage of 2 IU/ampoule. This material is intended for use as a reference preparation in assays used to measure diphtheria antibody levels in human serum.

The above-mentioned international standards and reference materials listed are held by the National Institute for Biological Standards and Control, Medicines and Healthcare Products Regulatory Agency, Potters Bar, Hertfordshire, EN6 3QG, England.¹ As reference materials mentioned may be superseded by replacement standards, the WHO catalogue of international reference preparations should be consulted for the latest list of established standards.²

¹ See: http://www.nibsc.org/

² See: http://www.who.int/bloodproducts/catalogue/en/index.html

International reference materials are intended for use in the calibration of national, regional or other secondary standards that are used for the production and quality control of diphtheria vaccines. They may also be suitable for use as a primary reference preparation for some assays.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name should be diphtheria vaccine (adsorbed). The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 **Descriptive definition**

Diphtheria vaccine (adsorbed) is a preparation of diphtheria toxoid prepared by treating diphtheria toxin using chemical means to render it nontoxic without destroying its immunogenic potency. The toxoid is adsorbed onto a suitable adjuvant. The preparation should satisfy the recommendations formulated below.

The most common method of preparing toxoid from toxin is by using formaldehyde.

In some countries, the adsorbent is precipitated in the presence of the toxoid.

A.2 General manufacturing recommendations

The general manufacturing recommendations contained in WHO good manufacturing practices for pharmaceutical products: main principles (15) and Good manufacturing practices for biological products (16) apply to the production of diphtheria vaccines. These practices include demonstrating the purity and quality of the production strain and seed lots, implementing in-process quality control testing, testing for process additives and process intermediates, and developing and establishing lot-release tests.

A written description of the procedures used in the preparation and testing of the diphtheria vaccine, together with appropriate evidence that each production step has been validated, should be submitted to the NRA for approval. Proposals for modifying the manufacturing process or quality control methods should also be submitted to the NRA for approval before such modifications are implemented.

A.3 Production, processing and control

A.3.1 **Production precautions**

The general production precautions, as formulated in Good manufacturing practices for biological products (16), apply to the manufacture of diphtheria vaccine.

Suitable methods for the production of diphtheria vaccine are given in the WHO *Manual for the production and control of vaccines: Diphtheria toxoid* (17).

Personnel employed in production and quality control should be adequately trained, should have completed a course of immunization against diphtheria, and should have received appropriate booster immunization(s). Appropriate health surveillance should also be carried out.

A.3.2 Production strain and seed lots

A.3.2.1 Strains of C. diphtheriae

Strains of *C. diphtheriae* used in preparing diphtheria toxoid should be identified using a record of their history and of all tests made periodically to verify the strain's characteristics. The strain should be approved by the NRA and should be maintained as a freeze-dried culture or as frozen stock in liquid glycerol.

A highly toxigenic strain of *C. diphtheriae* should be used. A strain that has proved satisfactory in many laboratories is the Park Williams 8 strain.

A.3.2.2 Seed-lot system

The preparation of seed lots should comply with the recommendations in Part A, section A.3.1, of this document. The production of diphtheria toxin should be based on a well defined seed lot system in which toxigenicity is conserved. Cultures of the working seed should have the same characteristics as those of the strain from which the master seed lot was derived. Detailed records of the origin, passage history, purification and characterization procedures, and storage conditions should be provided to the NRA when new master seeds or working seeds are introduced. Working seeds that are in use should be characterized at defined intervals that have been approved by the NRA on the basis of prior production history and experience. The maximum number of passages of each seed lot used for production should be specified based on the number shown to produce a safe and effective product.

When possible, a combination of validated biochemical, molecular and genetic tests should be used for identification and characterization of seed lots. Suitable methods include multilocus enzyme electrophoresis (MEE),

matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and restriction fragment length polymorphism (RFLP) analysis.

A.3.2.3 Culture medium for production of toxin

C. diphtheriae should be cultured in media that are suitable to support growth and to ensure a good yield of diphtheria toxin. Examples of suitable growth media that support the production of diphtheria toxin are given in the WHO *Manual for the production and control of vaccines: Diphtheria toxoid (17).*

Semisynthetic culture media consisting of enzymatic digests of protein (such as casein) have been shown to be suitable to support the growth of *C. diphtheriae*, but toxin yield is highly dependent on the level of available iron in the growth medium (17–19), and the tox gene is regulated at the transcriptional level by iron (19).

The culture media should be free from adventitious agents, and components that are known to cause allergic reactions in humans should be avoided. Human blood or blood products should not be used. If the medium is prepared from a protein digest (e.g. casein hydrolysate or digested muscle), precautions should be taken to ensure that digestion has proceeded sufficiently. Materials or components of animal origin should be identified and approved by the NRA, and their use should comply with the WHO Guidelines on transmissible spongiform encephalopathies (20). The methods for detecting these substances should be approved by the NRA.

Any change in the media used should be submitted to the NRA for approval.

A.3.3 Single harvests

The consistency of production should be demonstrated. This process may include using measurements of culture purity, growth rate, pH and rate of toxin production. Acceptance specifications with defined limits should be approved by the NRA.

Any culture showing anomalous growth characteristics should be investigated and should be shown to be satisfactory before being accepted as a single harvest. Contaminated cultures must be discarded.

Suitable methods for the production of diphtheria toxin are given in the WHO *Manual for the production and control of vaccines: Diphtheria toxoid* (17).

Single harvests that meet the acceptance criteria may be pooled to prepare the bulk purified toxoid. Storage times should be supported by data obtained from appropriate stability studies, and should be approved by the NRA.

A.3.3.1 Control of bacterial purity

Samples of cultures used for preparing single harvests should be tested for bacterial purity by microscopic examination of stained smears, and by inoculation into appropriate culture media. Single harvests should be discarded if contamination has occurred at any stage during their production.

A.3.3.2 Filtration

After the culture medium has been sampled to control for purity, filtration should be used to separate the medium aseptically from the bacterial mass as soon as possible. A preservative may be added, but phenol should not be used for this purpose.

To facilitate filtration, cultures may be centrifuged, provided that suitable precautions have been taken to avoid the formation of potentially hazardous aerosols. A filter aid may be added beforehand. A filter that does not shed fibres should be used.

A.3.3.3 Determination of crude toxin concentration

Prior to inactivation, the toxin content of the culture supernatant should be determined using a method approved by the NRA.

The flocculation test is suitable for the measurement of toxin content, and is described in the WHO Manual for the production and control of vaccines: Diphtheria toxoid (17) and the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (21). A reference material calibrated against the International Standard for diphtheria toxoid for flocculation test should be included, and the results should be expressed in Lf.

The measurement of toxin content is a good indicator of the consistency of production, and acceptance limits should be defined for monitoring purposes.

It is preferable that culture filtrates used to prepare purified toxoid contain at least 50 Lf/ml.

A.3.3.4 **Detoxification and purification**

Detoxification of diphtheria toxin may be performed using crude toxin (culture filtrate) or purified toxin. Detoxification of purified toxin results in a purer product, although particular care must be taken to avoid a reversion to toxicity; reversion may also occur when crude toxin is used for detoxification. The method of purification should be such that no substance is incorporated into the final product that is likely to cause adverse reactions in humans.

The method of purification and the agent used for detoxification should be suitably validated, and should be approved by the NRA. The rate of

detoxification may vary, and in-process monitoring of the detoxifying process should be performed.

Formaldehyde is most commonly used as the detoxifying reagent, and amino acids such as lysine or glycine may be added during detoxification to facilitate cross-linking of toxin molecules, and to help prevent reversion. The detoxification conditions should be well defined and controlled with respect to temperature, time, concentration of the detoxifying reagent, toxin concentration and any other critical parameters.

The method used for purification should be approved by the NRA.

Crude toxoid can be concentrated using ultrafiltration prior to purification by fractionation with ammonium sulfate, dialysis, gel filtration, ionexchange chromatography, or a combination of these methods.

Bioburden testing may also be performed after purification to ensure that potential levels of contamination have been minimized for subsequent steps that are not performed aseptically.

When measured in the final bulk vaccine, the amount of residual free detoxifying agent remaining after detoxification and purification have been completed should not exceed the limit stated in section A.3.5.2.7.

Harvests should be treated as potentially toxic, and subject to the appropriate safety restrictions until the detoxification has been shown to be complete by performance of a specific toxicity test (as detailed in section A.3.4.4) or any other suitably validated in vivo or in vitro method.

Detoxification can be confirmed by subcutaneous inoculation of the toxin into guinea-pigs, or by intradermal injection into guinea-pigs or rabbits. A cell culture assay, such as the Vero cell assay, is also suitable.

Storage times should be supported by data obtained from appropriate stability studies, and should be approved by the NRA.

A.3.4 **Bulk purified toxoid**

Preparation A.3.4.1

The bulk purified toxoid should be prepared from either a single harvest or a pool of single harvests, and should be sterile. If the NRA approves, a preservative may be added, provided that the preservative has been shown not to adversely affect the safety and immunogenicity of the toxoid. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity of diphtheria vaccines.

It is advisable to sterilize the bulk purified toxoid by filtration.

A.3.4.2 **Sterility**

Each bulk purified toxoid should be tested for bacterial and mycotic sterility in accordance with the requirements in Part A, section 5, of General requirements for the sterility of biological substances (22) or by a method approved by the NRA. The sterility test is performed using at least 10 ml of each bulk purified toxoid. If a preservative has been added to the purified bulk, appropriate measures should be taken to prevent it from causing any interference in the sterility test.

A.3.4.3 Antigenic purity

Each bulk purified toxoid should be tested for antigenic purity by determining the antigen concentration in Lf and the concentration of protein (nondialysable) nitrogen. The antigen concentration should be determined by comparing it with a reference material calibrated against the International Standard for diphtheria toxoid for flocculation test or against an equivalent reference preparation approved by the NRA. The method of testing should be approved by the NRA. The bulk purified toxoid passes the test if it contains at least 1500 Lf/mg of protein (nondialysable) nitrogen.

The flocculation (Ramon) assay is suitable for measuring antigen content, and is described in the WHO Manual for the production and control of vaccines: Diphtheria toxoid (17) and in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (21).

Physicochemical analysis, using methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) may be used to monitor antigenic purity and to provide additional information on antigen integrity and the extent of aggregation and proteolysis. These additional characterization tests should be performed whenever a new working seed is introduced.

A.3.4.4 **Specific toxicity**

Each bulk purified toxoid should be tested for the presence of diphtheria toxin. The test may be performed in vivo using guinea-pigs or in vitro using a suitable cell culture assay, such as the Vero cell assay.

A suitable in vivo test consists of injecting the toxoid into at least five guinea-pigs, each weighing 250–350 g. The guinea-pigs should not have been used previously for experimental purposes. Each guinea-pig should be given a subcutaneous injection of 1 ml of a dilution of purified toxoid containing at least 500 Lf of toxoid. The diluted toxoid is prepared in such a way that the chemical environment is comparable to that found in the final vaccine except for the absence of adjuvant. Animals are observed for 42 days, and any animals that die should undergo necropsy and be examined for symptoms of diphtheria

intoxication (e.g. red adrenal glands). The bulk purified toxoid passes the test if no guinea-pig shows symptoms of specific toxicity and if at least 80% (i.e. four fifths) of the animals survive the test period. If more than one animal dies from nonspecific causes, the test should be repeated. If more than one animal dies during the retest, then the bulk purified toxoid does not comply with the test.

Some manufacturers carry out an alternative test for determining whether diphtheria toxin is present: they inject intradermally into rabbits or guinea-pigs at least 20 Lf of purified toxoid and observe the injection sites for specific erythema. Erythema with a diameter greater than 5 mm is typically considered to be positive.

Alternatively, an in vitro cell culture test, such as the Vero cell assay, may be used, provided that the sensitivity of the test has been demonstrated to be not less than that of the guinea-pig test. For the Vero cell assay, a dilution of bulk purified toxoid is prepared so that the chemical environment is comparable to that present in the final bulk vaccine except for the absence of adjuvant, preservative and other excipients, which may cause nonspecific toxicity in Vero cells. A duplicate titration of toxoid is performed in the presence of diphtheria antitoxin to confirm that any signs of cytotoxicity are specific and due to the presence of diphtheria toxin. So that the sensitivity of the assay can be confirmed, a purified preparation of diphtheria toxin should be included in the test, diluted in a purified bulk diphtheria toxoid that has previously been shown to be nontoxic to Vero cells. The test procedure and the interpretation of results should be approved by the NRA. An example of the Vero cell method is included in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (21).

A.3.4.5 Reversion to toxicity

Each bulk purified toxoid should be tested to ensure that reversion to toxicity does not take place during storage. The test may be performed in vivo using guinea-pigs or in vitro using a suitable cell culture assay, such as the Vero cell assay. The test employed should be approved by the NRA, and should be sufficiently sensitive to detect very small amounts of toxin. For the in vivo assay, the bulk purified toxoid should be diluted in order to obtain the same concentration and chemical environment as present in the final bulk vaccine, except for the absence of adjuvant.

For bulk toxoid that will be used in the preparation of more than one final-bulk formulation, the test should be performed using dilutions of the bulk toxoid that represent the lowest and highest concentrations of toxoid that will be present in the final formulations.

To determine whether reversion has occurred, diluted toxoid that has been stored at 34–37 °C for six weeks should be tested. At the end of the incubation period,

groups of five guinea-pigs are each given a subcutaneous injection of the diluted toxoid sample. A total injection volume of 5 ml should be used (using multiple injection sites where necessary (such as two injections of 2.5 ml each), which is the equivalent of 10 SHDs. The animals are observed for 42 days for signs of ill health. No toxicity should be detected.

Similar dilutions of toxoid held at $2-8\,^{\circ}\text{C}$ during the same period of time as those held at $34-37\,^{\circ}\text{C}$ may be tested as controls.

Intradermal tests in guinea-pigs are considered to be suitable provided that the dose has been adjusted accordingly, and the sensitivity of the test has been shown to be not less than that of the subcutaneous test.

Alternatively, an in vitro cell culture test, such as the Vero cell assay, may be used, provided that the sensitivity of the test has been demonstrated to be not less than that of the guinea-pig test. For the Vero cell assay, a dilution of bulk purified toxoid is prepared in such a way that the chemical environment is comparable to that present in the final bulk vaccine, except for the absence of adjuvant, preservative and other excipients, which may cause nonspecific toxicity in Vero cells.

For bulk toxoid that will be used in the preparation of more than one final bulk formulation, the test should be performed using dilutions of the bulk toxoid that represent the lowest and highest concentrations of toxoid that will be present in the final formulations.

The diluted toxoid is stored at 34–37 °C for six weeks, and a duplicate sample is stored at 2–8 °C for the same period. So that the sensitivity of the assay can be confirmed, a purified preparation of diphtheria toxin should be included in the test, diluted in a purified bulk diphtheria toxoid that has previously been shown to be nontoxic to Vero cells. The test procedure and the interpretation of the results should be approved by the NRA. An example of the Vero cell method is included in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (21).

A.3.4.6 Storage of bulk purified toxoid

Storage times for the bulk purified toxoid should be supported by data from appropriate stability studies, and should be approved by the NRA (23).

A.3.5 Final bulk

A.3.5.1 **Preparation**

The final bulk is prepared from bulk purified toxoid adsorbed onto a suitable adjuvant. The final formulation of the vaccine should be based on formulations

that have been shown to be safe and effective in clinical use. The number of Lf per SHD should be approved by the NRA.

It is recommended that the diphtheria antigen content in vaccines intended for primary immunization should not exceed 30 Lf/SHD.

In vaccines intended for use as booster vaccines, the quantity of diphtheria toxoid in the vaccine should be approved by the NRA. It should be shown that the vaccine does not cause adverse reactions in people from the age groups for which the vaccine is intended.

In some countries it is recommended that the diphtheria antigen content of diphtheria vaccines intended for boosting should not exceed 2.5 Lf/SHD.

A.3.5.2 Control tests

A.3.5.2.1 Preservative

If the vaccine is to be dispensed into multidose containers, a suitable antimicrobial preservative should be added. The amount of preservative in the final bulk should be shown to have no deleterious effect on the toxoid or on other vaccine components with which the toxoid may be combined; the preservative should also be shown to cause no unexpected adverse reactions in humans. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity of diphtheria vaccines. The preservative and its concentration should be shown to be effective, and should be approved by the NRA. The WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines should be followed (24).

Determine the amount of preservative by using a suitable chemical method. The amount should be at least 85% and not more than 115% of the intended amount.

A.3.5.2.2 Adjuvants

The nature, purity and concentration of the adjuvant used in the formulation should be determined by methods approved by the NRA. When aluminium compounds are used as adjuvants the concentration of aluminium should not exceed 1.25 mg/SHD. If other adjuvants are used, specifications should be established by the manufacturer and approved by the NRA.

In some countries these recommended limits for adjuvant concentrations are considered too high, and lower limits have been approved and shown to be safe and effective.

A.3.5.2.3 Degree of adsorption

The degree of adsorption should be measured and should be shown to be comparable to that measured in vaccine lots used in clinical studies to support licensing. The measurement of antigen content and the degree of adsorption to adjuvant are good indicators of the consistency of production; in-house acceptance limits can be established once a suitable number of lots have been tested.

Suitable methods for determining the degree of adsorption in diphtheria vaccines are described in the WHO *Manual for quality control of diphtheria*, tetanus and pertussis vaccines (21).

These tests may be omitted provided they are performed on the final lot.

A.3.5.2.4 Sterility

Each final bulk should be tested for bacterial and mycotic sterility in accordance with the recommendations in Part A, section 5, of the revised General requirements for the sterility of biological substances (22) or by a method approved by the NRA. The sterility test should be performed using at least 10 ml of each final bulk. If a preservative has been added to the final bulk, appropriate measures should be taken to prevent it from causing any interference in the sterility test.

A.3.5.2.5 Specific toxicity

Each final bulk should be tested for specific toxicity in at least five guinea-pigs; each guinea-pig should weigh 250–350 g and not have been used previously for experimental purposes. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least 5 SHDs, and is observed for 42 days. Animals that die should undergo necropsy and be examined for symptoms of diphtheria intoxication (e.g. red adrenal glands). The final bulk passes the test if no guinea-pig shows symptoms of specific intoxication within six weeks of injection, and if at least 80% (i.e. four fifths) of the animals survive the test. If more than one animal dies from nonspecific causes, the test should be repeated. If more than one animal dies during the retest, then the final bulk does not comply with the test.

If the NRA approves, the specific toxicity test used on the final bulk may be omitted from routine lot-release procedures once consistency in production has been demonstrated.

A.3.5.2.6 *Potency*

The potency of each final bulk (or final lot) should be determined by comparison with a suitable reference preparation that has been calibrated in IUs against the Fourth WHO International Standard for Diphtheria Toxoid Adsorbed. Appropriate statistical methods should be used to calculate the potency of the final bulk (21). The NRA should approve the assay method and the method used for calculating the results. Details on methods to be used for the potency testing

of diphtheria vaccines can be found in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (21).

The minimum potency specifications introduced in the 1978 WHO Recommendations have helped to ensure the production and use of safe and effective diphtheria vaccines, as evidenced by the satisfactory performance of these products in clinical studies and the low incidence of diphtheria in populations with good immunization coverage. Therefore, it is recommended that the potency of a diphtheria vaccine used for the primary immunization of children should significantly exceed 30 IU/SHD (based on data showing that the lower 95% confidence limit of the estimated potency is at least 30 IU/SHD).

The minimum potency specification for diphtheria vaccine intended for booster immunization in older children, adolescents and adults should be approved by the NRA.

In some cases it is recommended that the lower 95% confidence limit of the estimated potency of diphtheria vaccines intended for boosting should be not less than 2 IU/SHD.

Product-specific minimum requirements for diphtheria potency are acceptable, provided that they have been justified and are based on potency values obtained for the vaccine in question. A suitable number of lots should be tested in order to define the minimum requirement for potency. Vaccine lots used for the establishment of the potency specification should include lots that have been shown to be safe and effective in clinical studies. Product-specific minimum requirements should be approved by the NRA. Once defined and approved, the potency of the vaccine should be shown to exceed the minimum requirement significantly (based on data showing that the lower 95% confidence limit of the estimated potency is at least that of the minimum requirement).

The following criteria should be met in order for the potency estimate to be statistically valid:

- the statistical analysis should show a significant regression (P < 0.05) of the log dose–response lines without significant deviation from linearity and parallelism (P > 0.05);
- for subcutaneous challenge assays, the 50% protective dose should lie between the smallest and largest vaccine doses for intradermal challenge assays, the mean score obtained for the smallest vaccine dose should be less than 3, and the mean score obtained for the largest vaccine dose should be more than 3.

When more than one assay is performed, the results of all statistically valid tests should be combined into a geometric mean estimate, and the confidence limits calculated.

Manufacturers are also encouraged to monitor the potency of different vaccine bulks and lots by setting minimum and maximum alert criteria once a suitable number of lots have been tested.

Calibration of reference preparations

Secondary reference preparations (regional, national, working or product-specific standards) should be calibrated using a multiple-dilution assay to immunize guinea-pigs with appropriate dilutions of both the international standard and the proposed reference preparation; immunization should be followed by challenge with diphtheria toxin (via the subcutaneous or intradermal route) or titration of immune serum samples using an in vivo TNT (in guinea-pigs) or an in vitro TNT (i.e. a Vero cell assay). Adequate controls should be in place to ensure and monitor the stability of all secondary standards; where possible, replacement lots should be calibrated against the international standard (25).

Potency test for routine lot release

For routine testing, the potency of diphtheria vaccine may be determined using guinea-pigs or mice. When potency tests are carried out in mice instead of guinea-pigs, transferability should be demonstrated for the product being tested (21).

To determine the potency of a diphtheria vaccine, guinea-pigs or mice are immunized with appropriate dilutions of the calibrated reference preparation and the product being tested. Care should be taken to ensure that the diluents are inert (e.g. phosphates might interfere with the adsorption of toxoid) and not pyrogenic. Guinea-pigs may be challenged with diphtheria toxin or bled for titration of immune serum. Mice should be bled for titration of immune serum. Titration of immune serum samples may be performed using an in vivo or in vitro TNT – such as a Vero cell assay – or using another in vitro method, such as the enzyme-linked immunosorbent assay (ELISA), subject to validation. If in vitro serological assays are used, they should show that the product induces an appropriate antibody response in animals when compared with the reference preparation.

The ELISA assay or another suitable in vitro method may be used to measure the antibody response to diphtheria toxoid, provided that these assays have been validated against the challenge assay or the TNT using the particular product in question. A minimum of three assays with a suitable dose–response range is likely to be required for validation of a particular product (26). These methods require precise definition of the characteristics of reagents (such as the antigen, and positive and negative control serum samples) that are critical for the successful performance of the testing method.

Potency assay modifications: reduced dilution schemes

Consistency limits for diphtheria potency should be established once a suitable number of lots has been tested using a multiple-dilution assay. Once consistency in production has been demonstrated for the vaccine, the potency assay (using the challenge or serological model) may, with the approval of the NRA, be performed using a reduced number of animals or doses, or both. Production consistency should be demonstrated using vaccine potency expressed in IUs and obtained for at least 10 consecutive vaccine lots derived from different toxoid bulks; the expectations of linearity and parallelism must be consistently satisfied, and the potency must be consistently higher than the minimum requirement. Once approved, fewer doses of the test and reference vaccines may be used, and the assumptions of linearity and parallelism need not be tested for each assay. When vaccine lots consistently give the lower limit of the 95% confidence interval for the estimated potency values (i.e. well in excess of the minimum requirement), onedilution tests may offer advantages. If one-dilution assays are not advantageous, a reduction in animal usage may nevertheless be achieved by using two-dilution assays or another suitable design modification.

A one-dilution assay is based on the same principles for evaluating the response as three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 30 IU (or the minimum requirement for the product expressed as an SHD), that elicits a minimum protective effect (or antibody response) in immunized animals; the effect of the reference vaccine is compared with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P \le 0.05$), the potency of the test vaccine is satisfactory.

One-dilution assays provide assurances that the potency significantly exceeds the minimum requirement. A disadvantage of this approach is that it is not possible to obtain strictly quantitative estimates of vaccine potency. Therefore, in order to ensure the overall consistency of production, there is a need to support the data generated by a simplified potency assay with data from physicochemical methods or other in vitro assays. When a one-dilution assay is used with serological analysis, measurement of the geometric mean antibody response in a group immunized with the test vaccine can provide some information about production consistency on a continual basis, provided that the in vitro assay used to measure antibody titres contains suitable internal controls.

Lot release based upon the use of a simplified approach requires periodic review to ensure that the validity of all procedures (including assumptions of linearity and parallelism) is maintained. The timing of the review should be decided on a case by case basis, depending on the number of lots of vaccine

produced annually, or by time schedule (at least every two years), and should be approved by the NRA. It should be noted that if there is a significant change in the production process, testing should revert to the full multiple-dilution assay, and production consistency should be reconfirmed before the reduction scheme is reintroduced.

A.3.5.2.7 Amount of residual free detoxifying agent

The amount of residual free detoxifying agent in each final bulk should be determined. The method used and the acceptable limits should be approved by the NRA.

If formaldehyde has been used, the residual content should not exceed 0.2 g/l. The colorimetric determination of the reaction product of formaldehyde and fuchsin–sulfurous acid is a suitable method for detecting residual free formaldehyde.

Where applicable, appropriate tests should be performed for the quantification of other detoxifying agents. The tests used and the maximum residual content of such chemicals should be approved by the NRA.

A.3.5.2.8 pH

The pH of the final bulk should be measured and should be within the range of values measured in vaccine lots shown to be safe and effective in clinical use.

A.3.5.3 Storage of final bulk

The final bulk may be stored in a single container or in multiple containers. When multiple storage containers are used, the contents must be pooled into a single container for filling into the final containers. Storage times for the final bulk should be supported by stability studies, and approved by the NRA.

A.4 Filling and containers

The requirements concerning filling and containers given in WHO good manufacturing practices for pharmaceutical products: main principles (15) and Good manufacturing practices for biological products (16) apply.

Single-dose or multiple-dose containers may be used. Vaccine in multidose containers should contain a suitable antimicrobial preservative (see section A.3.5.2.1).

The filling process should be suitably validated by comparing key parameters measured in the final bulk and in the final lot. Such studies should include measurement of the degree of adsorption.

A.5 Control of final product

Quality-control procedures and tests should be validated and approved by the NRA to ensure that the final containers hold the antigen and formulation appropriate for the intended use of the final product.

Unless otherwise justified and authorized, the following tests should be performed on labelled containers from each final lot by means of validated methods approved by the NRA.

A.5.1 **Identity**

An identity test should be performed on at least one container from each final lot using a validated method approved by the NRA.

The method used should be based on the specific interaction between the diphtheria antigen in the vaccine and diphtheria antitoxin. Suitable detection methods include flocculation (Ramon and light-scattering methods), immunoprecipitation assays and ELISA (17, 21). Tests on toxoid adsorbed on to an aluminium carrier should be performed after the carrier has been dissolved or the adsorbed toxoid has been wholly or partially eluted by sodium citrate or ethylenediaminetetraacetic acid (EDTA) (17, 21).

A.5.2 **Sterility**

Final containers should be tested for bacterial and mycotic sterility using a method approved by the NRA.

Many countries have regulations governing the sterility testing of the final product. Where these regulations do not exist, the requirements published by WHO should be met (22). If a preservative has been added to the purified bulk, appropriate measures should be taken to prevent it from causing any interference in the sterility test.

A.5.3 Potency

A potency test should be carried out on each final lot as described in Part A, section A.3.5.2.6, if such a test has not been performed on the final bulk.

A.5.4 Innocuity

Each final lot should be tested for innocuity by intraperitoneal injection of 1 human dose (but not more than 1 ml) into each of five mice (weighing 17–22 g) and by intraperitoneal injection of at least 1 SHD (but not more than 1 ml) into each of two guinea-pigs (weighing 250–350 g). The tests should be approved by the NRA. The final product is considered to be innocuous if the animals survive

for at least seven days without showing significant signs of toxicity. This test is also referred to as the abnormal toxicity test or the general safety test.

If the NRA approves, the innocuity test on the final lot may be omitted from routine lot release once the consistency of production has been demonstrated.

A.5.5 Adjuvant content

The adjuvant content of each final lot should be determined using a method approved by the NRA (see Part A, section A.3.5.2.2).

The formulation should be such that after shaking, the vaccine remains suspended as a homogeneous solution for a defined period (to allow sufficient time for administration).

A.5.6 **Degree of adsorption**

A test for the degree of adsorption should be carried out on each final lot as indicated in Part A, section A.3.5.2.3.

A.5.7 Preservative content

The preservative content of each final lot should be determined as described in section A.3.5.2.1. The method used should be approved by the NRA.

If the NRA approves, this test may be performed only on the final bulk.

A.5.8 **pH**

The pH of the final lot should be measured and should be within the range of values measured in vaccine lots shown to be safe and effective in clinical use.

In some cases, determination of osmolality may also be required.

A.5.9 Extractable volume

For vaccines filled into single-dose containers, the extractable content should be checked and shown to be not less than the intended dose.

For vaccines filled into multidose containers, the extractable content should be checked and should be shown to be sufficient for the intended number of doses.

A.5.10 Inspection of final containers

Each container in each final lot should be inspected visually or mechanically, and those containers showing abnormalities (e.g. improper sealing, clumping or the presence of particles) should be discarded.

A.6 Records

The requirements given in WHO good manufacturing practices for pharmaceutical products: main principles (15) and Good manufacturing practices for biological products (16) apply. Written records should be kept of all tests, irrespective of their results. The records should be of a type from which annual trends can be determined.

A model of a suitable summary protocol for diphtheria vaccines (adsorbed) is given in Appendix 1.

A.7 Retained samples

Vaccine samples should be retained, as recommended in WHO good manufacturing practices for pharmaceutical products: main principles (15) and Good manufacturing practices for biological products (16).

A.8 Labelling

The label printed on or affixed to each container, and the label on the carton enclosing one or more containers, should be approved by the NRA. The labels should be easily readable and should show as a minimum:

- the words "diphtheria vaccine (adsorbed)" or the proper name of the product, or both
- the licence number of the product
- the name of the manufacturer.
- the number of the final lot
- the identity of any preservative or adjuvant
- the amount of antigen in Lf or the minimum potency in IU/SHD, or both
- the recommended storage temperature and the expiry date if kept at that temperature
- the recommended SHD and route of administration.

In addition, the label printed on or affixed to the container, or the label on the cartons, or the leaflet accompanying the container should contain the following:

- a statement that the vaccine satisfies the recommendations of this document:
- the address of the manufacturer:
- the recommended temperature for transport;

- a warning that the adsorbed vaccine should not be frozen;
- a warning that the adsorbed vaccine should be shaken before use;
- instructions for the use of the vaccine, and information on contraindications and the reactions that may follow vaccination.

A.9 **Distribution and transport**

The requirements given in WHO good manufacturing practices for pharmaceutical products: main principles (15) and Good manufacturing practices for biological products (16) apply.

A.10 Stability, storage and expiry date

A.10.1 Stability

Stability evaluation is a critical part of quality assessment, and the general principles of stability evaluation are described in the WHO Guidelines on stability evaluation of vaccines (23). The purpose of stability studies is to confirm that at the end of its shelf-life (or other defined storage period) the vaccine has the required characteristics to ensure its quality, safety and efficacy. The stability of the vaccine in final containers maintained at the recommended storage temperature should be demonstrated to the satisfaction of the NRA. Containers from at least three consecutive final lots (each derived from unique toxoid bulks) should be tested. The vaccine should be tested up until its expiry date to demonstrate its stability during storage.

The vaccine should be manufactured in such a way that reversion to toxicity does not occur during the defined shelf-life, provided that the vaccine is stored under the conditions recommended on the label. To confirm that the vaccine does not revert to toxicity during storage, the specific toxicity test described in Part A, section A.3.5.2.5, should be scheduled up until the expiry date as part of the stability studies. In addition, at the time of the expiry date, the vaccine should meet the requirements or acceptance limits for the final product in terms of sterility, potency, adjuvant content, degree of adsorption, preservative content and pH (see Part A, sections A.5.2, A.5.3 and A.5.5–A.5.8), provided that it has been stored at the recommended temperature. The frequency of testing should be approved by the NRA.

When any changes that may affect the stability of the product are made in the production process, the vaccine produced by the new method should be shown to be stable.

Stability studies performed at temperatures other than those recommended for storage may be useful in providing information about transporting the vaccine at different temperatures for a limited time.

A.10.2 Storage conditions

The recommended storage conditions and the defined maximum duration of storage should be based on stability studies, as described in section A.10.1, and should be approved by the NRA. For diphtheria vaccines, a temperature of 2–8 °C is considered to be satisfactory and should ensure that the minimum potency specified on the label of the container or package will be maintained after release and until the end of the shelf-life, provided that the vaccine is stored under the recommended conditions.

Adsorbed vaccines must not be frozen.

A.10.3 Expiry date

The statement concerning the expiry date that appears on the label, as required in Part A, section A.8, should be based on experimental evidence, and approved by the NRA on the basis of data obtained during the stability studies referred to in section A.10.1. The date of manufacture (i.e. blending or filling) or the start date of the last satisfactory potency determination (i.e. the date on which the test animals were immunized with the vaccine) performed in accordance with Part A, section A.5.3 (or section A.3.5.2.6), is taken as the start date for the shelf-life.

In some cases, the date of the first satisfactory potency determination is used as the start date for the shelf-life.

Part B. Nonclinical evaluation of diphtheria vaccines

B.1 Introduction

The nonclinical testing of vaccines and their related intermediates is an essential part of the development of candidate vaccines, and is a prerequisite for the initiation of clinical trials in humans. Within the scope of this document, nonclinical evaluation means all in vivo and in vitro testing performed before and during the clinical development of the vaccine. Studies are aimed at defining the in vitro and in vivo characteristics of candidate vaccines, and such studies include evaluations of safety and immunogenicity. The recommendations included in this document are intended for new manufacturers of diphtheria vaccine, and should also be referred to if a significant change to the production process or product formulation is made by a manufacturer already producing diphtheria vaccine.

These recommendations refer only to products based on those that are currently licensed and in clinical use – i.e. vaccines based on the use of chemically detoxified diphtheria toxin as the antigen adsorbed onto an aluminium-based or calcium-based adjuvant. Diphtheria vaccines based on novel antigens or formulations that have not previously been evaluated for safety and efficacy in

clinical trials are likely to require more extensive nonclinical characterization, which is beyond the scope of this document.

The nonclinical evaluation of diphtheria vaccines should be based on WHO guidelines on nonclinical evaluation of vaccines (27) which incorporate definitions for commonly used terms related to nonclinical evaluations. Nonclinical evaluations of vaccine intermediates and the final product should be performed in accordance with the principles of good laboratory practice (GLP). Adhering to the principles of GLP promotes the development of high-quality test data, and provides a tool to ensure that a sound approach is taken to the management of laboratory studies, including how they are conducted, and how their data are reported and archived (28).

The nonclinical characterization of vaccine intermediates and in-process materials should be based on the use of adequately characterized, homogenous starting materials of defined origin and acceptable quality, including the bacterial strain and production seed lots. Demonstrating consistency in production may not be applicable during the early stages of nonclinical evaluation, but adequate validation of the production process is required to demonstrate that the manufacturing conditions are reproducible.

B.2 Nonclinical testing and characterization of intermediates and in-process materials

Intermediates and in-process materials must be tested and characterized to confirm that they meet the recommendations in Part A of this document. The source and quality of all starting materials should be documented and should include detailed descriptions of the characterization of the strain, master seed lot and working seed lot. Defined procedures should also be shown for the preparation of new working seeds from the master seed. Seed lots should be shown to retain the characteristics of the parent strain throughout seed lot production, and should be characterized whenever a new master seed or working seed is introduced. Seed lots should be identified and characterized using a combination of validated biochemical, molecular and genetic tests. Methods such as MEE, MALDI-TOF mass spectrometry, PFGE, MLST and RFLP analysis should be considered. The maximum number of passages of each seed lot used for production should be specified and based on the number shown to result in the production of a safe and effective product; the maximum number of passages should be approved by the NRA.

The toxigenicity of the *C. diphtheriae* strain used for production should be confirmed by titration of crude toxin harvested from the culture supernatant using an appropriate in vivo or in vitro method. The culture medium used for toxin production should be well defined, and any animal components present in the medium should be identified and documented. Protein contaminants derived from the bacterium or from components of the culture medium may increase

the potential for adverse reactions to immunization with the toxoid, and the detoxification and purification processes used should minimize the presence of any substances likely to cause adverse reactions in humans. The methods used for the detoxification and purification of crude toxin should be adequately described and should be supported by appropriate validation data.

B.2.1 Safety evaluation

The detoxification step of the production process should be validated to confirm that the detoxification of diphtheria toxin is complete and irreversible. Both the specific toxicity test (section A.3.4.4) and the reversion-to-toxicity test (A.3.4.5) should be performed on the bulk purified toxoid. Where possible, in vivo methods should be performed during nonclinical evaluations of the vaccine, but in vitro alternatives may be included as part of the validation studies.

B.2.2 Immunogenicity and/or potency

The adsorbed bulk vaccine should be tested for immunogenicity and/or potency during the nonclinical evaluation as described in section B.3.3.

B.2.3 **Stability**

Stability studies should be based on the WHO Guidelines on stability evaluation of vaccines (23). The stability of all intermediates not used within a short period of time should be evaluated and demonstrated using suitable methods. The choice of stability-indicating parameters as well as the frequency of testing should be justified to and approved by the NRA. Storage periods proposed for intermediates produced during the manufacturing process should be based on data obtained from the stability studies.

B.2.4 Adjuvants

Where appropriate, adjuvants should be characterized in terms of chemical composition, physical form and adsorption capacity, purity, endotoxin content and sterility. The interaction between the adjuvant and antigen should also be evaluated; this evaluation should include measurement of the degree of adsorption. This should be shown to be consistent from lot to lot and throughout the intended storage period, and quality specifications should be established once a sufficient number of lots have been produced.

Nonclinical characterization of formulated vaccine

Lots of the final formulated vaccine used in nonclinical studies should be adequately representative of those intended for clinical investigation, and, ideally, should be the same lots as those intended for clinical use. Manufacturers should

make every effort to keep some of this characterized material for future reference. As a minimum, candidate vaccines should be prepared under conditions of good manufacturing practice (GMP) for clinical trial material (29), and full implementation of the principles of GMP will be required during the later stages of clinical development (15, 16).

The final formulated vaccine should be evaluated using a combination of immunological and physicochemical approaches to determine key product characteristics including sterility, pH, antigen content and degree of adsorption, immunogenicity/potency, and safety, as described in Part A of this document. Particular attention should be paid to the assessment of safety, toxicology, immunogenicity and stability. In some cases, comparability testing should be performed (e.g. after a significant change in the manufacturing process or at the time of scale-up following licensure). Comparator studies may also be required when a new manufacturer produces a diphtheria vaccine. The requirement for and extent of comparative studies, and the choice of the comparator vaccine, should be approved by the NRA.

B.3.1 Safety

The vaccine should be tested to confirm the absence of specific toxicity and general toxicity using the in vivo methods described in Part A (the specific toxicity test and innocuity test). In vitro methods are not suitable for toxicity testing of the final vaccine formulation due to the presence of adjuvant.

B.3.2 Toxicology

Nonclinical toxicology studies should be such that reasonable assurance is obtained that it is safe to proceed to clinical evaluation. The potential toxic effects of the vaccine should be evaluated in at least one animal species; this evaluation should include histopathology of important organs. The study should investigate the potential for local inflammatory reactions, systemic toxicity and effects on the immune system. The animal species used should be sensitive to the biological effects of the vaccine and to the toxin. Where feasible, the highest dose to be used in the proposed clinical trial should be evaluated in an animal model. Further information on considerations related to dose, route of administration, controls, and parameters to be monitored can be found in the WHO guidelines on nonclinical evaluation of vaccines (27). A full toxicology assessment may not be required in all cases (e.g. when a manufacturer already producing the vaccine changes the production process), although any decision not to perform toxicology studies should be approved by the NRA. Diphtheria vaccines produced using a novel antigen or adjuvant, or both, are likely to require a full toxicology assessment, which is described elsewhere (27).

For diphtheria vaccines intended to be used in adolescents and adults (e.g. as booster vaccines or to manage diphtheria outbreaks), the need to perform developmental toxicology studies should be considered unless scientific and clinically sound arguments can be made that such studies are not necessary. Further information about developmental toxicity studies can be found in the WHO guidelines on nonclinical evaluation of vaccines (27).

Immunogenicity and/or potency B 3 3

Immunization studies in appropriate animal models can provide valuable proof-of-concept information during the preclinical development stages. For diphtheria vaccines, immunogenicity studies should include measurement of toxin neutralizing antibody responses in serum samples from vaccinated animals.

The potency of the vaccine should be determined, and those lots that have been shown to meet the recommendations described in Part A, section A.3.5.2.6, are likely to induce adequate immune responses in clinical trials. The measurement of vaccine potency by comparison with a suitable reference vaccine calibrated in IUs is useful for assessing production consistency. During nonclinical evaluations, the potency test should consist of a multiple-dilution assay (with at least three dilutions of each test vaccine and the reference preparation), should be performed using guinea-pigs or mice and should have a functional end-point (i.e. a challenge with diphtheria toxin when guinea-pigs are used, or titration of immune serum samples by TNT when guinea-pigs or mice are used). More details on the methods used for the potency testing of diphtheria vaccines can be found in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (21).

Stability **B34**

Stability testing should be seen as a continual process occurring from the development of the vaccine through licensing and on to post-licensure monitoring. Stability studies should be based on the WHO Guidelines on stability evaluation of vaccines (23). During the early stages of clinical trials, the amount of real-time stability data may be limited, but sufficient data should be generated to support the stability of the vaccine for the intended duration of the trial. For licensure, however, studies should be carried out under the proposed storage conditions, and should be performed in real time. Accelerated stability studies of products stored for limited periods at temperatures that may affect stability could support preliminary data from continuing real-time stability studies but should not replace them. Following licensure, continuing assessments of stability are recommended to support the shelf-life specifications. The cumulative nature of the actual age of the antigen at the end of the shelf-life of the final vaccine product

should be considered, and data covering the cumulative age of the antigen should be collected and reported to the NRA.

Stability studies should confirm that the production process results in a final product that does not revert to toxicity during long-term storage. As a result, safety testing, using both the specific toxicity test and the innocuity test, should be performed on the expiry date of the product. Additional tests that may be used to demonstrate stability include the potency test, and physical and chemical characterization; as a minimum, tests for potency, sterility, adjuvant content, degree of adsorption, preservative content and pH should be performed. Final containers from at least three vaccine lots, each of which has been derived from different bulks should be tested on their expiry date to demonstrate that stability has been maintained during storage at the recommended temperature. The time points selected for testing should be appropriate for the vaccine being evaluated, should be supported by validation data and should be approved by the NRA.

Part C. Clinical evaluation of diphtheria vaccines

C.1 Introduction

This section addresses issues that are relevant during the clinical development of diphtheria vaccines. Progression through the phases of clinical development should follow the principles outlined in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (30) which contains definitions for commonly used terms during clinical evaluations. Only those vaccines assessed by the NRA as having an adequate nonclinical evaluation should be considered for clinical evaluation.

Clinical evaluation is required for new diphtheria vaccines, and may also be required for existing vaccines if a significant change to the manufacturing process has been proposed. The content and extent of the clinical programme will vary according to each possible scenario. It is strongly recommended that early dialogue is established between the vaccine manufacturer and the NRA to clarify requirements for clinical studies as well as for marketing approval.

C.1.1 General considerations for clinical studies

All clinical trials on pharmaceutical products should adhere to the standards of good clinical practice set out by WHO (31). Vaccines have special aspects that demand careful consideration during clinical evaluation because they are given to healthy people, mostly in the paediatric population, and are given to prevent disease rather than to cure it, which limits the tolerance to adverse events.

It is expected that at least some clinical studies, including those in the primary target population, will be conducted with different lots of vaccine manufactured using the same process as the vaccine intended for marketing.

Consistency in the manufacture of the vaccine lots used in clinical trials should be demonstrated and well documented. Although a formal clinical trial to evaluate lot consistency may not always be needed, in some instances clinical data may be required to provide evidence to validate manufacturing consistency (e.g. if there is a particular concern about consistency). Vaccine lots used in clinical studies should ideally be the same lots that have been evaluated in nonclinical studies, and should be adequately representative of the formulation intended for marketing. Where this is not feasible, the lots used clinically should be comparable to those used in the nonclinical studies with respect to the manufacturing process, immunogenicity and potency, safety, stability and other relevant characteristics of quality. The number of different vaccine lots evaluated as part of the clinical studies should be approved by the NRA but should be more than one. It is important to note that clinical data used to provide evidence of production consistency do not replace the need to demonstrate consistency in the manufacturing process during nonclinical evaluations.

C.1.1.1 Scope of the studies

The size and design of the studies, and the selection of end-points for evaluation, require justification; they should provide reasonable assurance of the clinical benefit and safety of the candidate vaccine. Studies should include evaluations of the performance of the investigational vaccine when co-administered with other vaccines routinely recommended for the target population. Of particular interest in the evaluation of diphtheria vaccines are any effects on safety and immunogenicity found during co-administration with polysaccharide conjugate vaccines containing CRM197 or diphtheria toxoid as the carrier protein.

Where vaccines containing the same antigen(s) are already in common use, or the incidence of disease is very low – as is the case for diphtheria vaccines – it may not be feasible to perform a study based on protective efficacy. In the case of diphtheria, for which there are generally accepted serological correlates of protection, studies are based on the evaluation of antidiphtheria antibody responses in addition to a thorough assessment of vaccine safety. The primary immunogenicity end-point(s) should be those most relevant to the target population, and these will differ for evaluations of priming and booster doses of diphtheria toxoid.

C.1.1.2 Comparator vaccine

Immunological correlates of protection are well established for diphtheria vaccines (see section C.2.2), and it should be acknowledged that comparison studies, where used, do not bridge to efficacy but to serological correlates. In some cases, it may be decided to perform immunogenicity analyses that are not comparative, although in most cases comparative studies are recommended. The inclusion of a

comparator aids interpretation of the results of the trial, particularly if the expected seroprotection rate in the investigational group is not observed (e.g. if the study is conducted in a population where high levels of maternal antibody suppress the response to immunization in infants). Decisions about whether to include a comparator vaccine, and the selection of a comparator, should be justified by the manufacturer, and approved by the NRA. In studies performed to support major changes to the manufacturing process for a licensed vaccine, including a change in formulation or antigen dose, the candidate vaccine should be compared with the existing product (i.e. one manufactured according to the licensed production process). In this case, a comparative study is particularly useful for directly evaluating the effect of the change on the process or formulation. In studies of a new vaccine, the comparator is typically a licensed vaccine.

In some comparative studies, safety end-points may also be considered primary end-points (e.g. where the antigen content of a vaccine has been reduced with the intention of lowering the frequency of local reactions).

C.2 Assessment of immunogenicity in humans

C.2.1 Assays to assess antibody responses

Assays to measure the antibody response to diphtheria vaccine can be divided into functional assays (which demonstrate the capacity of diphtheria antibody in a serum sample to prevent the toxic effects of diphtheria toxin) and nonfunctional binding assays (which demonstrate the capacity of diphtheria antibody in a serum sample to bind to diphtheria toxin or toxoid).

The Vero cell assay is an in vitro toxin neutralization microneutralization) test that can be used to measure neutralizing antibodies in serum (32); it is considered to be the gold standard for measuring responses to diphtheria vaccines. This method can be used to confirm the relevance and performance of other in vitro serological assays as part of validation studies. However, the Vero cell assay is not commercially available, and it requires cell culture facilities and a relatively large volume of serum compared with other in vitro serological assays. Therefore, other in vitro serological assays may be preferred if they use a lower sample volume, are faster, and can be automated, all of which make it easier to screen large numbers of samples. These nonfunctional binding assays include ELISA, double-antigen ELISAs (DAEs), the dual doubleantigen time-resolved fluorescence immunoassay (dDA-DELFIA), the passive haemagglutination assay (PHA) and the toxin-binding inhibition (ToBI) test. Nonfunctional in vitro serological assays show variable degrees of correlation with the Vero cell assay, particularly when the levels of functional antibody are low (33, 34); the nonfunctional in vitro assays should be validated against the Vero cell TNT.

The method chosen to measure antibody response should be approved by the NRA. Where feasible, an assay that measures functional antibody responses should be used at some stage during the clinical evaluation of the vaccine (e.g. to analyse a subset of the clinical trial samples).

The International Standard for diphtheria antitoxin human, calibrated in IUs of diphtheria antitoxin, can be used in the TNT and in vitro immunoassays. Secondary reference materials intended for use with ELISA and other in vitro serological assays should be calibrated against the international standard using a TNT, but the results of all assays must be expressed in IU per ml.

C.2.2 Criteria for evaluation of immune responses

The end-points and criteria used to evaluate immune responses require justification, and must take into account the assay used to measure diphtheria antibody responses, the intended use of the vaccine (i.e. for primary or booster immunization) and established immunological correlates of protection.

Immunological correlates of protection are well established for diphtheria vaccines, and are recommended for use as primary or secondary end-points, depending on the scenario. When measured using a TNT, antidiphtheria antibody levels of less than 0.01 IU/ml are considered to indicate that a person is highly susceptible to the disease; an antibody level of 0.01 IU/ml is considered to be the minimum required for some degree of protection; levels of 0.1 IU/ml or higher are considered to confer full protection against the disease; and levels 1.0 IU/ml or higher are associated with long-term protection against diphtheria (1). However, it should be acknowledged that there is no sharply defined level of antitoxin above which all persons can be considered to be fully protected. When an ELISA is used, the minimum level of antibody considered to confer some degree of protection is usually 0.1 IU/ml, and at this level of response there is a good correlation with the Vero cell assay (34). The end-points and evaluation criteria proposed by the manufacturer should be approved by the NRA.

C.2.2.1 **Primary immunization of infants**

The proportion of subjects with a diphtheria antibody concentration above a prespecified threshold (indicating seroprotection) should be determined approximately one month following the last priming dose. When the Vero cell assay is used to measure antidiphtheria antibodies, the proportion of subjects with a post-vaccination level of 0.01 IU/ml or higher may be acceptable as the primary immunogenicity end-point. In countries where a booster dose of diphtheria toxoid is not routinely administered during the second year of life, a level of 0.1 IU/ml or higher may be recommended as the primary end-point (even when the Vero cell assay is used). When a nonfunctional assay such as an ELISA is used to measure antidiphtheria antibodies, the proportion of subjects

with a post-vaccination level of 0.1 IU/ml or higher is typically used as the primary end-point.

The noninferiority of the investigational vaccine relative to a comparator vaccine should be evaluated. Noninferiority is demonstrated if the upper limit of the 95% confidence interval for the difference in the seroprotection rates (i.e. the comparator vaccine minus the investigational vaccine) is less than the prespecified margin (usually 10%, although a margin of 5% may be recommended if the expected rates of seroprotection are very high). The specified threshold antibody concentration, noninferiority margin and bleeding time should be approved by the NRA.

In studies performed without a comparator vaccine, an acceptance criterion should be used for the proportion of subjects (usually 90% or 95%) that reaches the prespecified threshold antibody concentration as measured approximately 1 month following the last priming dose. The acceptance criterion should be based on the 95% confidence interval for the proportion of subjects achieving the prespecified antibody concentration (e.g. the lower limit of the 95% confidence interval for the observed proportion should be greater than 90% or 95%). The NRA should approve the specified antibody threshold, acceptance criterion and bleeding time.

In addition to the level of antibody specified as the primary end-point, it is recommended that secondary analyses of the proportion of subjects achieving other clinically relevant thresholds of diphtheria antibody (see section C.2.2) are also performed for the investigational vaccine and, where used, the comparator vaccine. The geometric mean titre (GMT) of the antidiphtheria antibody response should also be evaluated, and presented as a secondary end-point. In comparative studies, the GMT ratio of the investigational vaccine to the comparator vaccine may be evaluated using a predefined margin of noninferiority (e.g. the lower limit of the 95% confidence interval of the observed ratio of the investigational vaccine to the comparator vaccine should be greater than 0.67). The presentation of reverse cumulative distribution (RCD) curves, which show the accumulated proportion of subjects with an antibody concentration greater than or equal to a given level, may also provide useful information for comparison.

C.2.2.2 Primary immunization of adolescents or adults

In some countries it may be desirable to evaluate a diphtheria vaccine to be used for primary immunization in adolescents or adults. In these instances, it may be necessary to conduct pre-enrolment screening to identify previously unvaccinated (i.e. naive) subjects. Criteria to identify naive subjects for enrolment might include a diphtheria antibody level less than 0.01 IU/ml as measured by Vero cell assay prior to and seven days after receipt of the first dose of diphtheria vaccine. In some cases, it may be difficult to identify sufficient numbers of naive individuals, and the choice of study design (i.e. comparative or noncomparative)

may therefore be dependent on the number of naive subjects that can be identified in the target population. If sufficient numbers of naive subjects in older age groups cannot be identified, consideration may be given to extrapolating the effectiveness of primary immunization from infants to older age groups.

The end-points for the evaluation of the primary immune response in adolescents or adults are the same as those recommended for the primary immunization of infants (see section C.2.2.1). As with studies of primary immunization in infants (see section C.2.2.1), noninferiority criteria should be specified for comparative studies; acceptability criteria should be specified for noncomparative studies.

C.2.2.3 Booster immunization of pre-school-age children, school-age children, adolescents and adults

For the evaluation of diphtheria vaccines intended for booster immunization, the age of the participant and the interval since the last dose of diphtheria vaccine should be taken into account when designing and analysing the studies, since these factors may have a significant impact on the response to a booster dose. Criteria for the evaluation of booster doses of diphtheria vaccines should reflect the fact that prior to booster vaccination, a substantial proportion of the study population may have diphtheria antibody levels equal to or above those that may have been specified to evaluate responses to primary immunization. Assessing antibody levels both prior to and following immunization is recommended to optimize the interpretation of the data. In some cases, analysing the proportion of subjects who achieve a specified booster response (based on a comparison of pre-vaccination and post-vaccination antibody levels) may be more meaningful than using criteria based on the proportion of subjects reaching a prespecified antibody level. Decisions about whether to use an antibody threshold or booster response as the primary end-point should take into consideration the expected proportion of subjects who may have antibody levels that exceed the threshold prior to vaccination. If this proportion is high, the booster response may be a more meaningful primary end-point.

Where an antibody threshold is specified as a primary end-point for evaluating booster vaccination, it is recommended that the threshold value of 0.1 IU/ml should be used, even when analyses use the Vero cell assay. In comparative studies, noninferiority should be evaluated; noninferiority is demonstrated if the upper limit of the 95% confidence interval of the difference in seroprotection rates (i.e. for the comparator vaccine minus the investigational vaccine) is less than the prespecified margin (usually 10%, although a margin of 5% may be recommended if the expected rates of seroprotection are very high). In noncomparative studies, an acceptability criterion for the proportion of subjects who achieve the specified threshold post-vaccination (e.g. 90% or 95%) should be used, based on the 95% confidence interval for the observed proportion.

If the booster response is used as the primary end-point, the definition of booster response, based on an increase in diphtheria antibody concentration from pre-vaccination to post-vaccination, should be prespecified. For subjects with low pre-vaccination levels of antibody, the definition of booster response should include a requirement that the post-vaccination level exceeds an appropriate threshold by a specified amount. For instance, in subjects with a pre-vaccination antibody level less than 0.1 IU/ml, a booster response might be defined as a post-vaccination concentration of 0.4 IU/ml or greater (i.e. at least 4 times higher than the clinically relevant threshold of 0.1 IU/ml). In subjects with a pre-vaccination concentration of 0.1 IU/ml or greater, a booster response might be defined as a post-vaccination concentration that is at least 4 times higher than the pre-vaccination concentration. Using a lower-fold rise in antibody concentration to define the booster response in persons with specified high levels of pre-existing antibody may be appropriate, but this lower level should be prespecified and justified.

In comparative studies, booster responses should be compared between groups, and should be evaluated using a predefined noninferiority limit (e.g. the upper limit of the 2-sided 95% confidence interval of the observed difference – that is, the comparator vaccine minus the investigational vaccine – should be less than a prespecified margin, which is usually 10%). In noncomparative studies, an acceptability criterion should be used for the proportion of subjects who achieve a booster response (e.g. 80%), and this should be based on the 95% confidence interval of the observed proportion (see section C.2.2.1).

As an indicator of long-term protection, the proportion of subjects with a post-vaccination antibody level of 1.0 IU/ml or greater may be evaluated as a secondary end-point. The post-vaccination GMT of antidiphtheria antibody may be evaluated as a secondary end-point. In comparative studies, the GMT of the ratio of the investigational vaccine to the comparator vaccine may be evaluated using a predefined margin of noninferiority (e.g. the lower limit of the 95% confidence interval of the observed ratio of the investigational vaccine to the comparator vaccine should be greater than a prespecified limit, which is usually 0.67).

The choice of end-points and criteria for evaluation (including the need for a comparator vaccine) should be justified by the manufacturer, and approved by the NRA.

C.2.3 Antibody persistence

Where possible, subsets of subjects should be identified for longer-term followup of the persistence of immunity in order to determine the need for booster doses. Alternatively, population surveillance studies should be carried out to determine the prevalence of diphtheria antibody in different age groups, and to guide recommendations on the need for booster doses.

C.3 Safety evaluation

The clinical assessment of diphtheria vaccines should include a thorough assessment of the vaccine's safety using comparative prelicensure studies. In some cases, the evaluation of safety may be the primary (or coprimary) objective of a clinical study (e.g. when a change to the vaccine's formulation has been made to lower the antigen dose with the intention of reducing the frequency of local reactions). The assessment of safety should follow the general principles outlined in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (30). Safety data should be collected throughout the duration of clinical development. For a new vaccine, it is generally recommended that the overall safety database should consist of a minimum of 3000 subjects who have received the investigational vaccine. This number allows for the detection of adverse events that occur at a rate of 1 in 1000 subjects. However, the minimum acceptable size of the safety database needed to support licensure will vary according to several factors, including the formulation of the vaccine and prior experience with vaccines that have the same or similar composition. The size of the database should be justified by the manufacturer, and approved by the NRA. For diphtheria vaccines, in cases in which efficacy has been inferred from serological correlates of protection, the number of subjects that should be evaluated for safety is likely to exceed the number required for the evaluation of immunogenicity.

The frequency of adverse reactions following diphtheria immunization may vary according to the vaccine's formulation (e.g. according to the dose of the diphtheria antigen) and subjects' characteristics (e.g. prior vaccination history, time since previous dose and pre-vaccination level of diphtheria antibody). Careful attention should be paid to documenting and evaluating safety associated with the administration of booster doses, since higher rates of local adverse reactions have been observed following booster immunization with diphtheria toxoid compared with primary immunization. Consideration should also be given to the potential for the increased reactogenicity that may occur when diphtheria vaccines are administered at the same time as, or shortly after, polysaccharide conjugate vaccines containing CRM197 or diphtheria toxoid as the carrier protein.

Commonly occurring adverse reactions expected after diphtheria immunization include pain, redness and swelling at the injection site. Post-vaccination fever may also occur. Serious adverse reactions associated with diphtheria vaccine occur too infrequently to be reliably evaluated in most clinical trials. Although serious adverse events should be monitored during prelicensure clinical trials, post-marketing surveillance must also be performed to monitor such events.

C.4 Post-marketing studies and surveillance

Monitoring the effectiveness, safety and quality of licensed vaccines consists of post-marketing surveillance and post-marketing studies (phase IV studies). The purpose of post-licensure monitoring is to assess the performance of a vaccine in the target population under conditions of routine use, and to monitor rare adverse events. Post-marketing studies may also be useful for assessing antibody persistence and the need for booster doses. Marketing authorization holders should be committed to presenting a post-marketing surveillance programme at the time of licensure. The programme should be based on criteria for assessing the quality, safety and effectiveness of a particular vaccine to gain marketing approval.

In many cases, comprehensive post-marketing safety and effectiveness data cannot be collected by manufacturers alone, and close cooperation between manufacturers and public-health authorities is required. All data collected should be submitted to the NRA at regular intervals so that action can be taken if there are implications for the marketing authorization.

Post-marketing surveillance may be the only means of detecting rare adverse events that occur too infrequently to have been detected during clinical trials. For the collection of safety data, surveillance may be conducted by active or passive processes. Voluntary reporting of serious adverse events (passive surveillance) is most commonly used.

Part D. Recommendations for NRAs

D.1 **General**

The general recommendations for NRAs and national control laboratories (NCLs) given in Guidelines for national authorities on quality assurance for biological products (35) and Guidelines for independent lot release of vaccines by regulatory authorities (12) apply.

The details of production and quality control procedures, as well as any significant changes in them that may affect the quality, safety or efficacy of diphtheria vaccines, should be discussed with and approved by the NRA. For control purposes, the international standards currently in use (see the section on General considerations) should be obtained for the purpose of calibrating national, regional and working standards (25). The NRA may obtain the product-specific or working references from the manufacturer to be used for lot release until an international or national standard preparation has been established.

Consistency in production has been recognized as an essential component in the quality assurance of diphtheria vaccines. In particular, NRAs should carefully monitor production records and the results of quality control tests on clinical lots as well as results from tests on a series of consecutive lots of the vaccine.

D.2 Release and certification by the NRA

A vaccine should be released only if it fulfils all national requirements or satisfies Part A of these Recommendations, or both (12).

A protocol based on the model given in Appendix 1, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for the release of a vaccine for use.

A statement signed by the appropriate official of the NRA should be provided to the manufacturing establishment, and should certify that the lot of vaccine in question meets all national requirements as well as Part A of the present Recommendations. The certificate should provide sufficient information about the vaccine lot. A model certificate is given in Appendix 2. The official national release certificate should be provided to importers of the vaccine. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

Authors and acknowledgements

The first and second drafts of these Recommendations were prepared by Dr P. Stickings, National Institute for Biological Standards and Control, England, with contributions from other members of the drafting group: Dr M. Corbel, Consultant, England; Dr R. Dobbelaer, Consultant, Belgium; Dr. K. Farizo, United States Food and Drug Administration, United States; Dr M. Iwaki, National Institute of Infectious Diseases, Japan; Mrs T. Jivapaisarnpong, Institute of Biological Products, Ministry of Public Health, Thailand; Dr H. Lechner, Paul-Ehrlich-Institut, Germany; Dr D. Lei, World Health Organization, Switzerland; Dr F. Mawas, National Institute for Biological Standards and Control, England; Dr B. Meade, Meade Biologics, United States; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Dr S. Prieur, Agence Française de Sécurité Sanitaire des Produits de Santé, France; Dr M.P. Schmitt, Center for Biologics Evaluation and Research, United States Food and Drug Administration, United States; Dr T. Sesardic, National Institute for Biological Standards and Control, England; Dr M. Takahashi, National Institute of Infectious Diseases, Japan; Dr D. Xing, National Institute for Biological Standards and Control, England; Dr S. Zhang, State Food and Drug Administration, China.

The third and fourth drafts were prepared by Dr P. Stickings and Dr D. Lei following the consultation held in Beijing, China, 7–11 November 2011, with the following participants: Dr M. Baca-Estrada, Health Canada, Canada; Ms H. Bai, State Food and Drug Administration, China; Mr M. Contorni, Novartis Vaccines and Diagnostics, Italy; Dr P. Desmons, GlaxoSmithKline Biologicals, China; Dr R. Dobbelaer, Consultant, Belgium; Dr R. Dominguez Morales, World Health Organization, Switzerland; Mrs L. Du, Beijing Luzhu Biopharmaceutical Company, China; Dr S. Gairola, Serum Institute of India, India; Mr S. Goel, Central

Drug Laboratory Kasauli, India; Dr C. Hernandez, World Health Organization, Switzerland; Mrs Q. Hou, National Institutes for Food and Drug Control, China; Dr K. Farizo, Center for Biologics Evaluation and Research, United States Food and Drug Administration, United States; Dr K. Friedrich, Instituto Nacional de Controle de Qualidade em Saude, Brazil; Mrs H. Han, Crucell Korea, Republic of Korea; Dr L. Hiep, Institute of Vaccines and Medical Biologicals, Viet Nam; Dr X. Hong, Chinese Pharmacopoeia Commission, China; Mr A. Horita, The Chemo-Sero-Therapeutic Research Institute, Japan; Dr M. Iwaki, National Institute of Infectious Diseases, Japan; Mr Q. Jiang, Changchun Chang Sheng Life Sciences, China; Dr B. Kim, Korea Food and Drug Administration, Republic of Korea; Dr J. Kim, Korea Food and Drug Administration, Republic of Korea; Dr I. Knezevic, World Health Organization, Switzerland; Dr D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Mrs F. Lan, Chengdu Institute of Biological Products, China; Dr H. Langar, Regional Office for the Eastern Mediterranean, World Health Organization, Egypt; Dr H. Lechner, Paul-Ehrlich-Institut, Germany; Dr D. Lei, World Health Organization, Switzerland; Dr F. Li, National Institutes for Food and Drug Control, China; Mr J. Liu, Beijing Minhai Biotechnology Company, China; Dr J. Luo, Center for Drug Evaluation, China; Dr K. Markey, National Institute for Biological Standards and Control, England; Dr F. Mawas, National Institute for Biological Standards and Control, England; Dr B. Meade, Meade Biologics, United States; Mr P.V.V.S Murthy, Biological E. Limited, India; Dr S. Pakzad, Food and Drug Control Laboratory, Islamic Republic of Iran; Dr D. Pfeifer, Regional Office for Europe, World Health Organization, Denmark; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Mr M. Qin, Chengdu Institute of Biological Products, China; Mr M. Rahman, Panacea Biotec, India; Dr D. Sesardic, National Institute for Biological Standards and Control, England; Mrs J. Shi, Yuxi WALVAX Biotechnological Company, China; Dr. J. Shu, Sanofi Pasteur, China; Dr P. Stickings, National Institute for Biological Standards and Control, England; Mrs. C. Tan, Chengdu Institute of Biological Products, China; Dr J. Tresnabudi, BioFarma, Indonesia; Dr J. Wang, National Institutes of Food and Drug Control, China; Mrs X. Wang, Chinese Pharmacopoeia Commission, China; Mr H. Wei, Changchun Institute of Biological Products, China; Mrs Z. Xiao, Beijing Tiantan Biological Products Company, China; Dr D. Xing, National Institute for Biological Standards and Control, England; Dr M. Xu, National Institutes for Food and Drug Control, China; Mr B. Yang, Wuhan Institute of Biological Products, China; Mr M. Yang, Lanzhou Institute of Biological Products, China; Mr. H. Yin, Center for Drug Evaluation, China; and Dr S. Zhang, State Food and Drug Administration, China. Special acknowledgements are due to Dr K. Farizo, Center for Biologics Evaluation and Research, United States Food and Drug Administration, United States, for her critical review and comments on the revised Recommendations.

The fifth draft was prepared by Dr P. Stickings, National Institute for Biological Standards and Control, England, and Dr D. Lei, World Health Organization, Switzerland, on the basis of comments received from national regulators, the vaccine industry and the general public during a period of public consultation on the WHO web site.

References

- The immunological basis for immunization series. Module 2: Diphtheria update 2009. Geneva, World Health Organization, 2007 (http://whqlibdoc.who.int/publications/2009/9789241597869_eng.pdf, accessed 27 June 2013).
- 2. Diphtheria vaccine: WHO position paper. Weekly Epidemiological Record, 2006, 81:21–32.
- 3. Galazka A. Implications of the diphtheria epidemic in the former Soviet Union for immunization programs. *Journal of Infectious Diseases*, 2000, 181(Suppl. 1):S244–S248.
- Dittmann S et al. Successful control of epidemic diphtheria in the states of the former Union of Soviet Socialist Republics: lessons learned. *Journal of Infectious Diseases*, 2000, 181(Suppl. 1):S10– S22.
- 5. Requirements for diphtheria toxoid and tetanus toxoid (Requirements for biological substances No. 10). In: *WHO Expert Committee on Biological Standardization. Seventeenth report*. Geneva, World Health Organization, 1964 (WHO Technical Report Series, No. 293), Annex 1.
- Requirements for diphtheria toxoid, pertussis vaccines, tetanus toxoid, and combined vaccines.
 In: WHO Expert Committee on Biological Standardization. Thirtieth report. Geneva, World Health Organization, 1979 (WHO Technical Report Series, No. 638), Annex 1.
- 7. Requirements for diphtheria toxoid, pertussis vaccine, tetanus toxoid, and combined vaccines (Addendum 1986). In: *WHO Expert Committee on Biological Standardization. Thirty-seventh report.* Geneva, World Health Organization, 1987 (WHO Technical Report Series, No. 760), Annex 4.
- 8. Requirements for diphtheria, tetanus, pertussis and combined vaccines (revised 1989). In: WHO Expert Committee on Biological Standardization. Fortieth report. Geneva, World Health Organization, 1990 (WHO Technical Report Series, No. 800), Annex 2.
- 9. Recommendations for diphtheria, tetanus, pertussis and combined vaccines (Amendments 2003). In: WHO Expert Committee on Biological Standardization. Fifty-fourth report. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 5.
- 10. Assay of diphtheria vaccine adsorbed. In: *European Pharmacopoeia*, 7th ed. Strasbourg, European Directorate for Quality of Medicines & HealthCare, 2008.
- 11. *Minimum requirements: Diphtheria toxoid.* Fourth revision. Bethesda, MD, National Institutes of Health, 1947.
- 12. Guidelines for independent lot release of vaccines by regulatory authorities. In: WHO Expert Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 2.
- 13. Preneta-Blanc R. Calibration of replacement International Standards of diphtheria and tetanus toxoids for use in flocculation test. *Biologicals*, 2008, 36:315–326.
- Stickings P. Collaborative study for the calibration of a replacement International Standard for diphtheria toxoid adsorbed. *Biologicals*, 2010, 38:529–538.

- Good manufacturing practices: main principles for pharmaceutical products. In: WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 3.
- Good manufacturing practices for biological products. In: WHO Expert Committee on Biological Standardization. Forty-second report. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 1.
- Manual for the Production and Control of Vaccines: Diphtheria Toxoid. Geneva, World Health Organization, 1977 (BLG/UNDP/77.1) (http://whqlibdoc.who.int/hq/pre-wholis/BLG_ UNDP 77.1.pdf, accessed 27 June 2013).
- 18. Murphy JR, Michel JL, Teng M. Evidence that the regulation of diphtheria toxin production is directed at the level of transcription. *Journal of Bacteriology*, 1978, 135:511–516.
- 19. Griffiths E, Chart H. Iron as a regulatory signal. In: Bullen DJ, Griffiths E, eds. *Iron and infection*, 2nd ed. Chichester, John Wiley, 1999:213–253.
- WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products. Geneva, World Health Organization, 2003 (WHO/BCT/QSD/03.01) (http://www.who.int/biologicals/publications/en/whotse2003.pdf, accessed 22 June 2013).
- 21. Manual for quality control of diphtheria, tetanus and pertussis vaccines. Geneva, World Health Organization, 2013 (document WHO/IVB/11.11).
- General requirements for the sterility of biological substances. (Requirements for Biological Substances No. 6, revised 1973, amendment 1995). In: WHO Expert Committee on Biological Standardization. Forty-sixth report. Geneva, World Health Organization, 1998 (WHO Technical Report Series, No. 872), Annex 3.
- Guidelines on stability evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-seventh report. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 962), Annex 3.
- Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-third report. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 926), Annex 4.
- 25. WHO manual for the establishment of national and other secondary standards for vaccines. Geneva, World Health Organization, 2011 (WHO/IVB/11.03) (http://whqlibdoc.who.int/hq/2011/WHO_IVB_11.03_eng.pdf, accessed 12 July 2013).
- 26. A WHO guide to good manufacturing practice (GMP) requirements. Part 2: Validation. Geneva, World Health Organization, 1997 (WHO/VSQ/97.02).
- 27. WHO guidelines on nonclinical evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-fourth report. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.
- 28. Handbook: good laboratory practice (GLP). Quality principles for regulated non-clinical research and development, 2nd ed. Geneva, World Health Organization, 2009.
- Good manufacturing practice: supplementary guidelines for the manufacture of investigational pharmaceutical products for clinical trials in humans. In: WHO Expert Committee on Specifications for Pharmaceutical Preparations. Thirty-fourth report. Geneva, World Health Organization, 1996 (WHO Technical Report Series, No. 863), Annex 7.
- Guidelines on clinical evaluation of vaccines: regulatory expectations. In: WHO Expert Committee on Biological Standardization. Fifty-second report. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 924), Annex 1.

- 31. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products. In: *The use of essential drugs. Sixth report of the WHO Expert Committee.* Geneva, World Health Organization, 1995 (WHO Technical Report Series, No. 850), Annex 3.
- 32. Miyamura K et al. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. II. Comparison with the rabbit skin method and practical application for seroepidemiological studies. *Journal of Biological Standardization*, 1974, 2:203–209.
- 33. Walory J, Grzesiowski P, Hryniewicz W. Comparison of four serological methods for the detection of diphtheria anti-toxin antibody. *Journal of Immunological Methods*, 2000, 245:55–65.
- 34. Melville-Smith M, Balfour A. Estimation of Corynebacterium diphtheriae antitoxin in human sera: a comparison of an enzyme-linked immunosorbent assay with the toxin neutralization test. *Journal of Medical Microbiology*, 1988, 25:279–283.
- 35. Guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 2.

Appendix 1

Model protocol for the manufacturing and control of diphtheria vaccines (adsorbed)

The following protocol is intended for guidance. It indicates the information that should be provided as a minimum by a manufacturer to the NRA. Information and tests may be added or omitted as necessary, with the authorization of the NRA.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO Recommendations for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that will accompany the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot-release certificate from the NRA or from the NCL in the country where the vaccine was produced or released stating that the product meets national requirements as well as the recommendations in Part A of this annex.

1. Summary information on finished product (final lot)

International name:
Trade name/commercial name:
Product licence (marketing authorization) number:
Country:
Name and address of manufacturer:
Name and address of licence holder, if different:
Final packaging lot number:
Type of container:
Number of containers in this packaging:
Final container lot number:
Number of filled containers in this final lot:
Date of manufacture:
Description of final product (adsorbed):
Preservative, and nominal concentration:
Volume of each single human dose:
Number of doses per final container:

Summary of the composition (include a summary of the qualitative and quantitative composition of the vaccine in each human dose, including any adjuvant used and other excipients):
Shelf-life approved (months): Expiry date: Storage conditions:
The following sections are intended for recording the results of the tests performed during the production of the vaccine so that the complete document will provide evidence of consistency in production. If any test has to be repeated, this must be indicated. Any abnormal result must be recorded on a separate sheet.
2. Detailed information on manufacture and quality control
Starting materials
Identity of <i>C. diphtheriae</i> strain used for vaccine production:
Reference number of seed lot:
Date(s) of reconstitution (or opening) of seed lot ampoule(s):
Single harvests used for preparing the bulk purified toxoid
Name of the culture medium:
Date of inoculation:
Temperature of incubation:
Control of bacterial purity
Methods:
Result:
Date:
Date of harvest:
Volume of harvest:
Yield (Lf/ml):
Volume after filtration:
Toxin purification method:
Toxin content yield (Lf/ml):
Method of detoxification:
Date started:
Date finished:

Volume used for inactivation:
Temperature:
Concentration of detoxification agent:
ŭ
Confirmation of detoxification (before or after purification)
Method:
Dose of inoculation (Lf):
Route of inoculation (in vivo only):
Date of inoculation:
Date of observation:
Result:
Purification methods used for toxoid:
Yield of purified toxoid
Volume:
Toxoid content (Lf/ml):
Bulk purified toxoid
Reference number:
Volume and Lf/ml:
volume and El/IIII.
Sterility test
Tests for bacteria and fungi
Method:
Media:
Number of bulks tested:
Volume of inoculum per bulk:
Volume of medium per bulk:
Temperature of incubation:
Date of start of test:
Date of end of test:
Result:
Antigenic purity (Lf/mg of protein (nondialysable) nitrogen):
Method:
Date of test:
Specific toxicity test
Method:
Dose of inoculation (Lf):
Route of inoculation (in vivo only):

Test of reversion to toxicity If /ml of test toxoid solution	on:	
	on: n of toxoid:	
	d of incubation:	
	d of medoditon.	
	ivo only):	
Result:		
Final bulk Identification (lot number): Date of manufacture or blendin Volume: Lf/ml:	g:	
Blending:	Prescription (SHD)	Added
Toxoid (Lf):	r rescription (SITD)	Added
Adjuvant:		
Preservative (specify):		
Others (salt):		
Final volume (ml):		
1 111111 (0 1 1 1 1 1 1 1 1 1 1 1 1 1 1		
Preservative content		
Method:		
Date of test:		
Adjuvant content		
Method:		
Date of test:		
Result:		
Degree of adsorption		
Method:		
Date of test:		
Result:		

Sterility test
Tests for bacteria and fungi
Method:
Media:
Number of containers tested:
Volume of inoculum per container:
Volume of medium per container:
Temperature of incubation:
Date of start of test:
Date of end of test:
Result:
Specific toxicity test (when required)
Method:
Dose of inoculation (Lf):
Route of inoculation (in vivo only):
Date of inoculation:
Date of observation:
Result:
D-444
Potency test
Challenge method (multiple-dilution or single-dilution assay)
If single dilution, date of last satisfactory
multiple-dilution assay:
Species, strain and weight range
of animals:
Number of animals per dilution:
Reference vaccine used (IU):
Date of immunization:
Route of injection and volume of
dilutions administered:
Date of challenge:
Challenge method used (lethal or
intradermal challenge):
Challenge toxin used:
Challenge dose(s) used:
Date of end of observation:

Results (see Table 4.1 for an example of how to report the results from a lethal method, and see Table 4.2 for an example of reporting an intradermal method)

Table 4.1
Reporting results from a lethal challenge assay

Vaccine	Dilution	Lethal method No. survivors/No. tested	Median effective dose (ED ₅₀)	
Reference vaccine	1	/		
IU/ml	2	/	ml	
	3	/		
Test vaccine lot number	1	/		
	2	/	ml	
	3	/		
Potency of test vaccine in IU/SHD with 95% confidence limits:				
Single-dilution assays only: <i>P</i> value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD:				

Table 4.2
Reporting results from an intradermal challenge assay

Vaccine	Dilution	Mean score	
Reference vaccine	1		
IU/ml	2		
	3		
Test vaccine	1		
lot number	2		
	3		
Potency of test vaccine in IU/SHD with 95% confidence limits:			
Single-dilution assays only: <i>P</i> value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD:			

When a single-dilution assay is performed, only the responses or scores at the single dilution used are shown. For the intradermal challenge assay, the ED_{50} is not applicable.

200	
V	ī
1	
	٥
0	j
, ,	V
_	١,
\subset	٥
~	5
~	,
0	١
_	Š
_	•
2	,
	٦
$^{\circ}$	٦
0	j
. =	Ξ,
	٠,
Q	J
	١
	ð
-	
()
_	١
-	4
Q	J
0	7
_	
-	Ç
	٥
	J
. 5	5
	í
- C	
-	٦
>	,
υ,	J
	٠,
_	
(')
$\stackrel{\sim}{}$	′
_	ĺ
\geq	ĺ
\leq	

Serological method
Species, strain and weight range of animals:
Number of animals per dilution:
Reference vaccine used (IU):
Date of immunization:
Route of injection and volume of dilutions administered:
Date of bleeding:
Method for titration of immune serum samples:
Results (See Table 4.3 for an example of reporting results from a serological method)

Table 4.3
Reporting results from a serology assay

Vaccine	Dilution	Mean scores or response	
Reference vaccine	1		
IU/ml	2		
	3		
Test vaccine	1		
lot number	2		
	3		
Positive control			
Negative control			
Potency of test vaccine in IU/SHD with 95% confidence limits: 95% confidence limits expressed as % of potency estimate:			
Single-dilution assays only: <i>P</i> value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD:			

Test for	· amount	of r	esidua	l free	detoxifying agent	

Detoxifying agent:	
Method:	
Result (g/1):	
Date of test:	

pН
Result:
Date of test:
Final product
Identification:
Volume:
Identity test
Method:
Result:
Date of test:
Sterility test
Tests for bacteria and fungi
Method:
Media:
Number of containers tested:
Volume of inoculum per container:
Volume of medium per container:
Temperature of incubation:
Date of start of test:
Date of end of test:
Result:
Potency test
If this test was not performed on the final bulk, indicate this and report the data
obtained for the final product in the space provided for potency tests in the "final
bulk" section.
Innocuity test (when required)
Tests in mice
Date of start of test:
Date of start of test:
Number of animals tested:
Route of injection:
Volume of injection:
Observation period:
Results (give details of deaths):

4	4	
ı	N	
2	7	
ς		
(_	J
C	-	۹
7	₹	
2	△	j
ζ	2X2	١
	C	١
-	_	1
<	Nodiads	1
	×	į
ď	Й	J
	Z	
	0	ì
r	×	
,	-	Į
	t	ú
	7	
	C	
	õ	ζ
	ž	d
	đ	J
C	Kono	
	2	
	۲,	ť
	_	J
	2	
	DINNIN	
-	7	Į
	C	j
	Õ	ì
L	_	
(١
1	_	d
S	>	
4	₹	

Tests in guinea-pigs
Date of start of test:
Date of end of test:
Number of animals tested:
Route of injection:
Volume of injection:
Observation period:
Results (give details of deaths):
Test for adjuvant content
Nature and concentration of adjuvant/SHD:
Method of testing:
Result:
Date of test:
Test for degree of adsorption (when required)
Method:
Desorption method and reagent:
Result:
Date of test:
Test for preservative
Nature and concentration of preservative:
Method of testing:
Result:
Date of test:
Date of test:
pН
Method of testing:
Result:
Date of test:
Extractable content
Result:
Date of test:
Inspection of final containers
1
Organoleptic characteristics:
Number of containers inspected:
Va or containers rejected:

3. Certification by the manufacturer

Name of the manufacturer	
Name of head of production (typed	i)
	the control laboratory of the manufacturing e production and control of the vaccine
appears on the label of the final co	of diphtheria vaccine, whose number ntainer, meets all national requirements and/ecommendations to assure the quality, safety (adsorbed) (2014). ²
Signature	
Name (typed)	
Date	

4. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 3), a label from a final container and an instruction leaflet for users.

¹ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

² WHO Technical Report Series, No. 980, Annex 4.

Appendix 2

Model certificate for the release of diphtheria vaccines (adsorbed) by NRAs

Lot release certificate	
Certificate no	
The following lot(s) of diphtheria vaccine (adsorbed) produ	
abels of the final containers, complies with the relevant national speci and provisions for the release of biological products ³ and Part A ⁴ of the	fications
Recommendations to assure the quality, safety and efficacy of diphtheria	
(adsorbed) (2014)5, and complies with WHO good manufacturing p	
main principles for pharmaceutical products;6 Good manufacturing	practices
for biological products;7 and Guidelines for independent lot release of	vaccines
by regulatory authorities.8	
The release decision is based on	9

The certificate may include the following information:

- name and address of manufacturer
- site(s) of manufacturing
- trade name and common name of product
- marketing authorization number
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)

¹ Name of manufacturer.

² Country of origin.

³ If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.

⁴ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

⁵ WHO Technical Report Series, No. 980, Annex 4.

⁶ WHO Technical Report Series, No. 961, Annex 3.

⁷ WHO Technical Report Series, No. 822, Annex 1.

⁸ WHO Technical Report Series, No. 978, Annex 2.

⁹ Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.

- type of container used
- number of doses per container
- number of containers or lot size
- date of start of period of validity (e.g. manufacturing date) and/or expiry date
- storage conditions
- signature and function of the person authorized to issue the certificate
- date of issue of certificate
- certificate number.

The Director of the NRA (or other appropriate authority)

Name (typed) .	
Signature	
Date	

Annex 5

Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed)

Replacement of Annex 2 of WHO Technical Report Series, No. 800, and Annex 5 of WHO Technical Report Series, No. 927

introa	uction	2/3
Scope	of the Recommendations	279
Gener	General considerations	
Part A	. Manufacturing recommendations	284
A. A. A. A. A.	General manufacturing recommendations Production, processing and control Filling and containers Control of final product Records Retained samples Labelling	28 ² 28 ⁴ 28 ⁵ 29 ⁷ 29 ⁹ 29 ⁹ 29 ⁹
	9 Distribution and transport 10 Stability, storage and expiry date	300 300
Part B. Nonclinical evaluation of tetanus vaccines		302
В. В. В.	Nonclinical testing and characterization of intermediates and in-process materials	302 303 305
Part C.	Clinical evaluation of tetanus vaccines	307
C.	 1 Introduction 2 Assessment of immunogenicity in humans 3 Safety evaluation 4 Post-marketing studies and surveillance 	307 309 313 314
Part D	Part D. Recommendations for NRAs	
D. D.		315 315
Autho	rs and acknowledgements	315
References		317

Appendix 1

Model protocol for the manufacturing and control of tetanus vaccines (adsorbed)	322
Appendix 2	
Model certificate for the release of tetanus vaccines (adsorbed) by NRAs	332

Recommendations published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes recommendations for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments or examples intended to provide additional guidance to manufacturers and NRAs.

Introduction

Tetanus vaccines are among the most frequently used vaccines worldwide and have been remarkably successful products. Their widespread use in routine immunization programmes has resulted in a significant decrease in the incidence of tetanus in both developed and developing countries. Ensuring good coverage of childhood vaccinations and appropriate booster immunization of adults are essential to maintaining protection against tetanus (1).

Single-antigen tetanus toxoid vaccine (i.e. containing only tetanus toxoid) is almost never used in infants and children. Instead, tetanus toxoid is delivered as part of a primary immunization series in which it is generally presented in combination with diphtheria toxoid alone, or with diphtheria and whole-cell pertussis (DTP), or with diphtheria and acellular pertussis antigens (DTaP). Tetanus toxoid is also present in other combined vaccines that may contain, in addition to diphtheria and pertussis antigens, inactivated poliovirus, hepatitis B surface antigen or Haemophilus influenzae type b capsular polysaccharide conjugates, or some combination of these. Monovalent tetanus toxoid may be used more frequently for immunizations in adults and adolescents to reinforce immunity, which is essential because immunity to tetanus is induced only by vaccination. However, even for booster immunizations in adults, tetanus vaccines are often combined with other vaccines, and are generally formulated with a lower amount of antigens, particularly of diphtheria and acellular pertussis, when compared with vaccines intended for primary immunization (1). The potent immunogenicity of tetanus toxoid has also led to its use as a carrier protein in polysaccharide-protein conjugate vaccines such as Haemophilus influenzae type b conjugate vaccines (2).

The need for and use of tetanus vaccine will continue since immunity to tetanus is induced only by immunization because the pathogenic dose is lower than the immunogenic dose, and recovery from clinical tetanus does not result in protection against further episodes. The widespread use of tetanus vaccine, combined with improved standards of hygiene, has significantly reduced the incidence of tetanus disease in developed countries. However, neonatal tetanus remains a problem in developing countries, with infections occurring primarily through the unhealed umbilicus, and the mortality rate is high even with hospital care (3). The impact of neonatal tetanus is substantial and accounts for the most deaths from vaccine-preventable diseases, with an estimated 59 000 babies dying from tetanus in 2008 (4, 5).

Widespread coverage has not been achieved in low-income countries owing to the prohibitive cost of vaccination, which results from the requirements for multiple doses, parenteral administration by trained medical staff, and the need for a cold chain. Efforts are thus needed to improve the vaccine in terms of lowering the number of doses – e.g. by developing slow-release products (6, 7),

delivering the vaccine without needles (8) and improving the formulation and stability to eliminate the need for cold-chain storage. Subunit vaccines produced by recombinant technology that include a nontoxic receptor binding domain, the Hc fragment of tetanus toxin, are in various stages of development, and have shown promising results during preclinical evaluations (9-11). However, there are no data available from clinical trials in humans.

History of WHO Requirements and Recommendations, and standardization

The first WHO Requirements for tetanus toxoid vaccines were published in 1964 (12). These were revised in 1978 (13), with addenda in 1986 (14) and 1989 (15), and with further amendments made to the potency section during a consultation in 2003 (16).

The development of tetanus toxoid vaccines, and the publication of requirements for their manufacture and quality control, was helped considerably by the availability of international standards and international reference preparations.

The first milestone in the global standardization of tetanus toxoid was the establishment of the International Standard for tetanus antitoxin, equine in 1928 (17), which was replaced in 1969 (18). The availability and use of this preparation enabled toxoids to be assessed in terms of their ability to produce tetanus antitoxin in humans, and allowed protective units for antitoxin to be defined in International Units (IUs).

The first standard for tetanus toxoid established by WHO was the First International Standard for tetanus toxoid, plain (established in 1951). The IU was defined as the immunizing activity of 0.03 mg of the international standard, and was approximately equivalent to the existing German protective unit, the Schutzeinheit (19). This unit was defined on the basis of results obtained in guineapig challenge assays. At the Expert Committee meeting in 1958, it was noted that in mice, adsorbed preparations of tetanus toxoid could not be assayed with validity against the nonadsorbed international standard, and a recommendation was made that a separate International Standard for tetanus toxoid, adsorbed was needed (20). Thus the First International Standard for tetanus toxoid, adsorbed was prepared for determining the potencies of vaccines containing tetanus toxoid (adsorbed), and was established in 1966 (21). This standard was assigned an activity of 120 IU per ampoule (where 1 mg was determined to be equivalent to 1.5 IU of a previously established international standard (22), based on the results obtained in guinea-pig challenge assays). The International Standard for tetanus toxoid, adsorbed has been replaced at 10-20-year intervals with IUs assigned by WHO that have been based solely on calibration in guinea-pig challenge assays relative to the existing standard (23–27).

The Requirements published in 1964 specified assays against an international standard vaccine but required no minimum potency. A study published in 1970 provided evidence of a positive correlation between the amount of adsorbed tetanus toxoid in IUs (determined in guinea-pigs) and the antitoxin response in infants immunized with different DTP vaccines (28).

It has been recognized that there are difficulties in providing evidence of a direct correlation between the estimated potency of a vaccine (in a biological assay) and the level and duration of protective immunity in humans. Despite this lack of direct evidence, the minimum requirement for tetanus potency – 40 IU per single human dose (SHD) – which was introduced into WHO guidance in 1978 (13), has helped to ensure the production and release of safe and effective tetanus vaccines, based on the satisfactory performance of the vaccines in clinical studies and on the low incidence of tetanus in populations with good immunization coverage. The recommendation of 40 IU per SHD as a minimum requirement for tetanus potency for primary immunization is therefore retained in this latest revision of the Recommendations. However, the use of product-specific minimum requirements for potency may be justified, provided they are based on the results of clinical and laboratory studies, and approved by the national regulatory authority (NRA).

At the time of the 1990 revision, it was internationally agreed that the potency of tetanus vaccines could be measured by an active challenge test, and that either guinea-pigs or mice could be used as well as either a lethal challenge or a paralytic challenge dose. It was further stated that in vitro methods could be used for the determination of antibody levels instead of an in vivo challenge method, provided that appropriate validation studies had been done using the vaccine being tested. The 1990 Requirements emphasized explicitly that it was important for countries to adopt the principle of expressing the potency of tetanus vaccines in IUs, but failed to clarify the use of IUs across diverse methods and with increasingly diverse formulations. However, it was noted that when whole-cell pertussis vaccine is mixed with tetanus toxoid, and when the potency assay is carried out in mice, there is a significant adjuvant effect due to the whole-cell pertussis component. Subsequently, an increase in tetanus potency was also noted with combination vaccines containing *Haemophilus influenzae* type b and tetanus toxoid (29).

A number of international consultations convened during the late 1990s identified the need to clarify WHO guidance on the introduction and use of alternative potency assays for the purpose of routine lot release, and the transferability of IUs when different methods are used. The main revisions in the 2003 amendments, published in 2005 (16), thus included a division of the section on potency testing to clearly distinguish the recommendations for licensing from those for routine lot release. However, as with previous versions of the Recommendations, it was acknowledged that difficulties remained in the global

harmonization of potency testing procedures, even when international standards were used, and that different approaches were taken by different countries. The approach taken by WHO, as well as by the European Pharmacopoeia (30), is based on determining the immunizing potency of each final bulk by comparing it with an appropriate reference material that has been calibrated in IUs against the International Standard for tetanus toxoid, adsorbed. In some countries, potency is determined using the United States National Institutes of Health assay. In this test, the vaccine is assessed according to its ability to induce a protective or functional antibody response in guinea-pigs that reaches a minimum threshold of 2 Units per ml, as measured by an in vivo toxin neutralization test (TNT) against a standard antitoxin preparation (31). Although data are available demonstrating that vaccines meeting such requirements can induce significant levels of antitoxin response in recipients, the limitations of assays performed in the absence of a reference vaccine have been well documented (32). The expression of tetanus potency in IUs defined by the reference vaccine thus remains the approach recommended by WHO. However, there is still no universally accepted method for potency testing, and the formulation of global requirements remains a challenge.

A number of studies have shown that different results may be obtained when potency tests are carried out in mice instead of guinea-pigs (33–35). It was acknowledged in the 2003 amendments that mouse-challenge assays could be used for the potency testing of tetanus vaccines provided that the transferability of IUs had been demonstrated. However, this is not an entirely satisfactory procedure in view of the decreasing number of laboratories with experience in performing challenge assays in guinea-pigs (27). In addition, such an approach is particularly resource-intensive since a demonstration of transferability (through calibration in IUs) may be required for each product or product type.

It has been noted that many laboratories, particularly in the WHO European Region, rely largely on mouse-protection assays, and have adopted IUs for use in mouse assays from mouse-challenge data, despite the lack of traceability back to the first International Standard for tetanus toxoid, plain (27). Further, it has been noted that different laboratories calibrate in-house working standards using diverse methods, some using guinea-pigs and some using mice (with IUs from guinea-pigs or with IUs from mice), and some even use mean values determined in the two assay models (36). At its 2010 meeting, during adoption of the Fourth WHO International Standard for tetanus toxoid, adsorbed, the Expert Committee recommended that a working group should review the issue of transferability among different assay models, and the use of mouse-potency assays for expressing tetanus vaccine potency in IUs. The working group noted that a number of studies had highlighted the lack of agreement among guinea-pig assays and mouse assays in terms of tetanus vaccine potency expressed in IUs (33–35), and also noted that (according to the

results of a survey by WHO of vaccine manufacturers and control laboratories) in many cases mouse assays are widely used for the routine quality control of tetanus vaccines and for calibrating secondary standards. The logistical and practical difficulties of performing studies using guinea-pigs in order to demonstrate transferability were also recognized. The working group proposed that tetanus vaccine standards (including international standards, and regional, national and other secondary standards) could be calibrated using a mouse-challenge assay, and assigned IU. It is therefore recommended that the Fourth International Standard for tetanus toxoid, adsorbed (NIBSC code 08/218) should be assigned a potency of 260 IU (on the basis of the results obtained in the international collaborative study) for use in mouse-potency assays (26, 27).

The working group acknowledged that the WHO minimum requirement for tetanus potency was originally based on results obtained in guinea-pig challenge assays, using standards calibrated in IUs in guinea-pigs. However, the working group also acknowledged that vaccines with demonstrated clinical safety and efficacy have been licensed and released in which in vivo potency has been determined using the mouse-challenge model, including assays for which the reference preparation has been calibrated using a mouse-challenge assay. The group therefore proposed that the specifications for the minimum requirements for potency assays should be retained. It is recommended that the impact of expressing vaccine potency relative to a reference preparation that has been calibrated in mice should be closely monitored.

The discussion about requirements for potency assays provided an opportunity to question the relevance of animal models for predicting an effective protective response in humans. The minimum specification for potency, as recommended by WHO, has served well over many years, and there is a long history of the successful use of tetanus vaccines. Whereas some clinical studies have confirmed comparable functional antibody levels resulting from different doses of tetanus vaccine in humans and guinea-pigs (37, 38), there have been other examples of clinical studies (39) in which both mice and guinea-pigs were reported to provide comparable information for the type of product investigated. However, the influences of mouse strains on tetanus potency have been well documented (40-42), and there is evidence that different ratios of functional to nonfunctional antibodies are induced in different species (43). Therefore, the potency assays for new vaccines should be able to detect functional antibodies at an early evaluation stage, and should ultimately be approved on the basis of their effective performance in clinical trials. This is of particular relevance for newer combinations and when additional specifications for indicating consistency are under consideration.

During the past decade, there has been much activity aimed at simplifying the multiple-dilution direct-challenge potency tests by reducing the number of animals used or refining the end-points (such as paralysis), or by using validated serology assays. Some studies have also considered the possibility of using the same animals to test the potency of several antigens (44). The 2003 amendments (published in 2005) emphasized that methods other than challenge tests may be preferred for evaluating the potency of tetanus vaccines on a routine basis, but the amendments also indicated that potencies calculated by methods other than the challenge test should not be assumed to be transferable without validation. Suitable alternative methods and technical advice on validating such methods are given in the revised WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (45).

Amendments adopted in 2003 noted that, despite many attempts to harmonize potency requirements globally, there are still no universally accepted methods, and recognized that this leads to problems in the international exchange of these vaccines due to difficulties in the mutual recognition of the results of testing. Recommendations made by the working group on IUs and transferability aim to simplify and harmonize approaches, but it has been recognized that global acceptance and adoption of these remain a challenge.

During the revision process for these Recommendations, WHO held a scientific consultation in Beijing, China, in November 2011. At that consultation, the option of harmonizing minimum potency requirements for tetanus vaccine with those recommended in the European Pharmacopoeia was discussed. It was acknowledged that amending the WHO minimum requirement for potency could improve harmonization and the international exchange of vaccines. As a result, the minimum requirement for the potency of tetanus vaccine, tested according to the methods described in these Recommendations, was amended such that the specification (dependent on product type and method – i.e. 40 IU/ SHD for vaccines intended for primary immunization in the absence of a whole-cell pertussis component) now applies to the lower 95% confidence limit, thus demonstrating that the vaccine potency significantly exceeds the defined minimum specification. Because the minimum potency requirement now applies to the lower limit of the 95% confidence interval, there is no requirement to achieve a 95% confidence interval narrower than 50-200%. However, the revised section on potency testing in Part A of these Recommendations includes information on criteria that should be met in order for the potency estimate to be statistically valid.

The main changes included in this latest revision comprise:

- a change of title from Requirements to Recommendations;
- an update of the section on international standards and reference preparations, which has been moved to the General considerations section;
- an update of the section on general manufacturing recommendations and control tests;

- amendment of the minimum requirement for the potency of tetanus vaccine, which now applies to the lower limit of the 95% confidence interval;
- provision for using the mouse-challenge assay for calibration of standard preparations for tetanus toxoid (adsorbed);
- inclusion of new sections to provide guidance on the clinical and nonclinical evaluations of tetanus vaccines to assess safety, quality and efficacy.

In order to facilitate the release process of vaccines made in accordance with these Recommendations, a model protocol is provided in Appendix 1.

Certain issues, such as recommendations for labelling and lot release, are covered in more detail by other WHO documents (46).

Scope of the Recommendations

These Recommendations apply to the production and quality control of adsorbed tetanus vaccines, and have been updated from the 1989 revision of the Requirements for diphtheria, tetanus, pertussis and combined vaccines (15) and the 2003 Amendments to those Requirements (16). The current Recommendations highlight advances made in the production and testing of tetanus vaccines and related intermediates. The recommendations for the quality control of tetanus vaccines included in this document are based on currently licensed vaccines. Other products (such as those containing a new type of antigen or produced using novel technology) may require additional considerations.

Although these Recommendations apply to the production and quality control of tetanus vaccines, most tetanus vaccines are presented in their final formulation with at least one other vaccine. Therefore, in addition to monovalent tetanus vaccine, these Recommendations also apply to tetanus vaccine used in combination vaccines, and the tests recommended for the final bulk or final fill also apply to combined vaccines where appropriate.

In some cases, one or more of the component vaccines may be presented in separate containers that are intended to be mixed prior to administration. In such instances, tests may not need to be performed on the final combination during routine testing, but any effect on assays would normally be confirmed during nonclinical evaluation and as part of the licensing process.

General considerations

The supply of effective tetanus vaccines depends on the use of well characterized and standardized production processes, together with extensive in-process quality control tests and monitoring of the product and its related intermediates using suitable and validated methods. A written description of detailed and clearly defined standard operating procedures used for the production and testing of the vaccine, together with evidence of appropriate validation for each critical production step and relevant control tests, should be submitted by vaccine manufacturers to the NRA for approval as part of the licensing application. Proposals for any variation to the manufacturing or quality control methods should be submitted to the NRA for approval before implementation and according to national regulatory requirements.

The production of tetanus toxoid vaccine by chemical inactivation of tetanus toxin with formaldehyde has remained virtually unchanged since it was first introduced in the 1920s, although methods to monitor the production process, and characterize key intermediates and the final product, have improved significantly.

The production of tetanus toxin, from which the toxoid is prepared, requires the cultivation of a highly toxigenic strain of *Clostridium tetani* (e.g. the Harvard strain) with a known origin and history. Seed cultures are managed in a defined seed lot system in which toxigenicity is conserved. The approach adopted by most manufacturers is to obtain the greatest possible quantity of toxin during the growth phase of the microorganism and to convert the toxin into stable toxoid by the most effective method. Formaldehyde is most commonly used for the toxin detoxification process.

The purity and yield of toxin is checked to monitor consistency. Generally, toxin is purified prior to detoxification with formaldehyde in order to remove components that are likely to cause adverse reactions during use. The inactivation method must be validated to ensure that the toxoid does not revert to toxicity on exposure to heat but retains its immunogenic properties. Some manufacturers prefer to inactivate the toxin before purification in order to reduce the risk of reversion to toxicity. In view of the risk of reversion to toxicity, especially when a toxin is detoxified after purification, the present Recommendations have been formulated to address this risk by retaining the recommended 6-week incubation period for diluted, purified toxoid stored at elevated temperatures during the irreversibility test.

The demonstration of safety and the confirmation of vaccine potency are the fundamental requirements for the production of tetanus toxoid vaccines. These Recommendations call for the production of purified toxoid to minimize adverse reactions to vaccination in humans. The antigenic strength and purity of tetanus toxoid, defined in flocculation units (Lf units), is an essential quality indicator, and the minimum requirements remain set at 1000 Lf units per mg of protein (nondialysable) nitrogen. In addition to the traditional Ramon flocculation method used to determine the purity of tetanus toxoid, additional physicochemical methods – e.g. high-performance liquid chromatography

(HPLC), circular dichroism (CD) spectroscopy or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) – should be considered for characterization of the product (and intermediates), and for monitoring the production process.

The measurement of antigen content and the degree of adsorption in the final bulk or final fill are important quality tests, and useful indicators of consistency. The antigen content measured in Lf per dose in products recommended for primary immunization may be higher than in vaccines recommended for reinforcing immunity in adults and adolescents.

Several studies conducted during the last decade have provided useful information on the value and potential use of in vitro immunochemical assays for measurement of the toxoid antigen content and degree of adsorption in the final bulk or final fill of tetanus vaccines (47, 48). The results obtained using these methods will not necessarily correlate with measurements of vaccine potency that have been determined in vivo, particularly for complex combinations of vaccines, but the value of these in vitro methods for monitoring trends (47) and stability (27) has been well documented.

When a new bulk lot of vaccine is made, it is essential to confirm its safety (i.e. the absence of toxin and reversion to toxicity) and potency. Potency is measured using an in vivo challenge test, or a validated alternative, and results are expressed in IUs by comparison with a suitable reference preparation that has been calibrated in IUs. The minimum requirements for tetanus potency depend on the animal model used and the composition of the vaccine being tested. In some countries, the minimum requirement for the potency of vaccines intended for boosting immunity in adults and adolescents is lower than that recommended for vaccines intended for primary immunization because of the reduced antigen content in these products relative to vaccines intended for primary immunization. In addition to the minimum requirements stated in these Recommendations, it is also recommended that manufacturers set consistency limits for the potency of the tetanus vaccine being produced. Such limits may be useful in supporting the evaluation of consistency.

Clinical studies should be performed to support the licensure of new tetanus vaccines. Because new tetanus vaccines are expected to be evaluated in populations with a low incidence of the disease, it is not possible to evaluate efficacy. Instead, the ability to induce levels of tetanus antibodies that are considered to be protective is assessed. It may also be feasible to conduct population-based surveys of the prevalence of antibody in a given population to guide recommendations regarding the need for and timing of booster doses, although these surveys are not necessarily a prelicensure requirement. Further details on clinical evaluation and on the determination of antibody response in humans are included in Part C of these Recommendations.

The stability evaluation of tetanus vaccines is addressed in section A.10.1 of these Recommendations, and emphasizes the importance of real-time stability studies conducted on the final product and under intended storage conditions.

In addition to these Recommendations, the general manufacturing requirements contained in Good manufacturing practices: main principles for pharmaceutical products (49) and Good manufacturing practices for biological products (50) apply to the production of tetanus vaccines.

Terminology

Definitions for some common terms used throughout this document are given below. They may have different meanings in other contexts.

Bulk purified toxoid: the processed, purified material that has been prepared from either a single harvest or a pool of single harvests. It is the parent material from which the final bulk is prepared.

Final bulk: the homogeneous final vaccine present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

Final lot: a collection of sealed final containers that is homogeneous in all respects. In principle, a final lot must have been filled from a single final bulk container and processed further (e.g. freeze-dried) in one continuous working session. Different final lots may be filled or processed from the same final bulk in different working sessions. These related final lots are sometimes referred to as sub-lots, filling lots or freeze-drying lots, and should be identifiable by a distinctive final lot number.

Master seed lot: a quantity of bacterial suspension that has been derived from a single strain, has been processed as a single lot, and has a uniform composition. It is used to inoculate media for preparation of the working seed lot. The master seed lot should be stored as frozen stock or as lyophilized stock at a temperature known to ensure stability.

Seed lot: a quantity of bacterial suspension that has been derived from one strain, has been processed as a single lot, and has a uniform composition. It is used to prepare the inoculum for the production medium.

Single harvest: the toxic filtrate or toxoid obtained from one batch of cultures that have been inoculated, harvested and processed together.

Working seed lot: a bacterial culture consisting of a single substrain derived from the master seed lot. Working seed lots are stored in aliquots under the conditions described above for master seed lots. The working seed lot should be prepared from the master seed lot using as few cultural passages as possible; it should have the same characteristics as the master seed lot. It is used to inoculate media for the preparation of single harvests.

International reference materials

Subsequent sections of this document refer to WHO reference materials that may be used in laboratory or clinical evaluations. Key standards used in the control of tetanus vaccines include the following.

- The Fourth WHO International Standard for Tetanus Toxoid, Adsorbed this material (NIBSC code 08/218) was established by the WHO Expert Committee on Biological Standardization in 2010, and was assigned a value of 490 IU per ampoule against the Third WHO International Standard for Tetanus Toxoid, Adsorbed on the basis of challenge assays in guinea-pigs (26); in 2012 the Expert Committee assigned a value of 260 IU per ampoule on the basis of challenge assays in mice (27). This standard is intended for use as a reference vaccine in tetanus vaccine potency assays.
- The Second WHO International Standard for tetanus toxoid for use in flocculation test this material (NIBSC code 04/150) was established by the Expert Committee in 2007 (51, 52), and was assigned an activity of 690 Lf per ampoule, replacing the First International Reference Reagent for tetanus toxoid for flocculation test. This standard is intended for use in flocculation tests to determine the specific antigen content of tetanus toxoid in Lf.
- The First WHO International Standard for tetanus immunoglobulin, human this material (NIBSC code TE-3) was established in 1992 (53, 54) with an assigned unitage of 120 IU per ampoule, replacing the Second International Standard for tetanus antitoxin, equine, which had been in use since 1969. The standard was assigned activity from in vivo TNT assays, and is intended for use as a reference preparation in TNT in vivo.

The material is also used as a reference preparation for measurements of tetanus antitoxin in human serum by in vitro methods.

The above-mentioned international standards and reference materials are held by the National Institute for Biological Standards and Control, Medicines and Healthcare Products Regulatory Agency, Potters Bar, Hertfordshire, EN6 3QG, England.¹ As reference materials may be superseded by replacement standards, the WHO catalogue of international reference preparations should be consulted for the latest list of established standards.² International reference materials

See: http://www.nibsc.org/

² See: http://www.who.int/bloodproducts/catalogue/en/index.html

are intended for use in the calibration of national, regional or other secondary standards (55) that are used for the production and quality control of tetanus vaccines. They may also be suitable for use as a primary reference preparation for some assays.

Part A. Manufacturing recommendations

A 1 Definitions

A.1.1 International name and proper name

The international name should be tetanus vaccine (adsorbed). The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

In some countries the proper name used to refer to a tetanus toxoidcontaining vaccine is tetanus toxoid adsorbed.

A.1.2 **Descriptive definition**

Tetanus vaccine (adsorbed) is a preparation of tetanus toxoid prepared by treating tetanus toxin using chemical means to render it nontoxic without losing its immunogenic potency. The toxoid is adsorbed on to, or precipitated with, a suitable adjuvant. The preparation should satisfy the requirements formulated below.

The most common method of preparing toxoid from toxins is by using formaldehyde.

A.2 General manufacturing recommendations

The general manufacturing recommendations contained in Good manufacturing practices: main principles for pharmaceutical products (49) and Good manufacturing practices for biological products (50) apply to the production of tetanus vaccine. These practices include demonstrating the purity and quality of the production strain and seed lots, implementing in-process quality control testing, testing for process additives and process intermediates, and developing and establishing lot-release tests.

A written description of procedures used in the preparation and testing of the tetanus vaccine, together with appropriate evidence that each production step has been validated, should be submitted to the NRA for approval. Proposals for modifying the manufacturing process or quality control methods should also be submitted to the NRA for approval before such modifications are implemented.

A.3 Production, processing and control

A.3.1 Production precautions

The general production precautions, as formulated in Good manufacturing practices for biological products (50), apply to the production of tetanus vaccines and to establishments manufacturing tetanus vaccine.

Suitable methods for the production of tetanus vaccine are given in the WHO *Manual for the production and control of vaccines: tetanus toxoid* (56).

Personnel employed in production and quality control should be adequately trained, should have completed a course of immunization against tetanus, and should receive appropriate booster immunization(s). Appropriate health surveillance should also be carried out.

A.3.2 Production strain and seed lots

A.3.2.1 Strains of Clostridium tetani

In order to verify strain characteristics, strains of *C. tetani* used in preparing tetanus toxoid should be identified by using a record of their history and of all tests made in accordance with NRA recommendations. The strain should be maintained as a freeze-dried culture or as a frozen-liquid seed stock.

A highly toxigenic strain of *C. tetani* of known origin, history and verification, should be used. The strain of *C. tetani* used should be approved by the NRA.

A strain that has proved satisfactory for many manufacturers is the Harvard strain, but other strains have also been used.

A.3.2.2 Seed-lot system

The production of tetanus toxin should be based on a well defined and validated seed lot system. The strain used to establish the master seed lot should be chosen for desirable characteristics in which toxigenicity is conserved. Cultures of the working seed should have the same characteristics as those of the strain from which the master seed lot was derived. Detailed records of the origin, passage history, purification and characterization procedures, and storage conditions should be provided to the NRA when new master seeds or working seeds are introduced. Working seeds in use should be characterized at defined intervals that have been approved by the NRA on the basis of prior production history and experience. The preparation of seed lots should comply with the requirements of Part A, section A.3.1. The maximum number of passages of each seed lot used

for production should be specified, should be based on the number shown to result in the production of a safe and effective product, and should be approved by the NRA.

Where possible, a combination of validated biochemical, molecular and genetic tests should be used for identification and characterization of seed lots. Suitable methods include multilocus enzyme electrophoresis (MEE), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, pulsed-field gel electrophoresis (PFGE), multilocus sequencing typing (MLST) and restriction fragment length polymorphism (RFLP) analysis.

A.3.2.3 Culture medium for production of toxin

C. tetani should be cultured in a suitable liquid medium known to support the growth of bacteria and ensure a good yield of tetanus toxin. The culture medium should be free from adventitious agents and components that are known to cause toxic or allergic reactions in humans. Human blood products must not be used. Materials or components of animal origin should be identified, and methods for detecting these substances should be approved by the NRA.

Meat-free medium should be used where possible. If the medium is prepared from a protein digest (e.g. casein hydrolysate or digested muscle), precautions should be taken to ensure that digestion has proceeded sufficiently. If any materials of animal origin are used in seed preparation, in the culture medium or in production, they should comply with the WHO *Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (57). Where appropriate and relevant, an upper limit should be established for mammalian protein in the final vaccine, and this limit should not be exceeded.

Any change in medium should be submitted for approval to the NRA.

A.3.3 Single harvests

The consistency of production should be demonstrated. This process may involve measurements of culture purity, growth rate, pH, incubation period, temperature range and rate of toxin production. The NRA should establish and approve acceptance specifications with defined limits (and, where relevant, alert limits) that will demonstrate the consistency of production.

Any culture showing anomalous growth characteristics should be investigated and should be shown to be satisfactory before being accepted as a single harvest. Contaminated cultures must be discarded.

Suitable methods for the production of tetanus toxin are given in the WHO Manual for the production and control of vaccines: tetanus toxoid (56).

Single harvests that meet the acceptance criteria may be pooled to prepare the bulk purified toxoid. Storage times should be supported by data obtained from appropriate stability studies, and should be approved by the NRA.

A.3.3.1 Control of bacterial purity

Samples of individual cultures used for preparing single harvests should be tested for bacterial purity by microscopic examination of stained smears, and by inoculation into appropriate culture media, or by another suitable procedure. Single harvests should be discarded if contamination has occurred at any stage during production.

Toxin-containing culture medium should be collected aseptically or in a way that minimizes the bioburden. Adequate measures and conditions should be in place to minimize the growth of microorganisms while low-bioburden materials are stored.

A.3.3.2 Filtration

After the culture medium has been sampled to control for purity, filtration should be used to separate the medium aseptically from the bacterial mass as soon as possible. A preservative may be added, but phenol should not be used for this purpose.

To facilitate filtration, cultures may be centrifuged, provided that suitable precautions have been taken to avoid the formation of potentially hazardous aerosols. A filter aid may be added beforehand. A filter that does not shed fibres should be used.

A.3.3.3 Determination of crude toxin concentration

Prior to detoxification, the toxin content of the culture supernatant should be determined using a suitable in vitro method approved by the NRA.

The flocculation test is suitable for the measurement of toxin content, and is described in the WHO Manual for the production and control of vaccines: tetanus toxoid (56) and the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (45). A reference material calibrated in Lf against the International Standard for tetanus toxoid for flocculation tests, or an equivalent reference material approved by the NRA, should be included, and the results should be expressed in Lf units.

The measurement of toxin content (defined in Lf per ml) is a good indicator of the consistency of production, and acceptance limits should be defined for monitoring purposes.

It is preferable for culture filtrates to contain at least 40 Lf/ml of toxin, although lower concentrations have been applied by some manufacturers with satisfactory results.

A.3.3.4 **Detoxification and purification**

Detoxification of tetanus toxin may be performed using crude toxin (culture filtrate) or purified toxin and a well defined and validated process. Purifying the toxin before detoxification results in a purer product and is expected to remove components that are likely to cause adverse reactions in humans, although more care may need to be taken during detoxification because the risk of reversion to toxicity may be increased. The method of purification should be such that no substances are incorporated into the final products that are likely to cause adverse reactions in humans.

The method of purification and the agent used for detoxification should be suitably validated, and should be approved by the NRA. The rate of detoxification may vary, and in-process monitoring of the detoxifying process should be performed.

Formaldehyde is most commonly used as a detoxifying reagent, and amino acids such as lysine or glycine may be added during detoxification to facilitate cross-linking of toxin molecules, and to prevent reversion. The detoxification conditions should be well defined and controlled with respect to temperature, time, concentration of detoxifying reagent, toxin concentration and any other critical parameter, in order to produce consistent, inactivated toxoid of the desired immunogenicity.

The method used for purification should be approved by the NRA.

Crude toxoid can be concentrated using ultrafiltration prior to purification by fractionation with ammonium sulfate, dialysis, gel filtration, ion-exchange chromatography, or a combination of these methods.

Bioburden testing may also be performed after purification to ensure that potential levels of contamination have been minimized for subsequent steps that are not done aseptically.

When measured in the final bulk vaccine, the amount of residual free detoxifying agent remaining after detoxification and purification have been completed should not exceed the limit stated in section A.3.5.2.7.

Harvests should be treated as potentially toxic, and subject to the appropriate safety restrictions until the detoxification has been shown to be complete by performance of a specific toxicity test (as detailed in section A.3.4.4) or any other suitable in vivo method.

The conditions of storage, including shelf-life, should be validated and supported by an appropriate stability programme, and should be approved by the NRA.

A.3.4 Bulk purified toxoid

A.3.4.1 **Preparation**

The bulk purified toxoid should be prepared from either a single harvest or a pool of single harvests, and should be sterile. A preservative may be added, provided that it has been shown not to adversely affect the safety and immunogenicity of the toxoid; this addition is subject to approval by the NRA. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity.

It is advisable to sterilize the bulk toxoid by filtration.

A.3.4.2 **Sterility**

Each bulk purified toxoid should be tested for bacterial and mycotic sterility in accordance with the requirements in Part A, section 5, of the General requirements for the sterility of biological substances (58) or by a method approved by the NRA. The sterility test is performed using at least 10 ml of each bulk purified toxoid. If a preservative has been added to the purified bulk, appropriate measures must be taken to prevent any interference in the sterility test.

A.3.4.3 Antigenic purity

Each bulk purified toxoid should be tested for antigenic strength and purity by determining the antigen concentration in Lf and the concentration of protein (nondialysable) nitrogen. The antigen concentration should be determined using a suitably standardized binding assay in solution (e.g. the Ramon assay) and compared with a reference material that has been calibrated against the International Standard for tetanus toxoid for flocculation test, or an equivalent reference preparation approved by the NRA. The method of testing should be approved by the NRA. The bulk purified toxoid passes the test if it contains at least 1000 Lf/mg of protein (nondialysable) nitrogen.

The flocculation (Ramon) assay is suitable for measuring antigen content, and is described in the WHO Manual for the production and control of vaccines: tetanus toxoid (56) and in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (45).

Physicochemical analysis, using methods such as SDS-PAGE and HPLC, may be used to monitor antigenic purity and to provide additional

information on antigen integrity and the extent of aggregation and proteolysis. These additional characterization tests should be performed whenever a new working seed is introduced.

A.3.4.4 Specific toxicity

Each bulk purified toxoid, diluted with the same buffer solution as used in the final vaccine, should be tested for the absence of tetanus toxin in guinea-pigs; the guinea-pigs should each weigh 250–350 g and not previously have been used for experimental purposes. At least five guinea-pigs should be injected subcutaneously with 1 ml of a dilution of purified tetanus toxoid containing at least 500 Lf of toxoid; they must be observed daily for signs of tetanic paralysis over a period of 21 days. A suitable method is outlined in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (45).

Animals that die, whatever the cause, will need to be examined by necropsy. The bulk purified toxoid passes the test if no guinea-pig shows symptoms of specific paralysis or any other signs of tetanus within 21 days of injection, and if at least 80% (i.e. four fifths) of the animals survive the test period. If more than one animal dies from nonspecific causes, the test must be repeated (using at least five more guinea-pigs).

If more than one animal dies in the second test, the toxoid sample does not comply with the test.

A.3.4.5 **Reversion to toxicity**

Each bulk purified toxoid must be tested to ensure that reversion to toxicity cannot take place during storage. The test employed should be approved by the NRA, and should be sufficiently sensitive to detect very small amounts of toxin. To determine whether reversion to toxicity has occurred, diluted toxoid that has been stored at an elevated temperature for six weeks should be tested. The diluted toxoid is prepared in such a way that the chemical environment is comparable to that found in the final vaccine except for the absence of adjuvant.

For bulk toxoid that will be used in the preparation of more than one final-bulk formulation, the test should be performed using dilutions of the bulk toxoid that represent the lowest and highest concentrations of toxoid that will be present in the final formulations.

The diluted toxoid sample is incubated at 34–37 °C for a period of six weeks (42 days). At the end of the incubation period, five guinea-pigs are each injected subcutaneously with 5.0 ml (i.e. 10 human doses, using multiple injection sites where necessary) of test sample. The animals are observed for 21 days for signs of ill health.

Similar dilutions of toxoid held at 2-8 °C during the same period of time as those held at 34-37 °C may be tested as controls.

No toxicity should be detected. The bulk purified toxoid passes the test if no guinea-pig shows symptoms of specific paralysis or any other signs of tetanus within 21 days of injection.

A.3.4.6 Storage of bulk purified toxoid

Storage times for bulk purified toxoid should be supported by data from appropriate stability studies, should comply with WHO Guidelines on stability evaluation of vaccines (59), and should be approved by the NRA.

A.3.5 Final bulk

A.3.5.1 **Preparation**

The final bulk is prepared from purified toxoid by adsorption on to, or precipitation with, a suitable quantity of a mineral carrier, such as hydrated aluminium phosphate or aluminium hydroxide (or other suitable adjuvant). The resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. The final formulation of the vaccine should be based on formulations that have been shown to be safe and effective in clinical use. The number of Lf in a single human dose should be approved by the NRA.

It is recommended that the tetanus antigen content in vaccines intended for primary immunization should not exceed 25 Lf/SHD. In vaccines intended for reinforcing the immunity of adults and adolescents, the number of Lf per SHD may be reduced, and in some countries it is recommended that it should not exceed 10 Lf/SHD.

A.3.5.2 Control tests

A.3.5.2.1 Preservative

If the vaccine is to be filled into multidose containers, a suitable antimicrobial preservative should be added. The amount of preservative in the final bulk should be shown to have no deleterious effect on the toxoid or on other vaccine components with which the toxoid may be combined in the final product; the preservative should also be shown to cause no unexpected adverse reactions in humans. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity of tetanus vaccines. The preservative and its concentration should be shown to be effective, and should be approved by the NRA. WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines should be followed (60).

Determine the amount of antimicrobial preservative by using a suitable chemical method. The amount should be at least 85% and not more than 115% of the intended amount.

A.3.5.2.2 Adjuvants

The nature, purity and concentration of the adjuvant used in the formulation should be determined by methods approved by the NRA. When aluminium compounds are used as adjuvants, the concentration of aluminium should not exceed 1.25 mg/SHD. If other (new) adjuvants are used, quality specifications should be set for the adjuvant used alone and in combination with the antigen, and should be approved by the NRA.

In some countries these recommended limits for adjuvant concentrations are considered too high, and lower limits have been approved and shown to be safe and effective.

A.3.5.2.3 **Degree of adsorption**

The degree of adsorption should be measured and should be shown to be comparable to that measured in vaccine lots used in clinical studies to support licensing. The measurement of antigen content and the degree of adsorption to adjuvant are useful indicators of consistency in production. In-house specifications, with appropriate acceptance limits, can be established once a suitable number of lots have been tested.

Suitable methods for determining antigen content and the degree of adsorption are provided in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (45).

These tests may be omitted provided they are performed on the final lot.

A.3.5.2.4 Sterility

Each final bulk should be tested for bacterial and mycotic sterility in accordance with the requirements in Part A, section 5, of the General requirements for the sterility of biological substances (58) or by a method approved by the NRA. The sterility test is performed using at least 10 ml of each final bulk. If a preservative has been added to the final bulk, adequate measures should be taken to prevent it from causing any interference in the sterility test.

A.3.5.2.5 *Specific toxicity*

Each final bulk should be tested for specific toxicity in at least five guinea-pigs; each guinea-pig should weigh 250–350 g and not have been used previously for experimental purposes. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least 5 SHD, and is observed daily for a period of 21 days. Animals that die from any cause should undergo necropsy and be

inspected for symptoms of tetanus paralysis. The final bulk passes the test if no guinea-pig shows symptoms of specific paralysis or any other signs of tetanus intoxication within 21 days of injection. If more than one animal dies, the test must be repeated (using at least five more guinea-pigs). If more than one animal dies during the retest, the final bulk does not comply with the test.

Subject to the approval of the NRA, the specific toxicity test used on the final bulk may be omitted from routine lot-release procedures once consistency in production has been established.

A.3.5.2.6 *Potency*

The potency of each final bulk (or final lot) should be determined by comparison with a suitable reference preparation that has been calibrated in IUs against the International Standard for tetanus toxoid, adsorbed. Appropriate statistical methods should be used to calculate the potency of the final bulk (44). The NRA should approve the assay method and the method used for calculating the results.

Details on methods suitable for potency testing of tetanus vaccines can be found in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (45).

The minimum potency specifications introduced into WHO guidance in 1978 have helped to ensure the production and use of safe and effective tetanus vaccines, as evidenced by the satisfactory performance of these products in clinical studies and the low incidence of tetanus in populations with good immunization coverage. Therefore, it is recommended that the potency of a tetanus vaccine used for the primary immunization of children should significantly exceed 40 IU/SHD (based on data showing that the lower 95% confidence limit of the estimated potency is at least 40 IU/SHD). Where the test is performed in mice for a vaccine containing a whole-cell pertussis component, the minimum requirement is 60 IU/SHD (based on data showing that the lower 95% confidence limit of the estimated potency is at least 60 IU/SHD). The minimum potency specification for tetanus vaccine intended for booster immunization in older children and adults may be lower and should be approved by the NRA.

Product-specific minimum requirements for tetanus potency are acceptable, provided they have been justified and are based on potency values obtained for the vaccine in question. A suitable number of lots should be tested in order to define the minimum requirement for potency. Vaccine lots used for the establishment of the potency specification should include lots that have been shown to be safe and effective in clinical studies. Product-specific minimum requirements should be approved by the NRA. Once defined and approved, the potency of the vaccine should be shown to exceed the minimum requirement significantly (based on data showing that the lower 95% confidence limit of the estimated potency is at least that of the minimum requirement).

The following criteria should be met in order for the potency estimate to be statistically valid:

- the statistical analysis should show a significant regression (P < 0.05) of the log dose–response curves without significant deviation from linearity and parallelism (P > 0.05);
- the 50% protective dose should lie between the smallest and largest vaccine doses

When more than one assay is performed, the results of all statistically valid tests should be combined into a geometric mean estimate, and the confidence limits calculated.

Manufacturers are also encouraged to monitor the potency of different vaccine bulks and lots by setting minimum and maximum alert criteria once a suitable number of lots have been tested.

Calibration of reference preparations

The potency of a tetanus vaccine is determined in comparison with a reference preparation that has been calibrated in IUs against the International Standard for tetanus toxoid, adsorbed. Secondary reference preparations (regional, national, working or product-specific standards) should be calibrated with a multiple-dilution protection assay (of at least three dilutions), using either guinea-pigs or mice. Standards calibrated in IUs in guinea-pigs are considered to be suitable for use in guinea-pig potency assays, and standards calibrated using mouse assays are considered to be suitable for use only in mouse-potency tests. Adequate controls should be in place to ensure and monitor the stability of all secondary standards and, where possible, subsequent replacement batches of secondary or working standards should be calibrated against the primary international standard rather than against the previous working standard (55).

Potency test for routine lot release

For routine testing, the potency of every new bulk of tetanus vaccine is determined by the immunization of guinea-pigs or mice with appropriate dilutions of the calibrated reference preparation and test vaccine. Care should be taken to ensure that dilutions are inert (phosphate may interfere with the adsorption of toxoid) and not pyrogenic. Between four and six weeks after immunization, animals may be directly challenged with tetanus toxin by the subcutaneous route, or they may be bled for titration of immune serum.

Titration of immune serum samples may be performed using an in vivo TNT or by using a suitably validated in vitro method, such as an enzyme-linked immunosorbent assay (ELISA), the toxin-binding inhibition (ToBI) test or the particle agglutination test (30, 54, 61, 62). If in vitro serological assays are used,

they should show that the product induces an appropriate antibody response in animals when compared with the reference preparation.

The ELISA assay or another suitable in vitro method may be used to measure the antibody response to tetanus, provided that these assays have been validated against the functional assays (challenge or TNT) using the particular product in question. A minimum of three assays with a suitable dose-response range is likely to be required for validation for each product (30, 63). These methods require precise definition of the characteristics of reagents (such as the coating antigen, and positive and negative control sera) that are critical for the successful performance of the testing method.

Potency assay modifications: reduced dilution schemes

Consistency limits (upper and lower) for tetanus potency should be established in house once a suitable number of lots has been tested using a multiple-dilution assay. Production consistency has been demonstrated if the vaccine potency expressed in IUs obtained for at least 10 consecutive vaccine lots produced from different toxoid bulks is within the defined consistency limits, and if the expectations of linearity and parallelism have been consistently satisfied. Once consistency in production has been demonstrated for the vaccine, the potency assay (using the challenge or serology model) may, with the approval of the NRA, be performed using a reduced number of animals or doses, or both. Once approved, fewer doses of the test and reference vaccines may be used, and the assumptions of linearity and parallelism need not be tested for each assay. When particular vaccine lots consistently give the lower limit of the 95% confidence intervals for the estimated potency values (well in excess of the minimum requirement), one-dilution assays may offer an advantage. If one-dilution assays are not advantageous, a reduction in animal usage may nevertheless be achieved by using two-dilution assays or another suitable design modification.

A one-dilution assay is based on the same principles for evaluating the response as three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 40 IU (or the minimum requirement for the product expressed as an SHD), that elicits a minimum protective effect (or antibody response) in immunized animals; the effect of the reference vaccine is compared with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P \le 0.05$), the potency of the test vaccine is satisfactory.

One-dilution assays provide assurances that the lower limit of the estimated potency exceeds the minimum requirement. A disadvantage of such an approach is that a strictly quantitative estimate of vaccine potency will not be obtained.

Therefore, in order to ensure the overall consistency of production, there is a need to support the data generated by a simplified potency assay with data from other suitable in vitro assays. When a one-dilution assay is used with serological methods of analysis, measurements of a geometric mean antibody response in the group of animals immunized with the test vaccine can provide some information about production consistency on a continual basis, provided that the in vitro assay used to measure antibody titres contains suitable internal controls.

Lot release based on the use of a simplified approach will require periodic review to ensure that the validity of all procedures (including assumptions of linearity and parallelism) is maintained. The timing of the review should be decided on a case by case basis, depending on the number of lots of vaccine produced annually or by time schedule (at least every 2 years), and should be approved by the NRA. It is important to note that testing should revert to multiple-dilution assays if there is a significant change in the production process, and production consistency should be reconfirmed before the reduction scheme is reintroduced.

A.3.5.2.7 Amount of residual free detoxifying agent

The amount of residual free detoxifying agent in each final bulk should be determined. The methods used and acceptable limits should be approved by the NRA.

If formaldehyde has been used as detoxifying agent, the residual content should not exceed 0.2 g/l. The colorimetric determination of the reaction product of formaldehyde and fuchsin–sulfurous acid is a suitable method for detecting residual free formaldehyde.

Where applicable, appropriate tests for the detection and quantification of other detoxifying agents should be performed. The tests used and the maximum permissible concentrations of such chemicals should be approved by the NRA.

A.3.5.2.8 pH

The pH of the final bulk should be measured and should be within the range of values measured in vaccine lots shown to be safe and effective in clinical use.

A.3.5.3 Storage of final bulk

The final bulk may be stored in a single container or in multiple containers. When multiple storage containers are used, the contents must be pooled into a single container for filling into the final containers. Storage times for the final bulk should be supported by stability studies, and approved by the NRA.

A.4 Filling and containers

The requirements concerning filling and containers given in Good manufacturing practices: main principles for pharmaceutical products (49) and Good manufacturing practices for biological products (50) apply to vaccine filled in the final presentation. Single-dose or multiple-dose containers may be used. Vaccines in multidose containers should contain a suitable antimicrobial preservative (see section A.3.5.2.1).

The filling process should be suitably validated by comparing key parameters measured in the final bulk and in the final lot. Such studies should include measurement of the degree of adsorption.

A.5 Control of final product

Quality control procedures and tests should be validated and approved by the NRA to ensure that the final containers hold the appropriate amounts of each of the intended vaccine constituents (active components and excipients) in a formulation suitable for the intended use of the final product.

Unless otherwise justified and authorized, the following tests should be performed on labelled containers from each final lot by means of validated methods approved by the NRA.

A.5.1 **Identity**

An identity test should be performed on at least one container from each final lot using a validated method approved by the NRA.

The method used should be based on the specific interaction between the tetanus antigen in the vaccine and tetanus antitoxin. Suitable detection methods include flocculation (Ramon and light-scattering methods), immunoprecipitation assays and ELISA (44, 56). Tests on toxoids adsorbed on to an aluminium carrier should be performed after the carrier has been dissolved or the adsorbed toxoid has been wholly or partially eluted by sodium citrate or ethylenediaminetetraacetic acid (EDTA) (44, 56).

A.5.2 Sterility

Final containers should be tested for bacterial and mycotic sterility using a method approved by the NRA. Many countries have regulations governing the sterility testing of the final product. Where such regulations do not exist, the criteria published by WHO for the sterility of biological and pharmaceutical products (58) should apply. If a preservative has been added to the vaccine, appropriate measures should be taken to prevent it from causing any interference in the sterility test.

A.5.3 **Potency**

A potency test should be carried out on each final lot as outlined in Part A, section A.3.5.2.6, if such a test has not been performed on the final bulk.

A.5.4 Innocuity

Each final lot should be tested for innocuity. This test is also referred to as the abnormal toxicity test or the general safety test. One human dose, but not more than 1 ml, of the final lot is injected by the intraperitoneal route into each of five mice (weighing 17–22 g) and each of two guinea-pigs (weighing 250–350 g). The test should be approved by the NRA. The final product is considered to be innocuous if the animals survive for at least seven days without showing significant signs of toxicity.

If the NRA approves, the innocuity test on the final lot may be omitted from routine lot release once the consistency of production has been demonstrated.

A.5.5 Adjuvant content

The adjuvant content of each final lot should be determined using a method approved by the NRA and as described in Part A, section A.3.5.2.2. The formulation should be such that after shaking, the vaccine remains suspended as a homogeneous solution for a defined period of time (to allow sufficient time for administration).

A.5.6 **Degree of adsorption**

A test for the degree of adsorption should be carried out on each final lot as described in Part A, section A.3.5.2.3.

A.5.7 Preservative content

The content of the preservative(s) in each final lot should be determined as described in Part A, section A.3.5.2.1. The methods used should be approved by the NRA.

In some cases, if the NRA approves, this test may be performed only on the final bulk.

A.5.8 **pH**

The pH of each final lot should be measured and should be within the range of values measured in vaccine lots shown to be safe and effective in clinical use. The test may be omitted during the final fill if it has been performed on the final bulk.

In some cases, determination of osmolality may also be required.

A.5.9 Extractable volume

For vaccines filled into single-dose containers, the extractable volume should be checked and should be shown to be not less than the intended dose.

For vaccines filled into multidose containers, the extractable volume should be checked and should be shown to be sufficient for the intended number of doses.

A.5.10 Inspection of final containers

Each container in each final lot should be inspected visually or mechanically, and those showing abnormalities – such as improper sealing, lack of integrity, clumping or the presence of particles – should be discarded.

A.6 Records

The requirements given in Good manufacturing practices: main principles for pharmaceutical products (49) and Good manufacturing practices for biological products (50) apply. Written records should be kept of all tests, irrespective of their results. The records should be of a type from which annual trends can be determined.

A model of a suitable summary protocol for tetanus vaccines is given in Appendix 1.

A.7 Retained samples

Vaccine samples should be retained, as recommended in WHO good manufacturing practices for pharmaceutical products: main principles (49) and Good manufacturing practices for biological products (50).

A.8 Labelling

The label printed on or affixed to each container, and the label on the carton enclosing one or more containers, should be approved by the NRA. The labels should be easily readable and should show as a minimum:

- the words "tetanus vaccine (adsorbed)" or the proper name of the product, or both
- the licence number of the product
- the name of the manufacturer
- the number of the final lot
- the identity of any preservative or adjuvant
- the amount of antigen in Lf/ml or the minimum potency in IU/SHD, or both

- the recommended storage temperature and the expiry date if kept at that temperature
- the recommended SHD and the route of administration.

In addition, the label printed on or affixed to the container, or the label on the cartons, or the leaflet accompanying the container should contain the following:

- a statement that the vaccine satisfies the requirements of this document;
- the address of the manufacturer;
- the recommended temperature for transport;
- a warning that the adsorbed vaccine should not be frozen;
- a warning that the adsorbed vaccine should be shaken before use;
- instructions for the use of the vaccine, and information on contraindications and the reactions that may follow vaccination.

A.9 **Distribution and transport**

The requirements given in Good manufacturing practices: main principles for pharmaceutical products (49) and Good manufacturing practices for biological products (50) apply.

A.10 Stability, storage and expiry date

A.10.1 Stability

Stability evaluations form a critical part of quality assessment. The purpose of stability studies is to ensure that the vaccine at the end of its shelf-life, and during the storage period (or other defined storage period for intermediates) or period of use, retains the required characteristics to support its quality, safety and efficacy. Current recommendations on the evaluation of vaccine stability, as provided in the WHO Guidelines on stability evaluation of vaccines, apply (59).

The stability of the vaccine in final containers maintained at the recommended storage temperature should be demonstrated to the satisfaction of the NRA. The vaccine should be manufactured in such a way that reversion to toxicity does not occur during the defined period for shelf-life, provided that the vaccine is stored under the conditions recommended on the label. Typically, containers from at least three consecutive final lots (each derived from different toxoid bulk intermediates) should be tested and included in real-time stability studies that are supported by evidence of potency and lack of specific toxicity at the expiry date. To confirm that the vaccine does not revert to toxicity during

storage, the specific toxicity test described in Part A, section A.3.5.2.5, should be scheduled up to the expiry date as part of the stability studies. In addition, at the time of the expiry date, the vaccine should meet the requirements for the final product in terms of sterility, potency, adjuvant content, degree of adsorption, preservative content, pH and extractable volume, where applicable (as described in Part A, sections A.5.2, A.5.3 and A.5.5–A.5.9), provided that the vaccine has been stored at the recommended temperature. The frequency of testing should be approved by the NRA.

When any changes that may affect the stability of the product are made to the production process, the stability of the vaccine produced by the new method should be demonstrated.

Stability studies performed at temperatures other than those recommended for storing the vaccine may be useful in providing information about transporting the vaccine at different temperatures for a limited time. Accelerated stability studies may provide additional evidence of product stability, but cannot replace real-time stability studies.

A.10.2 Storage conditions

The recommended storage conditions and the defined maximum duration of storage should be based on stability studies, as described in section A.10.1, and should be approved by the NRA. For tetanus vaccine (adsorbed), storage at a temperature of 2–8 °C is generally considered to be satisfactory and should ensure that the minimum potency specified on the label of the container or package will be maintained after release and until the end of the proposed shelf-life, provided that the vaccine is stored under the recommended conditions and in accordance with instructions on the label.

Adsorbed vaccines must not be frozen.

A.10.3 Expiry date

The statement concerning the expiry date that appears on the label, as required in Part A, section A.8, should be based on experimental evidence, and approved by the NRA on the basis of the data obtained during the stability studies referred to in section A.10.1. The date of manufacture (i.e. blending or filling) or the start date of the last satisfactory potency determination, as performed in accordance with section A.5.3 or section A.3.5.2.6 – i.e. the date on which the test animals were immunized with the vaccine – is taken as the start date for the shelf-life.

In some cases, the date of the first satisfactory potency determination is used as the start date for the shelf-life.

Part B. Nonclinical evaluation of tetanus vaccines

B.1 Introduction

The nonclinical testing of tetanus vaccines and their related intermediates is an essential part of the development of candidate vaccines, and is a prerequisite for the initiation of clinical trials in humans and for licensure. Studies are aimed at defining the in vitro and in vivo characteristics of candidate vaccines and, within the scope of this document, nonclinical evaluation means all in vitro and in vivo testing performed before and during the clinical development of the vaccine. The recommendations included in this document follow a sequential approach and include the nonclinical evaluations that may need to be considered at different points in the manufacturing process – including during the production of tetanus toxin, purification and detoxification of the toxin, blending with the adjuvant in the final bulk and formulation of the final product. The recommendations are intended for new manufacturers of tetanus vaccine, and should also be relevant if a significant change to the production process or product formulation is made by a manufacturer already producing tetanus vaccine.

The extent to which nonclinical studies will be required depends on existing clinical experience with similar product types or with products containing the same antigen in a different formulation or combination. These Recommendations refer only to products based on those that are currently licensed and in clinical use – i.e. vaccines based on the use of chemically detoxified tetanus toxin (toxoid) as the antigen adsorbed on to, or precipitated with, an aluminium-based adjuvant. Tetanus vaccines based on novel antigens or formulations that have not previously been evaluated for safety and efficacy in clinical trials are likely to require more extensive nonclinical characterization, which is beyond the scope of this document.

The nonclinical evaluation of tetanus vaccines should be based on WHO guidelines on nonclinical evaluation of vaccines (64) which also contain definitions of commonly used terms related to nonclinical evaluations. Studies related to the nonclinical evaluation of vaccine intermediates and the final product should be performed in accordance with the principles of good laboratory practice (GLP). Adhering to the principles of GLP promotes the development of high-quality test data, and provides a tool for ensuring that a sound approach is taken to the management of laboratory studies, including how they are conducted, and how their data are reported and archived (65).

The nonclinical characterization of vaccine intermediates and in-process materials should be based on the use of adequately characterized, homogenous starting materials of defined origin and acceptable quality. Demonstrating consistency in production may not be applicable during the early stages of nonclinical evaluation, but adequate validation of the production process is required to demonstrate that the manufacturing conditions are reproducible.

The vaccine lots used in preclinical studies should be adequately representative of the formulation intended for clinical investigation, and, ideally, preclinical testing should be performed using the same lot(s) as those proposed for clinical trial. Manufacturers should make every effort to keep some of this characterized material for future reference. Early communications between the vaccine manufacturer and the NRA are recommended in order to agree on the requirements for, and type of, nonclinical testing.

8.2 Nonclinical testing and characterization of intermediates and in-process materials

Intermediates and in-process materials must be tested and characterized to confirm that they meet the requirements in Part A of this document. The source and quality of all starting materials should be documented and should include detailed descriptions of the characterization of the strain, master seed lot and working seed lot. Defined procedures should also be shown for the preparation of new working seeds from the master seed. Working seeds must be shown to produce an adequate yield of active toxin under defined culture conditions. Characterization studies should include measurements of viability, growth rate, rate of toxin production, confirmation of toxin activity (using the specific toxicity test, and defined as the minimum lethal or paralytic dose per ml of culture medium) and standard microbiological identification techniques. Cultures of the working seed should have the same characteristics as those of the strain from which the master seed lot was derived. Tests that are performed as part of the characterization of seed lots should include a combination of validated biochemical, molecular and genetic tests. Methods such as MEE, MALDI-TOF mass spectrometry, PFGE, MLST, RFLP or N-terminal sequencing could be considered. The maximum number of passages of each seed lot used for production should be specified, based on those shown to result in the production of a safe and effective product, and should be approved by the NRA.

The culture medium used for toxin production should be well defined, and all animal components, if present in the medium, should be identified and documented. Protein contaminants derived from the bacterium or from components of the culture medium may increase the potential for adverse reactions to immunization with the toxoid, and the detoxification and purification processes used should minimize the presence of any substances likely to cause adverse reactions in humans. The methods used for the detoxification and purification of crude toxin should be adequately described and supported by appropriate validation data.

Safety evaluation

The production process should be validated to confirm that the detoxification of tetanus toxin has been completed without reversion to toxicity, particularly when the toxoid is exposed to heat. Both the specific toxicity test (section A.3.4.4) and the reversion-to-toxicity test (A.3.4.5) should be performed on the bulk purified toxoid.

Aside from studies to confirm the absence of specific toxicity and reversion to toxicity, additional toxicology studies may need to be undertaken. The nonclinical toxicology studies should be such that reasonable assurance is obtained that it is safe to proceed to clinical evaluation. The potential toxic effects of the purified, inactivated toxoid should be evaluated in at least one animal species; this assessment should include the histopathology of important organs. The study should investigate the potential for local inflammatory reactions, systemic toxicity and effects on the immune system. The animal species used should be sensitive to the biological effects of the vaccine and to the toxin. Where feasible, the highest dose to be used in the proposed clinical trial should be evaluated in an animal model. Reproductive toxicity studies may be considered, particularly for novel tetanus antigens, since tetanus vaccine is likely to be used in women of childbearing potential as well as in pregnant women.

Information on endotoxin content may be obtained during validation of the production process as part of the nonclinical evaluation. A bioburden test may be used to ensure that potential levels of contamination have been minimized during steps that are not performed aseptically.

B.2.2 Characterization

The purity of the inactivated toxin should be established by determining the Lf concentration in relation to the concentration of total protein (nondialysable) nitrogen. A range of protein purity-indicating methods – such as HPLC, SDS-PAGE and Western blotting – are useful in providing full characterization and for evaluating the integrity of tetanus toxoid vaccine antigen prior to further formulation with adjuvant.

B.2.3 Immunogenicity and/or potency

An assessment of the ability of purified tetanus toxoid or antigen to induce functional antibody responses may be performed as part of preclinical studies, but most studies will be performed on the adsorbed bulk vaccine, which should be evaluated as described in section B.3.1.

B.2.4 Stability

Stability studies should be based on the Guidelines on stability evaluation of vaccines (59). The stability of all intermediates not used within a short

period of time should be evaluated and demonstrated using suitable methods. Manufacturers are encouraged to assign a shelf-life to all materials during the vaccine-production process, and in particular to key intermediates, such as single harvests and bulk purified toxoid. The choice of stability-indicating parameters and the frequency of testing should be justified to and approved by the NRA. Storage periods proposed for intermediates produced during the manufacturing process should be based on data obtained from the stability studies.

B.2.5 Adjuvants

Where appropriate, the adjuvant should be characterized in terms of chemical composition, physical form, adsorption capacity, purity, endotoxin content and sterility. The interaction between the adjuvant and antigen should also be evaluated; this evaluation should include measurement of the degree of adsorption. This should be shown to be consistent from lot to lot, and quality specifications should be established once a sufficient number of lots have been produced. The stability of the adjuvant alone, as well as in combination with the antigen, should be established during development, and should be shown to remain stable throughout the intended storage period.

R3 Nonclinical characterization of formulated vaccine

Lots of the final formulated vaccine used in nonclinical studies should be adequately representative of those intended for clinical investigation, and, ideally, should be the same lots as those intended for clinical use. Manufacturers should make every effort to keep some of this characterized material for future reference. As a minimum, the candidate vaccine should be prepared under conditions of good manufacturing practice (GMP) for clinical trial material (66), and full implementation of the principles of GMP will be required during the later stages of clinical development (49, 50).

The final formulated vaccine should be evaluated using a combination of immunological and physicochemical approaches to determine key product characteristics including sterility, pH, antigen content, degree of adsorption, potency/immunogenicity, and safety, as described in Part A of this document. Particular attention should be paid to the assessment of safety, toxicology, potency and stability. In some cases, comparability testing should be performed (e.g. after a significant change in the manufacturing process or at the time of scale-up following licensure). The requirement for and extent of comparative studies, and the choice of the comparator vaccine, should be approved by the NRA.

B.3.1 Immunogenicity and/or potency

Immunogenicity and/or potency studies should be performed in appropriate animal models, and may include more than one animal model. These studies

should include a potency assay consisting of multiple dilutions (consisting of at least three dilutions of each test vaccine and the suitable reference preparation), which should be performed using guinea-pigs or mice, followed by challenge with tetanus toxin or by titration of immune serum samples to determine functional (i.e. toxin neutralizing) antibody responses. The potency of the vaccine should be determined and should meet the requirements of Part A, section A.3.5.2.6. More details on the methods used for potency testing of tetanus vaccines can be found in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (45).

Multiple-dilution potency assays should also be used to demonstrate consistency in the production of the vaccine, and for stability testing for the purpose of establishing shelf-life.

B.3.2 Safety and toxicology

Aside from the specific toxicity test and innocuity test described in Part A, additional toxicology studies to address reproductive toxicity may be needed in certain cases (e.g. if a new production process or new tetanus antigen is introduced) since tetanus vaccine will probably be used in women of childbearing potential as well as in pregnant women (to prevent neonatal tetanus). Toxicological studies should be undertaken in accordance with the WHO guidelines on nonclinical evaluation of vaccines (64). Nonclinical toxicology studies should be such that reasonable assurance is obtained that it is safe to proceed to clinical evaluation. The potential toxic effects of the final formulated vaccine should be evaluated in at least one animal species; this evaluation should include histopathology of important organs. The study should investigate the potential for local inflammatory reactions, systemic toxicity and effects on the immune system. The animal species used should be sensitive to the biological effects of the vaccine and to the toxin. Where feasible, the highest dose to be used in the proposed clinical trial should be evaluated in an animal model. Further information on considerations related to dose, controls and parameters to be monitored can be found in the WHO guidelines on nonclinical evaluation of vaccines (64).

B.3.3 **Stability**

Stability studies should be based on WHO Guidelines on stability evaluation of vaccines (59), and should be seen as a continual process occurring from the development of the vaccine through licensing and on to post-licensure monitoring. Stability studies should be performed as real-time studies under conditions intended for storing the final product. During the preclinical phase, the amount of real-time stability data may be limited, but sufficient data should be generated to support the stability of the vaccine for the intended duration of the trial. Tests must include those for potency and specific toxicity, and should

also include physical and chemical characterization. Immunogenicity assessment (including measurement of functional antibody responses) and antigenicity assessment (as part of evaluating the degree of adsorption of the antigen) may provide valuable information in support of potency testing. Accelerated stability studies of products stored for limited periods at temperatures that may affect stability could support preliminary data from continuing real-time stability studies but should not replace them. Final containers from at least three lots of vaccine derived from different bulks should be tested on their expiry date to demonstrate stability during storage.

Any modification to the duration of shelf-life requires additional stability data to support the proposed modification, and should be approved by the NRA. Following licensure, stability should be monitored throughout the proposed shelf-life.

Part C. Clinical evaluation of tetanus vaccines

Introduction

This section addresses issues that are relevant during the clinical development of tetanus vaccines. Progression through the phases of clinical development should follow the principles outlined in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (67). Only those vaccines with an adequate nonclinical evaluation, as described in Part B, should be considered for clinical evaluation, with the NRA taking responsibility for evaluating the adequacy of the nonclinical information.

Clinical evaluation is required for any new tetanus vaccines, and may also be required for existing vaccines if a significant change to the manufacturing process has been proposed. The content and extent of the clinical programme will vary according to each possible scenario. It is strongly recommended that early dialogue is established between the vaccine manufacturer and the NRA in order to clarify requirements for carrying out clinical trials as well as for marketing approval.

General considerations for clinical studies C11

All clinical trials on pharmaceutical products should adhere to the standards of good clinical practice set out by WHO (68). However, tetanus vaccines are special in some respects and so demand careful consideration during clinical evaluation because the vaccines are given to healthy people, mostly in the paediatric population, to prevent rather than cure disease, and this limits the tolerance to adverse events. Tetanus vaccine for booster immunization is also given to adults, including pregnant women.

It is expected that at least some of the clinical studies, including those in the primary target population, will be conducted with different lots of vaccine manufactured using the same process as the vaccine intended for marketing. Consistency in the manufacturing of the vaccine lots used in clinical trials should be demonstrated and well documented. Although a formal clinical trial to evaluate lot consistency may not always be needed, in some instances clinical data may be required to provide evidence to support manufacturing consistency (e.g. if there is a particular concern about the consistency of the product). Vaccine lots used in clinical studies should ideally be the same lots that have been evaluated in nonclinical studies, and should be adequately representative of the formulation intended for marketing. Where this is not feasible, the lots used in clinical studies should be comparable to those lots used in the nonclinical studies in terms of their manufacturing process, immunogenicity and potency, safety, stability and other relevant characteristics of quality. The number of different vaccine lots evaluated as part of the clinical studies should be approved by the NRA but should be more than one. It is important to note that clinical data used to provide evidence of production consistency do not replace the need to demonstrate consistency in the manufacturing process during nonclinical evaluations.

C.1.2 Scope of the studies

The size and design of the studies and the selection of end-points for evaluation require justification, and should be such that they provide reasonable assurance of the clinical benefit and safety of the candidate vaccine. Because of the widespread use of tetanus toxoid and the low incidence of disease, it is unlikely that clinical end-point efficacy trials would be feasible for tetanus vaccines. In the case of tetanus, for which there are generally accepted serological correlates of protection, studies are based on the evaluation of antitetanus antibody responses in addition to the evaluation of safety. The primary immunogenicity end-point(s) should be the most relevant for the target population, and will differ for the evaluation of priming and booster doses of tetanus vaccine. Generally, studies of new tetanus vaccines are expected to make comparisons with licensed vaccines, and to include a noninferiority evaluation of the immune response to the investigational vaccine compared with the control vaccine, as well as an evaluation of safety.

Studies should evaluate the safety and immunogenicity of the investigational vaccine when co-administered with other routinely recommended vaccines given on the same schedule to the target population. Some studies should include an evaluation of the immune response to concomitantly administered vaccines in order to ensure that the investigational vaccine does not interfere with responses to concomitantly administered vaccines. Of particular interest in the evaluation of tetanus vaccines are any effects on the safety and immunogenicity found during co-administration with conjugate vaccines containing tetanus toxoid as a carrier protein.

Clinical studies may be needed when substantial manufacturing changes are made to an existing, licensed vaccine. Any change in the formulation of a vaccine should be considered carefully, both by manufacturers and the NRA.

C.1.3 Comparator vaccine

Immunological correlates of protection are well established for tetanus vaccines (see section C.2.2), and it should be acknowledged that comparison studies do not bridge to efficacy but to serological correlates. In some cases, it may be decided to perform immunogenicity analyses that are not comparative, although in most cases comparative studies are recommended. The inclusion of a comparator aids interpretation of the results of the trial, particularly if the expected seroprotection rate in the investigational group is not observed (e.g. if the study is conducted in a population where high levels of maternal tetanus antibody suppress in infants the response to immunization). Decisions about whether to include a comparator vaccine, and the selection of a comparator, should be justified by the manufacturer, and approved by the NRA. In studies performed to support major changes to the manufacturing process for a licensed vaccine (including a change in formulation or antigen dose), the candidate vaccine should be compared with the existing product (i.e. one manufactured according to the licensed production process). In this case, a comparative study is particularly useful for directly evaluating the effect of the change in the process or formulation. In studies of a new vaccine, the comparator is typically a licensed vaccine.

C.2 Assessment of immunogenicity in humans

C.2.1 Assays to assess antibody responses

Assays to measure the antibody response to tetanus vaccine can be divided into functional assays and nonfunctional binding assays (which demonstrate the capacity of tetanus antibody in a serum sample to bind directly to, or compete with, tetanus toxin or toxoid). Fully functional assays include the in vivo TNT performed in guinea-pigs or mice. The in vivo assay is a moderately severe procedure involving the injection of toxin/antitoxin mixtures into animals by the subcutaneous route. The procedure requires specialized facilities, is expensive and requires relatively large volumes of serum. As a result, it is not typically used in vaccine clinical trials.

Validated in vitro serological assays are preferred and are more suited to screening large numbers of serum samples because they are faster, use lower sample volumes, are easy to use and can be automated. There is no suitably validated in vitro assay with the capacity to detect functional antibodies to tetanus and that can be used as a TNT. However, several in vitro serological assays have been developed and validated against the in vivo TNT and show excellent correlation, particularly when the level of antibody is high (54). Examples of

these include ELISA (69), the ToBI test (70) and the particle agglutination test (61, 62). Other methods include double-antigen ELISAs or dual double-antigen time-resolved fluorescence immunoassays, which are also used for assessing antibody responses to tetanus and at least one other antigen (71, 72) delivered at the same time. The passive haemagglutination assay has also been used by some laboratories, but it is considered to be more variable and shows a poorer correlation with the in vivo TNT (1, 54).

The method chosen for antibody assessment should be validated for the intended purpose, using relevant samples, and should be approved by the NRA. Where feasible, an assay that measures functional antibody responses may need to be used at some stages during the clinical evaluation (e.g. to analyse a subset of samples from the clinical trial).

C.2.2 Criteria for evaluation of immune responses

Clinical protection against tetanus correlates well with the presence and level of circulating tetanus antitoxin. The end-points and criteria used for the evaluation of tetanus antibody response require a justification that takes into account the assay used to measure antibody levels and the intended use of the vaccine (i.e. for primary or booster immunization). It is generally accepted that, when measured by TNT, an antitetanus antibody level of 0.01 IU/ml is the minimum protective level. Thus, subjects with antitetanus antibody levels greater than 0.01 IU/ml (as measured by TNT) are considered to be protected (1) and those with antitetanus antibody levels below 0.01 IU/ml are considered susceptible to disease. When a validated ELISA is used, the minimum level of antibody needed to provide protection against tetanus is usually considered to be 0.1 IU/ml. When other in vitro assays are used, these criteria for evaluation of immune responses to tetanus vaccination may not apply because they are specific to each assay. In some settings (e.g. in booster immunization studies), a high proportion of subjects may have a level of tetanus antibody that is higher than the minimum protective level prior to vaccination with the investigational vaccine. In such cases, the minimum protective level of antibody would not be a meaningful end-point for evaluation of the immune response to the investigational vaccine.

Information from phase II studies in the target population may help guide the determination of appropriate primary end-points for phase III studies. The end-points and evaluation criteria proposed by the manufacturer should be approved by the NRA.

C.2.2.1 **Primary immunization of infants**

The proportion of subjects with a tetanus antibody level above the minimum protective level, or above a prespecified threshold, should be determined approximately one month after the last primary dose. For primary immunization,

a typical end-point uses a validated ELISA to assess the proportion of subjects with an antitetanus antibody level of 0.1 IU/ml or higher post-vaccination.

Where noninferiority of the investigational vaccine relative to a comparator vaccine is evaluated, noninferiority is shown if the upper limit of the 95% confidence interval for the difference in the seroprotection rates (i.e. the comparator vaccine minus the investigational vaccine) is less than a prespecified margin (usually 10%, although a margin of 5% may be recommended if the expected rates of seroprotection are very high, as may be the case for tetanus). The specified threshold antibody concentration, noninferiority margin and bleeding times should be approved by the NRA.

If the studies are performed in the absence of a comparator vaccine, an acceptance criterion should be used for the proportion of subjects – usually 90% or 95% – that reach the prespecified threshold antibody level (e.g. 0.1 IU/ml or higher using a validated ELISA), measured approximately one month after the last primary dose. The acceptance criterion should be based on the 95% confidence interval for the proportion of subjects achieving the prespecified antibody level (e.g. the lower limit of the 95% confidence interval for the observed proportion should be greater than 90% or 95%). The NRA should approve the specified antibody threshold, acceptance criterion and bleeding time.

In addition to the level of tetanus antibody specified as the primary end-point, it is recommended that the geometric mean titre (GMT) of antitetanus antibody should be evaluated as a secondary end-point. In comparative studies, the GMT ratio of the investigational vaccine to the comparator vaccine may be evaluated using a predetermined margin of noninferiority (e.g. the lower limit of the 95% confidence interval of the observed ratio of the investigational vaccine to the comparator vaccine should be greater than 0.67). The presentation of reverse cumulative distribution curves, which show the accumulated proportion of subjects with an antibody concentration greater than or equal to a given level, may also provide useful information for the purposes of comparison.

C.2.2.2 Primary immunization of adolescents or adults

In some countries it may be desirable to evaluate a tetanus vaccine to be used for primary immunization in adults and adolescents, including women of childbearing age, who have no prior history of vaccination against tetanus. Pre-enrolment screening criteria to identify previously unvaccinated (naive) subjects may include a tetanus antibody level of less than 0.1 IU/ml when measured by validated ELISA (or less than 0.01 IU/ml if using TNT) prior to and seven days after receipt of the first dose of tetanus vaccine. In some cases, it may be difficult to identify sufficient numbers of naive individuals, and the choice of study design (i.e. comparative or noncomparative) may therefore be dependent on the number of naive subjects that can be identified in the target

population. If sufficient numbers of naive subjects in older age groups cannot be identified, consideration may be given to extrapolating the effectiveness of primary immunization from infants to older age groups.

The end-points and noninferiority or acceptance criteria for evaluation of the primary immune response to a tetanus vaccine in adolescents or adults are the same as those recommended for the primary immunization of infants (section C.2.2.1).

C.2.2.3 Booster immunization of children, adolescents and adults

For the evaluation of tetanus vaccines intended for booster immunization, the age of the subjects (e.g. preschool age, school age, adults, elderly) and the interval since the last dose of tetanus vaccine should be considered when designing the clinical trial, since these factors may influence the baseline level of antibody and may have a significant impact on the response to a booster dose. In designing tetanus-booster immunization studies, appropriate enrolment criteria should be developed regarding factors that may affect immunogenicity outcomes (e.g. the time since previous dose). Enrolment procedures should also be designed to ensure adequate representation across the age range for which the vaccine is intended.

Criteria for evaluation should reflect the prevaccination immunity level in the study population. Specifically, if a substantial proportion of subjects is expected to have tetanus antibody levels above the minimum protective level prior to booster immunization, other primary end-points (e.g. the booster response rate) should be used. Information from phase II studies in the target population may help in determining appropriate primary end-points. The assessment of antibody levels both prior to and after vaccination is recommended in order to facilitate the interpretation of the data.

Typically, for tetanus-booster immunization studies, the proportion of subjects with a booster response is considered to be the primary end-point. If booster response is used as a primary end-point, the definition of booster response (e.g. a four-fold rise in antibody concentration from prevaccination to post-vaccination) should be prespecified and should include a provision that subjects attain at least four times the protective level – e.g. in subjects with a prevaccination antibody concentration of < 0.1 IU/ml (by validated ELISA), a post-vaccination concentration of \geq 0.4 IU/ml would be acceptable or in subjects with a prevaccination concentration \geq 0.1 IU/ml, an increase of at least four times the prevaccination concentration would be acceptable. Alternative definitions for booster response (e.g. a two-fold rise for subjects with prespecified high levels of antibody prior to vaccination) may be considered if well justified. As for the comparative evaluation of seroprotection rates, booster response rates should be

compared between groups using an appropriate predefined noninferiority limit – e.g. the upper limit of the two-sided 95% confidence interval of the observed difference (i.e. the comparator vaccine minus investigational vaccine) should be less than a prespecified margin (usually 10%). In noncomparative studies, the acceptability criterion for the proportion of subjects who achieve a booster response (e.g. 80%) should be based on the 95% confidence interval of the observed proportion.

In booster immunization studies, secondary end-points may include antitetanus antibody GMTs and the proportion of subjects with antibody levels greater than or equal to the minimum protective level.

The choice of end-points and criteria for evaluation (including the need for a comparator vaccine) should be justified by the manufacturer, and approved by the NRA.

C.2.3 Antibody persistence

Where possible, it is recommended that subsets of subjects should be identified for longer-term follow-up of the persistence of immunity in order to determine the need for, and appropriate timing of, booster doses. Population surveillance studies conducted to determine the prevalence of tetanus antibody in different age groups can help guide recommendations on the need for booster doses.

C.3 Safety evaluation

The assessment of safety should follow the general principles outlined in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (67). The size of the prelicensure safety database required for a tetanus-toxoid-containing vaccine will depend on many factors including other vaccine components, the age group for which use is being sought, and manufacturing methods (e.g. the use of novel components versus common components). For vaccines in which efficacy is inferred from immunogenicity data without larger clinical end-point efficacy trials, the number of subjects that need to be evaluated for safety may exceed the number of subjects required for the end-points evaluating immunogenicity. The size of the prelicensure safety database should be justified by the manufacturer, and approved by the NRA.

The frequency and severity of adverse reactions following receipt of tetanus vaccine may vary by vaccine formulation (e.g. according to the amount of tetanus toxoid), subjects' characteristics (e.g. prior vaccination history, time since previous dose, age, prevaccination level of tetanus antibody) and use with concomitantly administered vaccines. Theoretically, there is the potential for increased reactogenicity when tetanus toxoid vaccines are administered at the same time as (or shortly after) polysaccharide conjugate vaccines containing

tetanus toxoid as the carrier protein. Higher rates of some local adverse reactions have been observed following booster immunization with tetanus vaccine when compared with primary immunization. In designing tetanus-booster immunization studies, appropriate enrolment criteria should be developed regarding factors that may affect safety outcomes (e.g. the time since previous dose). Enrolment procedures should also be designed to ensure adequate representation across the age range for which the vaccine is intended.

Safety data should be collected throughout the duration of clinical development. Prelicensure clinical safety assessment will generally include safety data from comparisons of the investigational vaccine with the licensed control vaccine(s). Subjects should be carefully monitored for commonly occurring adverse events as well as less common, serious adverse events. Commonly occurring adverse reactions expected after tetanus immunization include pain, redness and swelling at the injection site. Post-vaccination fever may also occur. Although serious adverse events should be monitored during prelicensure clinical trials, serious adverse events that have been associated with tetanus toxoid (e.g. arthus reactions, Guillain–Barré syndrome) occur too infrequently to be reliably evaluated in most clinical trials, and post-marketing surveillance must also be performed to monitor serious adverse events.

c.4 Post-marketing studies and surveillance

Monitoring the efficacy, safety and quality of licensed vaccines consists of post-marketing surveillance and post-marketing studies (phase IV studies). The purpose of post-licensure monitoring is to assess the performance of a vaccine in the target population under conditions of routine use, and to monitor rare adverse events. Post-marketing studies may also be useful for assessing antibody persistence and the need for booster doses. Marketing authorization holders should be committed to presenting a post-marketing surveillance programme at the time of licensure. The programme should be based on criteria for assessing the quality, safety and effectiveness of a particular vaccine to gain marketing approval. In many cases, comprehensive post-marketing safety and effectiveness data cannot be collected by manufacturers alone, and close cooperation between manufacturers and public-health authorities is required.

Post-marketing surveillance may be the only means of detecting rare adverse events that occur too infrequently to have been detected during clinical trials. For the collection of safety data, surveillance may be conducted by active or passive processes. Voluntary reporting of serious adverse events (passive surveillance) is most commonly used. All data collected should be submitted to the NRA at regular intervals so that action can be taken if there are implications for the marketing authorization.

Part D. Recommendations for NRAs

D.1 General

The general recommendations for NRAs and national control laboratories (NCLs) given in Guidelines for national authorities on quality assurance for biological products (73) and Guidelines for independent lot release of vaccines by regulatory authorities (46) apply.

The details of production and quality control procedures, as well as any significant changes in them that may affect the quality, safety and efficacy of tetanus vaccines, should be discussed with and approved by the NRA. For control purposes, the international standards currently in use (see the section on General considerations) should be obtained for the purpose of calibrating national, regional and working standards (55). The NRA may obtain the product-specific or working references from the manufacturer to be used for lot release until an international or national standard preparation has been established.

Consistency in production has been recognized as an essential component in the quality assurance of tetanus vaccines. In particular, NRAs should carefully monitor production records and the results of quality-control tests on clinical lots as well as results from tests on a series of consecutive lots of the vaccine.

D.2 Release and certification by the NRA

A vaccine should be released only if it fulfils all national requirements or satisfies Part A of these Recommendations, or both (46).

A protocol based on the model given in Appendix 1, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for the release of a vaccine for use.

A statement signed by the appropriate official of the NRA should be provided to the manufacturing establishment, and should certify that the lot of vaccine in question meets all national requirements as well as Part A of the present Recommendations. The certificate should provide sufficient information about the vaccine lot. A model certificate is given in Appendix 2. The official national release certificate should be provided to importers of the vaccine. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

Authors and acknowledgements

The first and second drafts of these Recommendations were prepared by Dr T. Sesardic, National Institute for Biological Standards and Control, England, with contributions from other members of the drafting group: Dr M. Corbel, Consultant, United Kingdom; Dr R. Dobbelaer, Consultant, Belgium; Dr K. Farizo, United States Food and Drug Administration Center for Biologics

Evaluation and Research, USA; Dr M. Iwaki, National Institute of Infectious Diseases, Japan; Mrs T. Jivapaisarnpong, Institute of Biological Products, Ministry of Public Health, Thailand; Dr H. Lechner, Paul-Ehrlich-Institut, Germany; Dr D. Lei, World Health Organization, Switzerland; Dr F. Mawas, National Institute for Biological Standards and Control, England; Dr B. Meade, Meade Biologics, USA; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Dr S. Prieur, Agence Française de Sécurité Sanitaire des Produits de Santé, France; Dr M.P. Schmitt, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr P. Stickings, National Institute for Biological Standards and Control, England; Dr M. Takahashi, National Institute of Infectious Diseases, Japan; Dr D. Xing, National Institute for Biological Standards and Control, England; Dr S. Zhang, State Food and Drug Administration, China.

The third and fourth drafts were prepared by Dr T. Sesardic and Dr D. Lei (with clinical input from Dr K. Farizo) after a consultation held on 7-11 November 2011 in Beijing, China attended by: Dr M. Baca-Estrada, Health Canada, Canada; Ms H. Bai, State Food and Drug Administration, China; Mr M. Contorni, Novartis Vaccines and Diagnostics, Italy; Dr P. Desmons, GlaxoSmithKline Biologicals, China; Dr R. Dobbelaer, Consultant, Belgium; Dr R. Dominguez Morales, World Health Organization, Switzerland; Mrs L. Du, Beijing Luzhu Biopharmaceutical Company, China; Dr S. Gairola, Serum Institute of India, India; Mr S. Goel, Central Drugs Laboratory, India; Dr C. Hernandez, World Health Organization, Switzerland; Mrs Q. Hou, National Institutes for Food and Drug Control, China; Dr K. Farizo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr K. Friedrich, Instituto Nacional de Controle de Qualidade em Saude, Brazil; Mrs H. Han, Crucell Korea, Republic of Korea; Dr L. Hiep, Institute of Vaccines and Medical Biologicals, Viet Nam; Dr X. Hong, Chinese Pharmacopoeia Commission, China; Mr A. Horita, Chemo-Sero-Therapeutic Research Institute, Japan; Dr M. Iwaki, National Institute of Infectious Diseases, Japan; Mr Q. Jiang, Changchun Chang Sheng Life Sciences, China; Dr B. Kim, Korea Food and Drug Administration, Republic of Korea; Dr J. Kim, Korea Food and Drug Administration, Republic of Korea; Dr I. Knezevic, World Health Organization, Switzerland; Dr D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Mrs F. Lan, Chengdu Institute of Biological Products, China; Dr H. Langar, Regional Office for the Eastern Mediterranean, World Health Organization, Egypt; Dr H. Lechner, Paul-Ehrlich-Institut, Germany; Dr D. Lei, World Health Organization, Switzerland; Dr F. Li, National Institutes for Food and Drug Control, China; Mr J. Liu, Beijing Minhai Biotechnology Company, China; Dr J. Luo, Center for Drug Evaluation, China; Dr K. Markey, National Institute for Biological Standards and Control, England; Dr F. Mawas-Kossaibati, National Institute for Biological Standards and Control, England; Dr B. Meade, Meade Biologics, USA; Mr P.V.V.S Murthy, Biological E. Limited, India; Dr S. Pakzad, Food and Drug Control Laboratory, Islamic Republic of Iran; Dr D. Pfeifer, Regional Office for Europe, World Health Organization, Denmark; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Mr M. Qin, Chengdu Institute of Biological Products, China; Mr M. Rahman, Panacea Biotec, India; Mrs J. Shi, Yuxi Walvax Biotechnological Company, China; Dr J. Shu, Sanofi Pasteur, China; Dr P. Stickings, National Institute for Biological Standards and Control, England; Mrs C. Tan, Chengdu Institute of Biological Products, China; Dr J. Tresnabudi, BioFarma, Indonesia; Dr J. Wang, National Institutes of Food and Drug Control, China; Mrs X. Wang, Chinese Pharmacopoeia Commission, China; Mr H. Wei, Changchun Institute of Biological Products, China; Mrs Z. Xiao, Beijing Tiantan Biological Products Company, China; Dr D. Xing, National Institute for Biological Standards and Control, England; Dr M. Xu, National Institutes for Food and Drug Control, China; Mr B. Yang, Wuhan Institute of Biological Products, China; Mr M. Yang, Lanzhou Institute of Biological Products, China; Mr. H. Yin, Center for Drug Evaluation, China; and Dr S. Zhang, State Food and Drug Administration, China.

The fifth draft was prepared by Dr T. Sesardic and Dr D. Lei on the basis of comments received from national regulators, the vaccine industry and the general public during a period of public consultation on the WHO web site.

References

- The immunological basis for immunization series. Module 3: Tetanus update 2006. Geneva, World Health Organization, 2007 (http://whqlibdoc.who.int/publications/2007/9789241595551_eng. pdf, accessed 24 June 2013).
- Recommendations for the production and control of Haemophilus influenzae type b conjugate vaccines. In: WHO Expert Committee on Biological Standardization. Forty-ninth report. Geneva, World Health Organization, 2000 (WHO Technical Report Series, No. 897), Annex 1.
- 3. Roper MH, Vandelaer JH, Gasse FL. Maternal and neonatal tetanus. *Lancet*, 2007, 370:1947–1959.
- Black RE et al. Global, regional, and national causes of child mortality in 2009: a systematic analysis. *Lancet*, 2010, 375:1969–1987.
- Ganatra HA, Zaidi AKM. Neonatal infections in the developing world 2010. Seminars in Perinatology, 2010, 34:416–425.
- Howard CR, Beale AJ, Aguado MT. The development of controlled release vaccines the role of the World Health Organization. In: Gander B, Merkle HP, Corradin G, eds. Antigen delivery systems: immunological and technological issues. Newark, NJ, Harwood Academic Publishers, 1997:1–15.
- 7. O'Hagan DT, Singh M, Gupta RK. Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines. *Advanced Drug Delivery Reviews*, 1998, 32:225–246.
- 8. Tierney R et al. Transcutaneous immunization with tetanus toxoid and mutants of *Escherichia coli* heat-labile enterotoxin as adjuvants elicits strong protective antibody responses. *Journal of Infectious Diseases*, 2003, 188:753–758.

- 9. Qazi O et al. Reduction of the ganglioside binding activity of the tetanus toxin HC fragment destroys immunogenicity: implications for development of novel tetanus vaccines. *Infection and Immunity*, 2006, 4:4884–4891.
- Johnston L et al. Transcutaneous delivery of tetanus toxin Hc fragment induces superior tetanus toxin neutralizing antibody response compared to tetanus toxoid. *Human Vaccines*, 2009, 5:230–236.
- 11. Tierney R et al. A single-dose cytomegalovirus-based vaccine encoding tetanus toxin fragment C induces sustained levels of protective tetanus toxin antibodies in mice. *Vaccine*, 2012, 30:3047–3052.
- 12. Requirements for diphtheria toxoid and tetanus toxoid (Requirements for biological substances No. 10). In: *WHO Expert Committee on Biological Standardization. Seventeenth report*. Geneva, World Health Organization, 1964 (WHO Technical Report Series, No. 293), Annex 1.
- Requirements for diphtheria toxoid, pertussis vaccines, tetanus toxoid, and combined vaccines.
 In: WHO Expert Committee on Biological Standardization. Thirtieth report. Geneva, World Health Organization, 1979 (WHO Technical Report Series, No. 638), Annex 1.
- 14. Requirements for diphtheria toxoid, pertussis vaccine, tetanus toxoid, and combined vaccines (Addendum 1986). In: WHO Expert Committee on Biological Standardization. Thirty-seventh report. Geneva, World Health Organization, 1987 (WHO Technical Report Series, No. 760), Annex 4.
- 15. Requirements for diphtheria, tetanus, pertussis and combined vaccines (revised 1989). In: WHO Expert Committee on Biological Standardization. Fortieth report. Geneva, World Health Organization, 1990 (WHO Technical Report Series, No. 800), Annex 2.
- 16. Recommendations for diphtheria, tetanus, pertussis and combined vaccines (Amendments 2003). In: *WHO Expert Committee on Biological Standardization. Fifty-fourth report*. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 5.
- 17. Sokhey S. Therapeutic substances in their international aspects. BMJ, 1950, 1:1037–1044.
- 18. WHO Expert Committee on Biological Standardization. Twenty-second report. Geneva, World Health Organization, 1970 (WHO Technical Report Series, No. 444).
- 19. WHO Expert Committee on Biological Standardization. Fifth report. Geneva, World Health Organization, 1952 (WHO Technical Report Series, No. 56).
- 20. WHO Expert Committee on Biological Standardization. Twelfth report. Geneva, World Health Organization, 1959 (WHO Technical Report Series, No. 172).
- 21. WHO Expert Committee on Biological Standardization. Eighteenth report. Geneva, World Health Organization, 1966 (WHO Technical Report Series, No. 329).
- 22. WHO Expert Committee on Biological Standardization. Nineteenth report. Geneva, World Health Organization, 1967 (WHO Technical Report Series, No. 361).
- 23. WHO Expert Committee on Biological Standardization. Thirty-second report. Geneva, World Health Organization, 1982 (WHO Technical Report Series, No. 673).
- 24. WHO Expert Committee on Biological Standardization. Fifty-first report. Geneva, World Health Organization, 2002 (WHO Technical Report Series, No. 910).
- 25. Sesardic D et al. Calibration of replacement international standard and European Pharmacopoeia biological reference preparation for tetanus toxoid, adsorbed. *Biologicals*, 2002, 30:49–68.
- 26. Reference preparations established by the Expert Committee on Biological Standardization, 2010. Biological substances: International standards and reference reagents. Additions. Geneva, World Health Organization, 2010 (WHO/BS/10.2150) (http://www.who.int/entity/biologicals/Reference_preparations_established_by_ECBS.pdf, accessed 24 June 2013).

- 27. Tierney R et al. Collaborative study for the calibration of a replacement International Standard for Tetanus Toxoid Adsorbed. *Biologicals*, 2011, 39:404–416.
- 28. Murata R, Someya S, Kaneko J. Standardization of tetanus toxoid combined with pertussis vaccine. *Japanese Journal of Medical Science and Biology*, 1970, 23:211–216.
- 29. Redhead K et al. Interaction of *Haemophilus influenzae* type b conjugate vaccines with diphtheria-tetanus-pertussis vaccine in control tests. *Vaccine*, 1994, 12:1460–1466.
- 30. Assay of tetanus vaccine (adsorbed). In: *European Pharmacopoeia*, 7th ed. Strasbourg, European Directorate for Quality of Medicines & HealthCare, 2011 (01/2011:20708).
- 31. *Minimum requirements: tetanus toxoid*. Fourth Revision. Bethesda, MD, National Institutes of Health, 1952.
- 32. Brown JH et al. Precision of potency assay of alum-precipitated tetanus toxoid in mice: an interinstitutional study. *Journal of Immunology*, 1953, 70:171–180.
- 33. Lyng J, Nyerges G. The second international standard for tetanus toxoid (adsorbed). *Journal of Biological Standardization*, 1984, 12:121–130.
- 34. Van Ramshorst JD, Sundaresan TK, Outschoorn AS. International collaborative studies on potency assays of diphtheria and tetanus toxoids. *Bulletin of the World Health Organization*, 1972, 46:263–276.
- 35. Mussett MV, Sheffield F. A collaborative investigation of methods proposed for the potency assay of adsorbed diphtheria and tetanus toxoids in the European Pharmacopoeia. *Journal of Biological Standardization*, 1973, 1:259–283.
- 36. Tierney R et al. Calibration of the Ph. Eur. Biological Reference Preparation (BRP) for tetanus vaccine (adsorbed) batch 3. *Pharmeuropa Bio and Scientific Notes*, 2011, 2011(1):1–26.
- 37. Kondo S, Kameyama S, Murata R. Standardization and control of toxoid components in the combined vaccine (DPT). *Japanese Journal of Medical Science and Biology*, 1974, 27:315–319.
- 38. Ikic D. Immunity in humans and guinea-pigs vaccinated with different doses of tetanus vaccine. *Proceedings of the International Symposium on Microbiological Standardization*, Opatija, 1960:415–422.
- 39. Ipsen J Jr. Bio-assay of four tetanus toxoids (aluminium precipitated) in mice, guinea-pigs and humans. *Journal of Immunology*, 1953, 70:426–434.
- 40. Hardegree MC, Pittman M, Maloney CJ. Influence of mouse strain on the assayed potency (unitage) of tetanus toxoid. *Applied Microbiology*, 1972, 24:120–126.
- 41. Cohen H, Van Ramshorst JD, Tasman A. Consistency in potency assay of tetanus toxoid in mice. *Bulletin of the World Health Organization*, 1959, 20:1133–1150.
- 42. Hardegree MC. Overview of tetanus toxoid: history and current status. In: *Tetanus vaccines for human use*. Pharmeuropa, October 2000: 5–28. (http://pharmeuropa.edqm.eu/PharmeuropaArchives/, accessed 9 September 2013).
- 43. Gupta RK et al. Evaluation of a guinea-pig model to assess interference in the immunogenicity of different components of a combination vaccine comprising diphtheria, tetanus and acellular pertussis (DTaP) vaccine and *Haemophilus influenzae* type b capsular polysaccharide conjugate vaccine. *Biologicals*, 1999, 27:167–176.
- 44. Stickings P et al. Animal refinement and reduction: alternative approaches for potency testing of diphtheria and tetanus vaccines. *Procedia in Vaccinology*, 2011, 5:200–212.
- 45. Manual for quality control of diphtheria, tetanus and pertussis vaccines. Geneva, World Health Organization, 2013 (WHO/IVB/11.11).

- 46. Guidelines for independent lot release of vaccines by regulatory authorities. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 2.
- 47. Coombes L et al. Development and use of a novel in vitro assay for testing of diphtheria toxoid in combination vaccines. *Journal of Immunological Methods*, 2009, 350:142–149.
- 48. Prieur S et al. Development of an in vitro potency test for tetanus vaccines: an immunoassay based on Hc fragment determination. *Developments in Biologicals*, 2002, 111:37–46.
- Good manufacturing practices: main principles for pharmaceutical products. In: WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 3.
- 50. Good manufacturing practices for biological products. In: WHO Expert Committee on Biological Standardization. Forty-second report. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 1.
- 51. WHO Expert Committee on Biological Standardization. Fifty-eighth report. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 963).
- 52. Preneta-Blanc R et al. Calibration of replacement International Standards of diphtheria and tetanus toxoids for use in flocculation test. *Biologicals*, 2008, 36:315–326.
- WHO Expert Committee on Biological Standardization. Forty-third report. Geneva, World Health Organization, 1994 (WHO Technical Report Series, No. 840).
- 54. Sesardic D et al. The First International Standard for Antitetanus Immunoglobulin, Human: pharmaceutical evaluation and international collaborative study. *Biologicals*, 1993, 21:67–75.
- 55. WHO manual for the establishment of national and other secondary standards for vaccines. Geneva, World Health Organization, 2011 (WHO/IVB/11.03).
- Manual for the production and control of vaccines: tetanus toxoid. Geneva, World Health Organization, 1977 (BLG/UNDP/77.2) (http://whqlibdoc.who.int/hq/pre-wholis/BLG_ UNDP_77.2.pdf, accessed 24 June 2013).
- 57. WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products. Geneva, World Health Organization, 2003 (WHO/BCT/QSD/03.01) (http://www.who.int/biologicals/publications/en/whotse2003.pdf, accessed 22 June 2013).
- 58. General requirements for the sterility of biological substances. (Requirements for Biological Substances No. 6, revised 1973, amendment 1995). In: WHO Expert Committee on Biological Standardization. Forty-sixth report. Geneva, World Health Organization, 1998 (WHO Technical Report Series, No. 872), Annex 3.
- 59. Guidelines on stability evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-seventh report.* Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 962), Annex 3.
- 60. Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-third report*. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 926), Annex 4.
- 61. Hendriksen C, Winsnes R. Serological methods for potency testing of tetanus toxoid vaccines for human use. *Developments in Biologicals*, 2002, 111:131–140.
- 62. Coplu N et al. Tetanus antibody assay combining in-house ELISA and particle agglutination test and its serosurvey application in a province in Turkey. *Japanese Journal of Infectious Diseases*, 2004, 57:97–102.

- 63. A WHO guide to good manufacturing practice (GMP) requirements. Part 2: Validation. Geneva, World Health Organization, 1997 (WHO/VSQ/97.02).
- 64. WHO guidelines on nonclinical evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-fourth report. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.
- 65. Handbook: good laboratory practice (GLP). Quality principles for regulated non-clinical research and development. 2nd ed. Geneva, World Health Organization, 2009.
- 66. Good manufacturing practice: supplementary guidelines for the manufacture of investigational pharmaceutical products for clinical trials in humans. In: WHO Expert Committee on Specifications for Pharmaceutical Preparations. Thirty-fourth report. Geneva, World Health Organization, 1996 (WHO Technical Report Series, No. 863), Annex 7.
- 67. Guidelines on clinical evaluation of vaccines: regulatory expectations. In: *WHO Expert Committee* on *Biological Standardization. Fifty-second report*. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 924), Annex 1.
- 68. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products. In: *The Use of Essential Drugs. Sixth report of the WHO Expert Committee.* Geneva, World Health Organization, 1995 (WHO Technical Report Series, No. 850), Annex 3.
- 69. Melville Smith ME, Seagroatt VA, Watkins JT. A comparison of enzyme-linked immunosorbent assay (ELISA) with the toxin neutralization test in mice as a method for the estimation of tetanus antitoxin in human sera. *Journal of Biological Standardization*, 1983, 11:137–144.
- 70. Hendriksen CFM et al. The toxin binding inhibition test as a reliable in vitro alternative to the toxin neutralization test in mice for the estimation of tetanus antitoxin in human sera. *Journal of Biological Standardization*, 1988, 16:287–297.
- Kristiansen M, Aggerbeck H, Heron I. Improved ELISA for determination of anti-diphtheria and/ or anti-tetanus antitoxin antibodies in sera. Acta Pathologica, Microbiologica et Immunologica Scandinavica, 1997, 105:843–853.
- 72. Reder S et al. Measuring immunoglobulin G antibodies to tetanus toxin, diphtheria toxin, and pertussis toxin with single-antigen enzyme-linked immunosorbent assays and a bead-based multiplex assay. *Clinical and Vaccine Immunology*, 2008, 15:744–749.
- 73. Guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 2.

Appendix 1

International name:

Model protocol for the manufacturing and control of tetanus vaccines (adsorbed)

The following protocol is intended for guidance. It indicates the information that should be provided as a minimum by a manufacturer to the NRA. Information and tests may be added or omitted as necessary, with the authorization of the NRA.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO Recommendations for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that will accompany the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot-release certificate from the NRA or from the NCL in the country where the vaccine was produced or released stating that the product meets national requirements as well as the recommendations in Part A of this annex.

1. Summary information on finished product (final lot)

Trade name/commercial name:					
roduct licence (marketing authorization) number:					
Country:					
Name and address of manufacturer:					
Name and address of licence holder,					
if different:					
Final packaging lot number:					
Type of container:					
Number of containers in this packaging:					
Final container lot number:					
Number of filled containers in this final lot:					
Date of manufacture:					
Description of final product (adsorbed):					
Preservative, and nominal concentration:					
Volume of each single human dose:					
Number of doses per final container:					

Summary of the composition (include a summary of the qualitative and quantitative composition of the vaccine in each human dose, including any adjuvant used and other excipients):
Shelf-life approved (months):
Expiry date:
Storage conditions:
The following sections are intended for recording the results of the tests performed during the production of the vaccine so that the complete document will provide evidence of consistency in production. If any test has to be repeated, this must be indicated. Any abnormal result must be recorded on a separate sheet.
2. Detailed information on manufacture and quality control
Starting materials
Identity of <i>C. tetani</i> strain used for
vaccine production:
Reference number of seed lot:
Date(s) of reconstitution (or opening) of seed lot ampoule(s):
Single harvests used for preparing the bulk purified toxoid
Name of the culture medium:
Date of inoculation:
Temperature of incubation:
Control of bacterial purity
Methods:
Result:
Date:
Date of harvest:
Volume of harvest:
Yield (Lf/ml):
Volume after filtration:
Toxin purification method:
Method of detoxification:
Date started:
Date finished:

Volume used for inactivation:
Temperature:
Concentration of detoxification agent:
Confirmation of detoxification (before or after purification)
Method:
Dose of inoculation (Lf):
Route of inoculation:
Date of inoculation:
Date of observation:
Result:
Purification methods used for toxoid:
Yield of purified toxoid
Volume:
Toxoid content (Lf):
Bulk purified toxoid
•
Reference number: Volume and Lf:
volume and Li:
Sterility test
Tests for bacteria and fungi
Method:
Media:
Number of bulks tested:
Volume of inoculum per bulk:
Volume of medium per bulk:
Temperature of incubation:
Date of start of test:
Date of start of test:
Result:
Antigenic purity (Lf/mg of protein (nondialysable) nitrogen):
Method:
Date of test:
Specific toxicity test
Method:
Dose of inoculation (Lf):
Route of inoculation:

Date of inoculation:					
Date of observation:					
Result:					
Test of reversion to toxicity Lf of test toxoid solution: Temperature of incubation Dates of beginning and en Method: Dose of inoculation (Lf): Route of inoculation: Date of inoculation:	n of toxoid: d of incubation:				
Final bulk					
Identification (lot number):					
Date of manufacture or blending					
Volume:					
Li.					
Blending:	Prescription (SHD)	Added			
Toxoid (Lf):					
Adjuvant:					
Preservative (specify):					
Others (salt):					
Final volume (ml):					
Preservative content					
Result:					
Result.					
Adjuvant content					
Method:					
Date of test:					
Result:					
Degree of adsorption					
Method: Date of test: Result:					

Sterility test
Tests for bacteria and fungi
Method:
Media:
Number of containers tested:
Volume of inoculum per container:
<u> </u>
Volume of medium per container:
Temperature of incubation:
Date of start of test:
Date of end of test:
Result:
Specific toxicity test (when required)
Method:
Dose of inoculation (Lf):
Route of inoculation:
Date of inoculation:
Date of observation:
Result:
Potency test
Challenge method (multiple-dilution or single-dilution assay)
If single dilution, date of last satisfactory
multiple-dilution assay:
Species, strain and weight range
of animals:
Number of animals per dilution:
Reference vaccine used (IU):
Date of immunization:
Route of injection and volume of
dilutions administered:
Date of challenge:
Challenge method used (lethal or
paralytic challenge):
Challenge toxin used:
Challenge dose(s) used:
Date of end of observation:

Results (See Table 5.1 for an example of how to report the results from a lethal method, and see Table 5.2 for an example of reporting a paralytic method)

Table 5.1
Reporting results from a lethal challenge assay

Vaccine	Dilution	Lethal method No. survivors/No. tested	Median effective dose (ED ₅₀)		
Reference vaccine	1	/			
IU/ml	2	/	ml		
	3	/			
Test vaccine	1	/			
lot number	2	/	ml		
	3	/			
Potency of test vaccine in IU/SHD with 95% confidence limits:					
Single-dilution assays only: <i>P</i> value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD:					

Table 5.2
Reporting results from a paralytic challenge assay

Vaccine	Dilution	Mean score		
Reference vaccine	1			
IU/ml	2			
	3			
Test vaccine	1			
lot number	2			
	3			
Potency of test vaccine in IU/SHD with 95% confidence limits:				
Single-dilution assays only: <i>P</i> value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD:				

When a single-dilution assay is performed, only the responses at the single dilution used are shown. For the paralytic challenge assay, the ED_{50} is not applicable.

4	
-	
	5
0	í
	ľ
0	5
00	٥
õ	ĺ
	5
	,
<	-
Š	١
Θ.)
~	1
0	j
6	١
+	j
~	
- C	٥
2	١
0	j
0	
_	1
C	5
Č	í
.=	
~	
4	
()
ρ,)
	١
\leq	′
Ĺ	
	Ĺ

Serological method
Species, strain and weight range of animals:
Number of animals per dilution:
Reference vaccine used (IU):
Date of immunization:
Route of injection and volume of dilutions administered:
Date of bleeding:
Method for titration of immune serum samples:
Results (See Table 5.3 for an example of reporting results from a serological method)

Table 5.3
Reporting results from a serology assay

Vaccine	Dilution	Mean scores or response			
Reference vaccine	1				
IU/ml	2				
	3				
Test vaccine lot number	1				
	2				
	3				
Positive control					
Negative control					
Potency of test vaccine in IU/SHD with 95% confidence limits:					
Single-dilution assays only: <i>P</i> value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD:					

Test	for	amount	of	residual	free	detox	ifvine	agent
1000	jui	WIII COUIT	v,	1 CSTOTOTOTI	jice	ucion	リノいち	ugenn

Detoxifying agent:	
Method:	
Result (g/1):	
Date of test:	

pН
Result:
Date of test:
Final product
Identification:
Volume:
Identity test
Method:
Result:
Date of test:
Sterility test
Tests for bacteria and fungi
Method:
Media:
Number of containers tested:
Volume of inoculum per container:
Volume of medium per container:
Temperature of incubation:
Date of start of test:
Date of end of test:
Result:
Potency test
If this test was not performed on the final bulk, indicate this and report the data
obtained for the final product in the space provided for potency tests in the "final
bulk" section.
Innocuity test (when required)
Tests in mice
Date of start of test:
Date of end of test:
Number of animals tested:
Route of injection:
Volume of injection:
Observation period:
Results (give details of deaths):

٩	7	1	
3			
5			
(`	,
ć		,	
7	ì	7	
)			
١			
	í		
4			
	ζ	/	
	(J	
	0013	7	
	(1	
(r	١
	4		
	77	1	
	(_	
	3		
	(J	
(Ľ	
•	-	٠,	
	Ş	7	
	5	=	۰
	ē	=	
	Ċ	i	
ŀ		_	
,			,
١	ı,		è
		Ę	
,		5	
2			

Tests in guinea-pigs
Date of start of test:
Date of end of test:
Number of animals tested:
Route of injection:
Volume of injection:
Observation period:
Results (give details of deaths):
Test for adjuvant content
Nature and concentration of adjuvant/SHD:
Method of testing:
Result:
Date of test:
Test for degree of adsorption (when required)
Method:
Desorption method and reagent:
Result:
Date of test:
Test for preservative
Nature and concentration of preservative:
Method of testing:
Result:
Date of test:
рH
Method of testing:
Result:
Date of test:
Extractable volume
Result:
Date of test:
Instruction of final containers
Inspection of final containers
Date of inspection:
Organoleptic characteristics:
Number of containers inspected:
% OF CONTAINERS PERCIPAL

3. Certification by the manufacturer

Name of the manufacturer
Name of head of production (typed)
Certification by the person from the control laboratory of the manufacturing company taking responsibility for the production and control of the vaccine
I certify that lot no of tetanus vaccine (adsorbed), whose number appears on the label of the final container, meets all national requirements and/or satisfies Part A¹ of the WHO Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed) (2014).²
Signature
Name (typed)
Date

4. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 2), a label from a final container and an instruction leaflet for users.

¹ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

² WHO Technical Report Series, No. 980, Annex 5.

Appendix 2

Model certificate for the release of tetanus vaccines (adsorbed) by NRAs

Lot-release certificate	
Certificate no.	
	vaccine (adsorbed) produced by,² whose numbers appear on the
-	s with the relevant national specifications ogical products ³ and Part A ⁴ of the WHO
Recommendations to assure the quali-	ty, safety and efficacy of tetanus vaccines
· · · · · · · · · · · · · · · · · · ·	th WHO good manufacturing practices:
1 1 1	roducts;6 Good manufacturing practices
for biological products;7 and Guideline	es for independent lot release of vaccines
by regulatory authorities.8	
The release decision is based on	9

The certificate may include the following information:

- name and address of manufacturer
- site(s) of manufacturing
- trade name and common name of product
- marketing authorization number
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)

¹ Name of manufacturer.

² Country of origin.

³ If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.

With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

⁵ WHO Technical Report Series, No. 980, Annex 5.

⁶ WHO Technical Report Series, No. 961, Annex 3.

⁷ WHO Technical Report Series, No. 822, Annex 1.

⁸ WHO Technical Report Series, No. 978, Annex 2.

⁹ Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.

- type of container used
- number of doses per container
- number of containers or lot size
- date of start of period of validity (e.g. manufacturing date) and/or expiry date
- storage conditions
- signature and function of the person authorized to issue the certificate
- date of issue of certificate
- certificate number.

The Director of the NRA	(or othe	r appropriate a	uthority
-------------------------	----------	-----------------	----------

Name (typed) _	
Signature	
Date	

Annex 6

Recommendations to assure the quality, safety and efficacy of DT-based combined vaccines

Replacement of Annex 2 of WHO Technical Report Series, No. 800

Introdu	ction	337
Scope o	f the Recommendations	339
General	considerations	339
Part A. N	Manufacturing recommendations	343
A.1	Definitions	343
A.2	General manufacturing recommendations	345
A.3	Control of vaccine production	345
	Filling and containers	350
	Control of final product	350
A.6		351
	Retained samples	351
	Labelling	351
	Distribution and transport	352
A.10	Stability, storage and expiry date	352
Part B. N	Ionclinical evaluation of DT-based combined vaccines	353
B.1	Introduction	353
B.2	Characterization of individual vaccines prior to formulation	355
B.3	Characterization of individual vaccines in the combined vaccine	355
B.4	Immunogenicity evaluation in animal models	356
B.5	Nonclinical safety studies	357
B.6	Toxicology studies	357
Part C. C	linical evaluation of DT-based combined vaccines	358
C.1	Introduction	358
C.2	Scenarios and clinical trial designs	360
	Assessment of immunogenicity in humans	365
C.4	Safety evaluation	374
C.5	Post-marketing studies	375
Part D. F	Recommendations for NRAs	376
D.1	General	376
D.2	Release and certification by the NRA	376
Authors	and acknowledgements	377

References	378
Appendix 1	
Model protocol for the manufacturing and control of DT-based combined vaccines	382
Appendix 1a	
Model protocol for the manufacturing and control of the Hib component of DT-based combined vaccines	395
Appendix 2	
Model certificate for the release of DT-based combined vaccines by NRAs	403
Appendix 3	
Examples of international names, proper names and abbreviations of combined vaccines	405

Recommendations published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes recommendations for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments or examples intended to provide additional guidance to manufacturers and NRAs.

Introduction

A combined vaccine may be defined as a vaccine that consists of two or more antigens, either combined by the manufacturer or mixed immediately before administration, that is intended to protect either against more than one infectious disease or against an infectious disease caused by different types or serotypes of the same organism.

Combined vaccines that allow simultaneous administration of diphtheria (D) and tetanus (T) toxoids with several other antigens have been in use since the middle of the 20th century. Some of the earliest DT-based combined vaccines included inactivated poliomyelitis vaccines (IPV) or whole-cell pertussis vaccine (wP), or both. These were followed by combinations with various acellular pertussis antigens (aP), which were used as an alternative to DTwP, and with combinations that included the addition of one or more of the *Haemophilus influenzae* type b (Hib) conjugates – Hib(conj) – and hepatitis B (HepB) surface antigen (HBsAg).

There are many DTwP-based and DTaP-based combined vaccines available worldwide that vary in the amounts of each antigen and the range of antigens according to the intended age range for use (i.e. infants, toddlers, older children, adolescents or adults). There are also DT-based vaccines available without pertussis components, some of which contain other antigens such as IPV. Hence, DT-based combined vaccines commonly include antigens derived from both bacteria and viruses. The most complex vaccines approved in some countries include DTaP, IPV, HBsAg and Hib(conj), but it is quite possible that more extensive combined vaccines may be developed in future (e.g. containing conjugated meningococcal polysaccharides).

The WHO Requirements for diphtheria, tetanus, pertussis and combined vaccines (1) incorporated guidance on the individual components of these vaccines (e.g. diphtheria vaccine, tetanus vaccine and whole-cell pertussis vaccine). In addition, a separate section on the manufacture of combined vaccines containing more than one of the individual vaccines (e.g. DT and DTwP) was included; this guidance commences at the stage of the final bulk vaccine and considers the specifics of manufacture of the final combined product. However, the section on the requirements for combined vaccines (adsorbed) stated that no attempt had been "made to include other combinations, including those with *Haemophilus influenzae* and *Neisseria meningitidis* polysaccharides and poliomyelitis vaccines". In addition, it is now WHO policy to include in new recommendations guidance on the nonclinical and clinical aspects of vaccine development. The 1990 WHO Requirements for diphtheria, tetanus, pertussis and combined vaccines (1) do not contain such guidance.

Since the publication of the first Requirements for diphtheria, tetanus, pertussis and combined vaccines (1), new and revised WHO recommendations

on single vaccines that are directly relevant to DT-based combined vaccines have been established or are under development. Published documents include:

- Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (2);
- Recommendations to assure the quality, safety and efficacy of tetanus vaccines (3);
- Recommendations for whole-cell pertussis vaccine (4);
- Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines (5);
- Recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines (6);
- Recommendations for the production and control of poliomyelitis vaccine (inactivated) (7);
- Recommendations for the production and control of *Haemophilus* influenzae type b conjugate vaccines (8);
- Recommendations for diphtheria, tetanus, pertussis and combined vaccines (Amendments 2003) (9);
- the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (10).

WHO convened two meetings on the revision of its Recommendations to assure the quality, safety and efficacy of diphtheria vaccines, tetanus vaccines and DT-based combined vaccines. The first meeting was held in Geneva, Switzerland, from 21-22 June 2011, and the second was held in Beijing, China, from 7-11 November 2011. At these meetings, scientific experts, regulatory professionals and other stakeholders met to develop the revisions. The recommendations in this document are intended to provide background and guidance to national regulatory authorities (NRAs) and vaccine manufacturers on the production, quality control and evaluation of the safety and efficacy of diphtheria vaccines, tetanus vaccines and DT-based combined vaccines. Part A of this document sets out the guidance on the manufacture and quality assessment of DT-based combined vaccines. Guidance specific to the nonclinical evaluation of DT-based combined vaccines is provided in Part B; and guidance on the clinical evaluation of these vaccines is contained in Part C. This document should be read in conjunction with all relevant WHO guidelines, including those on the nonclinical (11) and clinical evaluation (12) of vaccines. This guidance is based on experience with the products developed so far, as described below, and may need to be updated in response to future developments.

Taking into account the history of guideline development, it was considered appropriate to replace Annex 2 of WHO Technical Report Series, No. 800 (*I*) with a revised version in order to take into account the developments that have occurred since 1990. The main changes introduced in this revision are:

- a change of title from Requirements to Recommendations;
- the abbreviations for DT-based combined vaccines and their components;
- the replacement of self-standing sections on the components of DT-based combined vaccines with references to the recommendations for individual vaccines wherever relevant;
- the inclusion of considerations for all DT-based combined vaccines that had been developed at the time the revision was drafted (e.g. combined vaccines that include HBsAg, IPV and Hib(conj);
- the inclusion of new sections on the clinical and nonclinical evaluation of DT-based combined vaccines;
- a revision of the model protocol for the information to be provided for the lot release of DT-based combined vaccines.

Scope of the Recommendations

The scope of this document is to provide guidance on the data needed to ensure the quality, safety and efficacy of DT-based combined vaccines. These Recommendations mention a number of combined vaccines as examples. The principles expressed in this document may also apply to combined vaccines that are not explicitly mentioned.

General considerations

In addition to WHO recommendations for an individual component vaccine of a combined vaccine, the following considerations are specific to combined vaccines.

Vaccines that can offer protection against several infectious diseases allow for the simplification of vaccination programmes, improved acceptance by parents and vaccinees, and increased vaccine coverage. However, the development, production, control and use of combined vaccines present a number of challenges to both manufacturers and NRAs, as well as national control laboratories (NCLs).

Important issues for ensuring the quality of DT-based combined vaccines include:

- the development of optimal formulations (including the choice of compatible adjuvants) and formulation conditions that lead to vaccines of adequate immunogenicity, acceptable reactogenicity, and stability, and that are appropriate for the intended use;
- the applicability of testing methods originally established for monocomponent vaccines;
- the suitability of using monocomponent reference materials in evaluating combined vaccines;
- the corresponding release and stability criteria.

Specific issues regarding the suitability of combined vaccines intended for prequalification – and therefore critical to the WHO Programmatic Suitability for Prequalification Standing Committee (13) and the Immunization Practices Advisory Committee (14) – include the use of the appropriate vaccine-vial monitor, the choice of effective antimicrobial preservatives for multiple-dose presentations in relation to the open-vial policy, and the need to demonstrate adequate in-use stability.

The nonclinical programme for the development of a new DT-based combined vaccine should follow the general guidance (11), but particular attention should be given to the choice of animal models used for the assessment of the clinical immunogenicity, efficacy and reactogenicity of the final product.

Important features of the clinical development programme include the assessment of the reactogenicity that results from the administration of multiple antigens simultaneously and via a single injection site, and the potential for a clinically important reduction in the immune response to one or more antigens when delivered in the combined product compared with the delivery of separate vaccines or administration in less complex vaccines. For example, the inclusion of a conjugated polysaccharide in a combined vaccine has sometimes been associated with lower antibody levels when compared with separate injections that are co-administered or separated in time. In addition, immunological interference resulting in a lower antibody response to a conjugate antigen may arise when more than one conjugate is included in the same DT-based combined vaccine or when conjugate-containing combined vaccine is co-administered with other conjugate vaccines (15, 16).

In addition, established vaccination schedules may have to be adapted to the simultaneous administration of several antigens, and the potential effect of delivering concomitant vaccinations on other vaccines (including those in the schedule for the Expanded Programme on Immunization) has to be taken into consideration.

On the other hand, extensive experience with licensed and WHO-prequalified DT-based combined vaccines has demonstrated that the above

concerns can be overcome, and that safe and effective combined vaccines can be developed.

In the process of drafting this document it was considered that, at least up to the stage of the production of purified bulk antigens, the quality aspects would be identical for monocomponent and combined vaccines. In addition, it was felt that many issues regarding the production of the formulated final bulk and the final lot, and some of the issues for the nonclinical and clinical programmes, would be similar for monocomponent and combined vaccines. Therefore, these Recommendations are – wherever possible and relevant – limited to the production and development of DT-based combined vaccines, with appropriate reference made to the corresponding WHO recommendations for the individual component vaccines.

Terminology

Definitions for some common terms used throughout this document are given below. They may have different meanings in other contexts.

Adverse event: any untoward medical occurrence affecting a participant in a clinical trial to whom a vaccine has been administered. The occurrence may not necessarily have a causal relationship with the vaccine or vaccination (12).

Adverse reaction: a response to a vaccine that is noxious and unintended, and that occurs at doses tested in humans for prophylaxis or during subsequent clinical use following licensure. The term "adverse reaction" is usually reserved for a true causal association with a medicine or a vaccine (12).

Booster vaccination: a vaccination given at a certain time interval (at least six months) after primary vaccination in order to induce long-term protection (12).

Bulk: processed purified material, prepared from either a single harvest or a pool of single harvests. It is the parent material from which the final bulk is prepared.

Combined vaccine: a vaccine that consists of two or more antigens, either combined by the manufacturer or mixed immediately before administration, that is intended to protect either against more than one disease or against one disease caused by different strains or serotypes of the same organism (11).

Comparator vaccine: an approved vaccine with established efficacy or effectiveness, or with traceability to a vaccine with established efficacy or effectiveness, that is tested in parallel with an experimental vaccine to serve as an active control during nonclinical or clinical testing (5). Examples of comparator vaccines that can be used in studies of combination vaccines can be found in Table 6.1 in section C.2.2.

Final bulk: the homogeneous final vaccine present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

Final lot: a collection of sealed final containers that is homogeneous in all respects. In principle, a final lot must have been filled from a single final bulk container and processed further (e.g. freeze-dried) in one continuous working session. Different final lots may be filled or processed from the same final bulk in different working sessions. These related final lots are sometimes referred to as sub-lots, filling lots or freeze-drying lots, and should be identifiable by a distinctive final lot number.

Functional antibody: an antibody that binds to an antigen and has a biological effect (e.g. toxin neutralization, viral inactivation, opsonic or bactericidal activity, or whole-cell agglutination) that can be demonstrated by laboratory testing.

Immunogenicity: the capacity of a vaccine to induce antibody-mediated or cell-mediated immunity, or immunological memory, or some combination of these (12).

Noninferiority margin or limit: a prespecified limit based on an appropriate confidence interval. Meeting this criterion may exclude a prespecified difference in immune response believed to be clinically meaningful.

Noninferiority trial: a trial that has the primary objective of showing that the response to a vaccine being investigated is not clinically inferior to the response to the comparator vaccine (12).

Primary end-points: the prespecified end-points that are considered most relevant for evaluating the outcome of a clinical trial (e.g. safety, efficacy or immunogenicity).

Primary vaccination: the first vaccination, or series of vaccinations, given within a predefined period, with an interval of less than six months between doses, to induce clinical protection (12).

Reactogenicity: reactions, either local or systemic, that are considered to have a causal relationship to vaccination (12).

Secondary end-points: prespecified end-points that are considered in addition to the primary end-points when evaluating the outcomes of a clinical trial.

Seroconversion: a predefined increase in antibody concentration that is considered to correlate with the transition from seronegative to seropositive and that provides information about the immunogenicity of a vaccine. If there are pre-existing antibodies, seroconversion is defined by a transition from a predefined low level to a significantly higher level, such as a four-fold increase in geometric mean antibody concentration (12).

Vaccine effectiveness: the protection rate conferred by vaccination in a specified population. Vaccine effectiveness measures both direct protection and indirect protection (i.e. protection of unvaccinated persons by the vaccinated

population). Vaccine effectiveness is also determined following the introduction of a vaccine in a population by measuring vaccination coverage, how well the vaccine strains correlate with circulating strains, and the incidence of disease caused by strains not included in the vaccine (12).

Vaccine efficacy: the reduction in the chance or odds of developing clinical disease after vaccination relative to the chance or odds when unvaccinated. Vaccine efficacy measures direct protection (i.e. protection induced by vaccination in the vaccinated population) (12).

Part A. Manufacturing recommendations

A.1 **Definitions**

A.1.1 International names, proper names and abbreviations

The international names and abbreviations of combined vaccines should follow the examples in Appendix 3. Other combined vaccines exist or may be developed in the future – e.g. combinations with meningitis antigens added – and their international names should follow the structure of the examples. The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 **Descriptive definition**

The descriptive definition of a combined vaccine should be based on the definitions stated in section A.1.2 of the recommendations for the individual vaccines; for example:

- DTwP-HepB is a combined vaccine composed of diphtheria toxoid, tetanus toxoid, whole-cell pertussis suspension and purified hepatitis B surface antigen presented with a suitable adjuvant (e.g. aluminium salts);
- DTaP-HepB-IPV-Hib_X or DTaP-HepB-IPV+Hib_X is a combined vaccine composed of diphtheria toxoid, tetanus toxoid, acellular pertussis components, purified hepatitis B surface antigen, inactivated poliomyelitis antigens and *Haemophilus influenzae* type b (X-) conjugate presented with a suitable adjuvant (e.g. aluminium salts). The product may be a mixture of all components or may be presented with the *Haemophilus influenzae* component in a separate container, the contents of which are mixed with the other components immediately before use.

A.1.3 International reference materials

There are no international standards or international reference preparations specifically designed for combined vaccines. For reference materials from WHO that may be used in laboratory or clinical evaluations of combined vaccines, refer to section A.1.3 of WHO recommendations for the individual vaccine. The WHO catalogue of international reference preparations¹ should be consulted for the latest list of appropriate standards and reference materials. The use of stable, monocomponent international, regional and national reference materials that have been calibrated against the international standard for assaying the potency of combined vaccines serves as the primary consideration; it has practical advantages and should be used whenever possible. The suitability of this approach should be carefully evaluated on a case by case basis for combined vaccines since it has been shown that, in some cases, qualitative differences in antigen or excipient composition, or both, between a monovalent reference preparation and a combined vaccine that is being tested may result in invalid test results (e.g. deviations from parallelism of the dose-response curves) or excessive variability within and between assays and between laboratories (17-21), or a combination of these. The suitability of using monovalent reference preparations is of particular significance for the NRA in terms of lot release. Therefore, in some laboratories, combined vaccines that have a composition that is close to that of the combined vaccine being tested have been used successfully as in-house reference material after suitable calibration of the components to the international standard, where this exists. Also, in some cases the need for a product-specific reference vaccine has been evident during specific toxicity monitoring of acellular pertussis vaccines in mice. Such in-house or homologous reference materials should be stable and should preferably have shown satisfactory performance in clinical trials, or should have the same composition and production process as a vaccine lot previously shown to have satisfactory performance in clinical trials. Appropriate procedures should be operational during the licensing process or to give official status to such process-specific or product-specific reference materials. This can be done through collaborative studies carried out by manufacturers and NCLs to assess the suitability and behaviour of such reference materials. Where calibration of such references in International Units (IUs) is not possible, the specifications stating the acceptable limits for the relevant tests and the conditions for their validity should be determined and validated by the individual manufacturers and approved by the NRA. Reference is made to the WHO manual for the establishment of national and other secondary standards for vaccines (22).

¹ See: http://www.who.int/bloodproducts/catalogue/en/index.html

A.2 General manufacturing recommendations

The general manufacturing requirements described in WHO good manufacturing practices for pharmaceutical products: main principles (23) and Good manufacturing practices for biological products (24) apply to the establishment of facilities that manufacture combined vaccines. Reference is made to the recommendations for any individual vaccine-specific additions.

A written description of procedures for preparing and testing a combined vaccine, together with appropriate evidence that each production step has been validated, should be submitted to the NRA for approval. Proposals for modifying the manufacturing process or quality control methods should also be submitted to the NRA for approval before such modifications are implemented.

A.3 Control of vaccine production

For all production stages, production control should be carried out in accordance with the corresponding sections and subsections of WHO recommendations for the individual vaccines. In addition, the following considerations apply for combined vaccines.

A 3.1 Control of the final bulk

It should be noted that, in general, formulation conditions that have been established as optimal for monocomponent vaccines may not be optimal for some combined vaccines. Important considerations include the choice and concentration of any preservative agent or adjuvant and their optimal ratio to the antigen(s), pH and ionic strength. Formulation conditions should be validated to ensure optimal clinical immunogenicity, reactogenicity and stability of the vaccine.

For vaccines containing a Hib vaccine, two types of formulation have been developed: vaccines with all components in the same container (known as fully liquid or all in one) and those with the Hib component in a separate container (known as a "lyo-liquid"). The specific testing conditions and issues for these two types differ, as described in the relevant sections on testing provided in this annex.

A.3.1.1 **Preparation**

The final bulk is prepared by blending all components of the combined vaccine. Suitable antimicrobial preservatives may be added. With the approval of the NRA, one or several component vaccine bulk materials may be adsorbed to or mixed with an adjuvant at an acceptable concentration prior to blending into the final vaccine bulk (these intermediates have been called preadsorbed bulks).

Such intermediates may be kept at validated storage temperatures for validated storage times. For the stability aspects of such intermediates, reference is made to the WHO Guidelines on stability evaluation of vaccines (25).

A.3.1.2 Preservatives

If the vaccine is to be dispensed into multidose containers, a suitable antimicrobial preservative should be added. The amount of preservative in the final bulk should have been shown to have no deleterious effect on any of the vaccine's components, and to cause no unexpected adverse reactions in humans. The preservative and the concentration used should be approved by the NRA. Certain antimicrobial preservatives, particularly those of the phenolic type, have been shown to adversely affect the antigenic activity of tetanus and diphtheria vaccines, and are not recommended for use in combined vaccines that fall within the scope of these Recommendations. Similarly, thiomersal is known to adversely affect the antigenic activity of IPV (26). In some vaccines, 2-phenoxyethanol has been shown to be a suitable alternative, but its compatibility with the antigens in the combined vaccine should be evaluated on a case by case basis. For the prequalification of multidose presentations, programmatic issues, such as the compatibility of the formulation with an open-vial policy, and the need for in-use stability data, should be taken into account (25, 27).

A.3.1.3 Adjuvants

The use of an adjuvant should be carefully evaluated to determine its effect on the safety, immunogenicity and efficacy of the combined vaccine. If adjuvants are used, their concentration and quality characteristics, demonstrating their suitability as an adjuvant and their compatibility with the component vaccines in the combined vaccine being considered, should be approved by the NRA.

Aluminium compounds are generally used as mineral carriers. The quality characteristics of aluminium hydroxide, hydrated, for adsorption have been described in the European Pharmacopoeia (28).

It should be noted that the concentration of aluminium may be higher in combined vaccines than in monocomponent vaccines due to the contribution of the individual preadsorbed component bulks during blending. The final bulk may also contain a mixture of adjuvants from the individual preadsorbed bulks. When aluminium compounds are used as adjuvants, the concentration of aluminium should not exceed 1.25 mg per single human dose (SHD). For combined vaccines, it is important to determine the degree of adsorption of each of the antigens as parameters for consistency, release and stability.

In some countries, upper limits for the concentration of mineral carriers are set at lower amounts (i.e. less than half) than that given above.

The formulation should be such that the vaccine remains suspended after shaking for a time that is considered adequate to allow a representative sample to be withdrawn from the container.

A.3.1.4 Consistency

The consistency of a combined vaccine should be evaluated at several stages (29). At the bulk antigen stage there should be at least three consecutive batches of each component – e.g. a new DTw/aP-HepB combination should be from D_1 , D_2 , D_3 plus T_1 , T_2 , T_3 plus w/aP₁, w/aP₂, w/aP₃ plus HepB₁, HepB₂ and HepB₃. At the level of the formulated final bulk, the combination would be D_1T_1 w/aP₁HepB₁, D_2T_2 w/aP₂HepB₂, and D_3T_3 w/aP₃HepB₃.

When a new vaccine is added (e.g. IPV) to an established, licensed combined vaccine (e.g. DTw/aP-HepB), and after demonstration of the consistency of the new vaccine at the level of the bulk antigen (if its production is new to the particular manufacturer), consistency is demonstrated as: D_1T_1w/aP_1IPV_1 , D_1T_1w/aP_1IPV_2 and D_1T_1w/aP_1IPV_3 .

A.3.2 Control tests on the final bulk

Each final bulk of the combined vaccine should be tested for sterility, the potency of each component vaccine and specific toxicity in accordance with the individual recommendations for each of the component vaccines. In general, the testing described in the corresponding sections of WHO recommendations for the individual vaccines is applicable to combined vaccines. Reference is also made to the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (10). A number of issues that are specific to combined vaccines are described below.

Multiple-dilution in vivo potency testing of combined vaccines requires a considerable number of laboratory animals. A significant reduction in the use of laboratory animals could be achieved through the development and use of simplified in vivo models (e.g. single-dilution models) and particularly through those that would allow for the concurrent serological testing of multiple components (e.g. concurrent testing of purified pertussis antigens and diphtheria and tetanus toxoids) (30-34). A laboratory that intends to introduce an alternative method should perform adequate validation studies to enable comparisons to be made with the multiple-dilution in vivo model (32-34).

A.3.2.1 Diphtheria potency testing

In general, potency values determined by a test in guinea-pigs, as described in the WHO Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (2), are significantly lower in the absence of a whole-cell pertussis

component than the values found in vaccines containing this component. This may hamper the implementation of a single-dilution assay for combined vaccines that do not contain a whole-cell pertussis component. In general, if the true potency of a vaccine antigen is close to the minimum required specification, a single-dilution model often gives inconclusive results.

In contrast, diphtheria potency values for all-in-one liquid vaccine or reconstituted combined vaccines with a Hib component produced with CRM197 as a carrier tend to be higher than the values observed for vaccines with a Hib component produced with a different carrier, and a single-dilution model may successfully replace the multiple-dilution model. In this case, a minimum specification of 30 IU/SHD is not useful for monitoring consistency since the observed potency estimates are always higher. Therefore, in addition to setting a minimum potency specification of 30 IU/SHD for vaccines used to immunize children, manufacturers, with NRA approval, should also set lower consistency limits and upper consistency limits that reflect the potency values found in practice for combined vaccines that have been demonstrated to be safe and effective in the clinical setting. Manufacturers and the NRA must closely monitor such limits and the trends in key consistency data (35).

A.3.2.2 **Tetanus potency testing**

Similar to diphtheria toxoid, potency values for tetanus toxoid determined by the tests described in the WHO Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed) (3) are significantly higher in the presence of a wP component and in the presence of a Hib component produced with a tetanus toxoid carrier than the values found in the absence of such components, particularly when assayed in mice. In such cases, a minimum specification of 40 IU/SHD, or 60 IU/SHD for vaccines containing wP when assayed in mice, are not useful for monitoring consistency since the observed potency estimates are always much higher. Therefore, in addition to setting a minimum potency specification of 40 IU/SHD for vaccines used for the primary immunization of children (or 60 IU for vaccines containing wP when assayed in mice), manufacturers, with NRA approval, should also set lower consistency limits and upper consistency limits that reflect the potency values found in practice for the combined vaccines that have been demonstrated to be safe and effective in the clinical setting. Manufacturers and the NRA must closely monitor such limits and the trends in key consistency data (35).

A.3.2.3 Hepatitis B potency testing

In principle, in vitro assays can be used for combined vaccines as outlined in the WHO Recommendations to assure the quality, safety and efficacy of recombinant

hepatitis B vaccines (6). However, some in vitro assays have been shown to work less well for combined vaccines with a wP component. If that is the case, an in vivo assay may have to be used. Furthermore, in vivo potency estimates for the HepB component have been shown to be significantly higher in some combined vaccines (e.g. those containing wP) than in vaccines containing only the HepB component. Specifications should be set accordingly, and manufacturers should set lower consistency limits and upper consistency limits, all of which should be approved by the NRA, that reflect the potency values found in practice for the combined vaccine and that have been demonstrated to be safe and effective in the clinical setting. Manufacturers and the NRA must closely monitor such limits and the trends in key consistency data (35).

A.3.2.4 Potency-related tests on combined vaccines with a Hib component (full liquid or all-in-one formulations) or reconstituted lyo-liquid formulations

For some vaccines it has proved difficult to perform potency-related and stability-indicating testing of the Hib component (i.e. measuring total saccharide content, molecular weight distribution, free saccharide content and free carrier protein). Manufacturers are encouraged to develop a method that allows such tests to be performed on the formulated vaccine, including at the final-lot stage. If justified, performing such tests at the bulk conjugate stage may, with NRA approval, be considered acceptable. Animal models (e.g. mice, rats, rabbits or guinea-pigs) – although used less often for routine lot release – may be useful in characterizing the protective potency or immunogenicity, the consistency and, if needed, for monitoring stability.

For combined vaccines with a separate freeze-dried Hib component, the testing required by WHO recommendations for the individual vaccines may be performed on the separate containers as described for the combined vaccine (see section A.5).

A.3.2.5 Safety-related testing of aP components (residual activity of pertussis toxin and reversion to toxicity)

In the presence of aluminium-based adjuvants, the in vitro Chinese hamster ovary (CHO) cell-based assay may not be applicable for testing the formulated product and for some chemically detoxified antigens. In addition, the in vivo test may be sensitive to other components in the formulation rather than to any residual native pertussis toxin (PT) (e.g. aluminium-based adjuvants or IPV). Proper standardization of the in vivo test, and the development and introduction of alternative test methods, are strongly encouraged. Further information can be found in section A.3.4.2.5 of the WHO Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines (5).

A.3.2.6 **wP potency tests**

For some vaccines, the suppression of in vivo wP potency has been observed in the presence of an IPV component. However, for such vaccines, the minimum specifications stated in the Recommendations for whole-cell pertussis vaccine (4) are maintained (i.e. an estimated potency of at least 4.0 IU in the volume recommended for an SHD, and the lower fiducial limit – P = 0.95 – of the estimated potency of at least 2.0 IU).

A.3.2.7 Endotoxins

For monovalent wP vaccines, the Recommendations for whole-cell pertussis vaccine (4) state that since "there is no agreement as to what constitutes an acceptable level of endotoxin in whole-cell pertussis vaccines, monitoring of endotoxin level on a lot-to-lot basis is encouraged as a monitor of consistency of production". This statement also holds true for combined vaccines containing a wP component. For combined vaccines, the wP component is by far the major contributor to the final endotoxin content. In general, for each component in a combined vaccine, the content of bacterial endotoxins should be less than the limit approved for the particular vaccine and, in any case, for combined vaccines that do not contain a wP component, the contents should be such that the final vaccine as administered contains less than 100 IU/SHD.

A.4 Filling and containers

The requirements concerning filling and containers given in WHO good manufacturing practices for pharmaceutical products: main principles (23) and Good manufacturing practices for biological products (24) apply to vaccine filled in the final form.

Single-dose and multiple-dose containers may be used. Vaccine in multiple-dose containers should contain a suitable antimicrobial preservative.

A.5 Control of final product

Each final lot of the combined vaccine should be tested to assess the identity of each component, and the sterility, pyrogenicity or endotoxin content, adjuvant content, preservative content, the potency of each component and innocuity in accordance with the recommendations for each individual vaccine. In general, the methods described in the corresponding sections of WHO recommendations for each individual vaccine are applicable to combined vaccines. A number of issues that are specific to combined vaccines are described in section A.3.

For routine release testing purposes it should be noted that when a combined vaccine is composed of two separate preparations that need to be reconstituted with each other at the time of administration (i.e. lyo-liquid

formulations) the full approval of release testing carried out on each of the two preparations separately is deemed to be sufficient. Repeating the tests – particularly those such as potency testing, which involve animals – on the reconstituted combined vaccine is not required provided that during development duly validated studies demonstrating the compatibility of the two components following reconstitution have been conducted by the manufacturer, and that due consideration has been given to issues of batch consistency, batch size and the frequency of production. These studies must show that the component vaccines and the final reconstituted combination are sufficiently comparable in terms of quality, innocuity and immunogenicity to meet the release specifications, and that any systematic effect associated with reconstitution is consistent between batches and compatible with the vaccine's clinical safety and effectiveness. Please refer to the note on potency-related tests in section A.3.

A.6 Records

The recommendations given in WHO good manufacturing practices for pharmaceutical products: main principles (23) and Good manufacturing practices for biological products (24) apply.

A model protocol to be used for DT-based combined vaccines is provided in Appendix 1.

A.7 Retained samples

Vaccine samples should be retained, as recommended in WHO good manufacturing practices for pharmaceutical products: main principles (23) and Good manufacturing practices for biological products (24).

A.8 Labelling

The recommendations given in WHO good manufacturing practices for pharmaceutical products: main principles (23) and Good manufacturing practices for biological products (24) apply with the addition of the following:

- the word "combined" or "combination" to be added
- the word "adsorbed" to be added, if applicable
- the name and address of the manufacturer
- the recommended storage temperature and the expiry date if kept at that temperature
- the recommended SHD and route of administration.

In addition, the label printed on or affixed to the container, or the label on the cartons, or the leaflet accompanying the container should contain the following information:

- a statement that the vaccine satisfies the recommendations of this document;
- the nature and amount of any preservative present in the vaccine (if there is no preservative in single-dose containers, this should be stated);
- the nature and amount of the adsorbing agent, if applicable;
- the nature and amount of any substances added to the vaccine;
- the recommended conditions for storage and transport;
- a warning that the vaccine should not be frozen;
- a warning that the vaccine should be shaken before use;
- instructions for the use of the vaccine, and information on contraindications and reactions that may follow vaccination.

A.9 **Distribution and transport**

The recommendations given in WHO good manufacturing practices for pharmaceutical products: main principles (23) and Good manufacturing practices for biological products (24) apply.

A.10 Stability, storage and expiry date

The stability-indicating parameters are those selected for the individual component vaccines. Stability studies should be performed in accordance with WHO Guidelines on stability evaluation of vaccines and in particular with the section of those Guidelines relevant to combined vaccines (25).

A.10.1 Stability testing

Stability evaluations are an important part of quality assessment. The purpose of stability studies is to ensure that at the end of the combined vaccine's shelf-life, and during the storage period or period of use, each of the component vaccines retains the characteristics necessary to support the combined vaccine's quality, safety and efficacy. If applicable, the desorption of antigens from the adjuvant, which may occur over time, should be investigated and limits should be agreed with the NRA.

The real-time stability of the vaccine in final containers maintained at the recommended storage temperature, should be demonstrated to the satisfaction of the NRA. In general, manufacturers should follow WHO Guidelines on stability evaluation of vaccines (25) when assessing a combined vaccine's stability for licensure, at different stages of the manufacturing process, and to gain approval for a clinical trial.

Accelerated stability studies may provide additional evidence of product stability, but cannot replace real-time studies.

When any changes that may affect the stability of the product are made to the production process, the stability of the vaccine produced by the new method should be demonstrated.

A.10.2 Storage conditions

The recommended storage conditions and the defined maximum duration of storage should be based on stability studies, as described in section A.10.1, and should be approved by the NRA. For DT-based combined vaccines, storage at a temperature of 2-8 °C is generally considered to be satisfactory. Storage at this temperature range should ensure that the minimum potency specified on the label of the container or package will be maintained after release and until the end of the product's shelf-life if the conditions under which the vaccine is stored are in accordance with the instructions on the label.

The manufacturer should recommend conditions of storage and transport that will ensure the vaccine satisfies the potency requirements until the expiry date stated on the label.

The vaccine must not be frozen.

Expiry date A.10.3

The expiry date should be defined based on a shelf-life that has been justified by stability studies as described in section A.10.1, and should be approved by the NRA.

Part B. Nonclinical evaluation of DT-based combined vaccines

Introduction B.1

Nonclinical testing is a prerequisite for the initiation of clinical studies in humans, and includes extensive product characterization, immunogenicity studies (known as proof-of-concept studies) and safety testing in animals. The extent to which nonclinical studies will be required depends on the type of antigen used, the complexity of the formulation, and clinical experience with the different individual vaccines, used alone and in combination. More extensive nonclinical testing is likely to be required when the combined vaccine includes novel antigens or novel adjuvant systems. Details of the design, conduct, analysis and evaluation of nonclinical studies are available in WHO guidelines on nonclinical evaluation of vaccines (11). The nonclinical studies performed should provide proof that: (i) the individual vaccine antigens and final product are well defined

and have been thoroughly characterized; (ii) the combined vaccine administered to humans is expected to be well tolerated and is unlikely to introduce new safety concerns; and (iii) the vaccine is reasonably likely, based on data from animal immunogenicity studies or protection studies, to provide an acceptable level of protection against the diseases targeted by each of the individual vaccines present in the final combined vaccine. These issues are discussed in detail below.

The following sections describe the types of nonclinical information that should be considered in the context of the development of a new combined vaccine, or when significant changes to the manufacturing process require reevaluation and re-characterization of the vaccine. The goal is to collect data that can be submitted to the NRA. The purpose of the submissions will vary during the product-development process. In some cases, nonclinical data will be submitted to support the initiation of a specific clinical study; in other cases, the nonclinical data will be included in an application for marketing authorization. The goal of preclinical testing, defined as the nonclinical testing done prior to the initiation of a clinical investigation, is to develop a package of supporting data and product information that justifies the move to clinical studies.

Many considerations influence the extent of the nonclinical testing required. New vaccine formulations that have not been evaluated previously for safety and efficacy require extensive characterization, including immunogenicity studies or challenge studies in animal models (known as proof-of-concept studies), and safety testing in animals. However, extensive nonclinical testing may not be required for vaccines that use antigens that are the same as those in vaccines that have already been approved (i.e. from the same manufacturer and produced by the same methods). New combined vaccines may require nonclinical testing if:

- they include a combination of two or more already approved products; or
- a new and not currently licensed vaccine antigen has been added to an existing vaccine; or
- one antigen in a combination vaccine has been replaced with an antigen used for the same indication; or
- an antigen has been removed from an approved combination; or
- changes have been made to the manufacturing process for one or more of the individual component vaccines; or
- changes have been made to the amount of one or more of the antigens or excipients; or
- changes have been made to the adjuvant, preservative or another excipient.

The specific questions to be addressed by nonclinical testing depend on the nature of the changes. However, the primary concerns relate to the compatibility of each of the vaccines with one another, the physicochemical and immunochemical integrity of each of the antigens in the combination, the stability of the individual components, the potential for immunological interactions when the individual vaccines are combined, and the potential for increased reactogenicity. Some of these evaluations will include testing in animal models, which is discussed below. Comprehensive toxicology studies (see section B.6) will not necessarily be required for all new combined vaccines. Prior to the initiation of toxicology studies, it is recommended that the NRA should be consulted regarding the need for and the design of toxicology studies for a new combined vaccine.

The vaccine lots used in nonclinical studies should be adequately representative of the formulation intended for clinical investigation, and, ideally, should be the same lots as those used in clinical studies. If this is not feasible, then the lots used clinically should be comparable to those used in nonclinical studies with respect to the manufacturing process, immunological activity or potency, purity, stability and other aspects of quality.

B.2 Characterization of individual vaccines prior to formulation

For vaccines based on novel antigens or on formulations for which one or more of the components have been produced using a new manufacturing process that is different from the established one, nonclinical testing should include detailed characterization and evaluation of the individual vaccines prior to formulation. A detailed discussion of this characterization is beyond the scope of this document; instead, one should refer to the product-specific WHO document dealing with that component as well as to the general guidance provided in the WHO guidelines on nonclinical evaluation of vaccines (11).

B.3 Characterization of individual vaccines in the combined vaccine

Creating a new combined vaccine using any of the scenarios described above leads to a change in environment for the antigens in each of the individual vaccines. For example, there could be a change in pH, diluent composition, adjuvant nature or concentration, or protein concentration. Any of these could lead to changes in the degree of adsorption on to the adjuvant, physicochemical or immunochemical integrity, or stability.

Thus, the combined antigens should be examined by appropriate means to evaluate possible changes in antigen properties that arise as a result of combining them. The compatibility of all of the antigenic components of the vaccine with one another should be demonstrated in nonclinical studies. Where

relevant, adsorption of all of the antigenic components present in the vaccine should be shown to be consistent from lot to lot. The possible desorption of an antigen during the shelf-life of the product should be evaluated and reported, and specifications should be set. More extensive studies will be required if a new adjuvant is proposed for use in a vaccine formulation. Preclinical studies should evaluate the combination of adjuvants and antigens as formulated for clinical use. Whenever possible, the properties of the individual antigens should be evaluated by comparison with the properties of the same antigens when used in uncombined licensed vaccines. In some situations, the licensed comparator vaccine or vaccines may be lower-order combined vaccines (e.g. DTP may be used as a comparator for a DTP-HepB combination).

B.4 Immunogenicity evaluation in animal models

Before the initiation of human clinical trials, new combinations produced either by formulation or by reconstitution should be studied for adequate immunogenicity in an appropriate animal model if available. The immune response to each of the antigens in the vaccine should be assessed including the quality of the response, the potential interference, and incompatibilities among combined antigens. When possible, it is preferable to study a new combination in comparison with the individual antigens (or an approved lower-order combined vaccine) in animals to determine whether augmentation or diminution of response occurs. The use of an animal model in which more than one of the individual vaccines can be evaluated is encouraged for such investigations.

Immunogenicity studies in animal models can provide important information with respect to optimizing adjuvant formulations and evaluating the immunological characteristics of the antigen including the ability to induce functional antibodies or protection from challenge. However, experience has shown that extrapolating data from animal models to human disease has to be approached with caution. The following issues should be considered when evaluating immunogenicity as part of a nonclinical programme.

- Preclinical studies should evaluate the combination of adjuvant and antigen as formulated for clinical use.
- The quantity of antibody directed towards each of the component antigens should be directly compared between the candidate vaccine and at least one licensed comparator, preferably a comparator that has been used extensively and for which data support its effectiveness in routine use. If testing is performed as a result of a significant change in the manufacturing process, the candidate vaccine should be compared with the corresponding licensed vaccine. Depending on the nature of the changes, the comparator could be the licensed individual component vaccine, a lower-order combined vaccine, or a

licensed vaccine with the same composition. For some combinations, more than one comparator will be required to allow for the assessment of each of the component antigens.

- The potential need to characterize the immune response in more depth should be assessed including, when possible, evaluating functional antibody responses or cellular immunity, or both.
- If a new candidate vaccine contains a new adjuvant, its inclusion should be supported by adequate immunogenicity data that in addition to measuring humoral antibodies, may include an assessment of the cellular immune response. Studies should compare the adjuvanted candidate vaccine with appropriate comparator vaccines. In the case of new adjuvants intended to replace well established aluminium adsorbants in a vaccine already in use, the selection of appropriate control groups of animals should be considered carefully. These groups may include one group receiving the antigen alone or a group receiving the antigen adsorbed to an aluminium compound, or both.

B.5 Nonclinical safety studies

Preclinical animal studies should be undertaken to determine the safety profile of the combination of adjuvant and vaccine. The safety of a new combination should be evaluated in an animal model on a case by case basis, especially if there is a concern that combining antigens or adjuvants may lead to toxicity problems (e.g. in the case of a novel adjuvant). For vaccines that contain one or more chemically inactivated toxins (e.g. diphtheria, tetanus and acellular pertussis), studies should specifically evaluate the presence of residual active toxin and the potential for reversion to toxicity in the final combination.

If a new additive, such as a preservative or excipient, is to be used, its safety should be investigated and documented. If a new preservative is used, its safety, efficacy and appropriateness for use in a particular product must be documented. The safety of new additives can be evaluated by using vaccine formulations without antigens. However, the compatibility of a new additive with all of the vaccine's antigens should be documented, in addition to documenting the toxicological profile of the particular combination of antigens and additives in animal models.

B.6 Toxicology studies

Toxicology studies on the final formulation, which includes the antigens and adjuvants, should be undertaken in accordance with the WHO guidelines on nonclinical evaluation of vaccines (11). When toxicology studies are needed, the design should take into consideration the intended clinical use of the vaccine.

This is of particular concern for vaccines that will be used in certain target populations, such as infants, young children, pregnant women or women of childbearing potential. As noted in section B.1, it is recommended that the NRA should be consulted prior to the initiation of toxicology studies.

If the vaccine has been formulated with a novel adjuvant, appropriate nonclinical toxicology studies should be conducted on the final vaccine formulation, which should include the adjuvant. Repeated-dose toxicity studies may be used to compare the safety profile of the novel adjuvant with the safety profile of an established vaccine formulation, taking into account existing guidelines. If no toxicological data exist for a new adjuvant, in some situations toxicity studies of the adjuvant alone may provide information that is useful for interpretation; however, the NRA should be consulted for guidance.

If a novel cell substrate (i.e. a substrate that has not been previously licensed or used in humans) is used for the production of one of the component antigens, safety aspects, such as potential immune responses elicited by residual host-cell proteins, should be investigated in a suitable animal model.

Variations to the route of administration may require re-evaluation of the immunogenicity of the vaccine as well as adequate studies of animal safety and toxicology, taking into account existing guidelines.

Part C. Clinical evaluation of DT-based combined vaccines

1 Introduction

Part C provides guidance on issues related to the design and evaluation of clinical studies for new combined vaccines and for existing vaccines for which a significant change to the manufacturing process has been proposed. Clinical trials should adhere to the general principles described in international guidelines on good clinical practice (36) and to the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (12). The clinical programme should be preceded by adequate nonclinical studies as discussed in Part B. The content and extent of the clinical programme will vary according to the specific combined vaccine being tested and according to previous clinical experience with the individual vaccines and similar vaccines. The vaccine-specific requirements for clinical studies should be discussed with the appropriate NRA.

These Recommendations specifically address the clinical evaluation of combined vaccines that contain diphtheria and tetanus toxoids. As of 2012, approved DT-based combined vaccines included one or more of the following additional components: pertussis (wP or aP); Hib(conj); inactivated poliovirus; and HepB. Although this document focuses on combinations currently in use, the general principles and procedures apply to new antigens that may be included in future DT-based combined vaccines. Many of the vaccines considered here are intended for infant immunization because immunizing infants is the most

effective prevention strategy for many diseases. However, catch-up and booster strategies, the vaccination of adults, and the vaccination of special populations are common. These Recommendations address issues that are relevant to the diverse indications and use of DT-based combined vaccines.

The main goals of a clinical development programme for a DT-based combined vaccine are to evaluate the safety of the combined vaccine and the immunogenicity of each individual vaccine in the combined vaccine. Generally, a clinical development programme should include comparative clinical trials. Section C.2 discusses the overall design of comparative clinical trials and how to choose a comparator vaccine or vaccines. Unless an alternative approach can be justified adequately, the safety and immunogenicity of a new combination should be compared in a randomized, controlled trial with the safety and immunogenicity of one or more approved vaccines that contain the antigens in the new combination. The value of randomized, controlled trials cannot be overemphasized. The inclusion of a control group receiving approved vaccines provides assurance of the adequacy of the trial's procedures and methods, including the immunoassays, and facilitates the interpretation of data in circumstances in which unexpected results (e.g. low immune response to one or more antigens, high rates of specific adverse events, or unexpected adverse events) are observed following immunization with the new combined vaccine.

The specific questions to be addressed during clinical testing depend on the nature of the new combined vaccine; however, the primary concerns usually relate to the potential for immunological interference and increased reactogenicity. Effects on both immunogenicity and safety have been observed as a result of combining antigens. Generally, safety studies should be designed to determine whether the combined vaccine is more reactogenic than the individual vaccines administered separately, and to obtain an adequate safety database, which is needed to assess risks and benefits prior to licensure. With respect to immunogenicity, the primary concern is typically to evaluate whether the presence of an antigen in a combination interferes with, or in some way influences, the response to any of the other antigens in the vaccine. For the antigens included in currently approved DT-based combined vaccines, direct measurement of clinical efficacy is, with rare exceptions, impractical or impossible. Thus, the evaluation of immunogenicity has been accepted as an appropriate approach for evaluating the adequacy of a DT-based combined vaccine in providing clinical benefit. The existence of established serological correlates of protection for some individual vaccines used in DT-based combined vaccines facilitates the selection of immunological end-points and the interpretation of immunogenicity data. The use of immunogenicity studies to infer clinical benefit for vaccines requires careful selection, proper design, and adequate validation of the assays (see section C.3). The NRA should be consulted when immunoassays are being selected and evaluated for use in clinical studies.

Although not unique to combined vaccines, data on the safety and immunogenicity of new combined vaccines when co-administered with other routinely used vaccines are essential in order to make recommendations regarding concomitant use (12). Concomitant administration may cause lower immune responses to one or more of the co-administered antigens (i.e. immune interference) (15), although the clinical significance of this phenomenon is not always clear. An exaggerated immune response has been observed in some situations in which the carrier protein used in a co-administered conjugate vaccine is related to one of the antigens in the combined vaccine (16). Due to the diversity of possible interactions, the initial assessment of the effects of concomitant vaccine administration should be evaluated at an early stage of clinical development. Nevertheless, data on the effects of co-administration will be accumulated throughout the duration of the clinical development programme and during post-approval studies.

C.2 Scenarios and clinical trial designs

C.2.1 Considerations for the clinical development programme

The clinical development programme should be developed in consultation with the NRA, and should follow available general guidance, including WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (12). A clinical development programme for a new vaccine typically begins with small safety and immunogenicity studies, and then progresses to larger studies. For paediatric vaccines containing novel antigens and formulations, it may be appropriate to conduct preliminary evaluations of safety and immunogenicity in adults and then progress in a stepwise fashion from older age groups to younger age groups. When evaluating such studies, it should be noted that safety and immunogenicity may be dependent on age, prior infection or prior immunization, or a combination of these.

Prior to the initiation of any clinical study, the manufacturer should provide justification for the choice of vaccine formulation and the design of the study. The amount of each antigen in each dose of a combined vaccine requires justification, which may be based on previous experience with each individual vaccine, as well as on nonclinical studies and formal dose-ranging clinical studies. In all cases, clinical studies should be initiated only for products for which there is adequate information on nonclinical testing and on manufacturing.

Consistency in manufacturing should be demonstrated and well documented for the vaccine lots used in clinical trials. Although a formal clinical trial to evaluate lot consistency may not always be needed, in some instances clinical data may be required to provide evidence to support manufacturing consistency (e.g. if there is a particular concern about the consistency of the product). Nevertheless, multiple lots of the combined vaccine formulation that

are intended for marketing and that have been manufactured using different bulk lots for each of the immunogens, should be used during the later stages of the clinical development programme. For components that are already licensed vaccines, fewer lots in combination may be needed than for components that are not licensed. Guidance should be sought from the NRA when determining the composition of lots to be used during the later stages of clinical development.

C.2.2 Overview of potential scenarios that may be encountered with new combined vaccines

New combined vaccines should be compared directly with one or more licensed vaccines with which there has been considerable clinical experience. During late-phase clinical development, the most appropriate study design is usually a randomized, controlled trial with participants from the target age group. The selection of the comparator vaccine or vaccines should be discussed with the NRA, and should take into account the study population, the proposed immunization schedule, the total antigen composition of the candidate vaccine, and previous clinical experience with the comparator vaccine. For some products, more than one comparator vaccine, administered concomitantly, may be required for adequate clinical evaluation of all component antigens. In this case, it is necessary to consider whether these licensed vaccines are recommended for co-administration at separate injection sites or whether there should be staggered administrations (i.e. occurring on different days).

Table 6.1 describes the most common scenarios that are likely to be encountered during the clinical evaluation of a new combined vaccine. New combinations could result from making changes to existing combined vaccines, including adding a new antigen, replacing one antigen with another antigen for the same indication, removing an antigen, or making a significant change to the manufacturing process or formulation. Additionally, a new manufacturer may wish to begin producing a vaccine that is similar in composition to an already approved combination. Although scenarios not specifically addressed here may be encountered, the general principles outlined here should be adaptable to other situations. For each trial, manufacturers should justify the choice of the comparator vaccine, the trial design, and the safety and immunogenicity end-points.

The comparative clinical trial should be designed to enable adequate evaluation of safety and immunogenicity, and should prespecify appropriate end-points related to the rates of adverse events and immune responses to each of the antigens in the vaccine. Issues related to the immunogenicity end-point are discussed in section C.3; issues related to safety are discussed in section C.4). Although the trial designs outlined below apply both to safety and immunogenicity assessments, Table 6.1 provides more detail on evaluating immune responses owing to their increased complexity.

WHO Technical Report Series No. 980, 2014

Table 6.1 Overview of potential scenarios that may be encountered during the clinical evaluation of new combination vaccines^a

Category	Scenario	Example	Suggested design ^b
	Combination of two or more already approved products (e.g. AB $+ C \rightarrow ABC$)	Licensed IPV is added to a licensed DTwP-HepB combination	Immune responses to ABC are compared with immune responses to the separately administered licensed vaccines, AB and C
Antigen addition	Combination of one already approved product (AB) and a new (and not currently licensed) vaccine antigen (C) (e.g. $AB + C \rightarrow ABC$)	A new and unlicensed antigen is added to a licensed DTaP-HepB combination	Immune responses to antigens A and B of ABC are compared with immune responses to the separately administered licensed vaccine, AB; responses to the new antigen C are based on criteria appropriate for C; if a vaccine comparable to C is already licensed, responses to C should be compared with responses to the licensed product
, , , , , , , , , , , , , , , , , , ,	One of the antigens in a combination is replaced by an already approved antigen (for the same vaccine component) (e.g. ABC → ABC*)	The wP component of a licensed DTwP combination is replaced by a licensed aP component	Immune responses to antigens A and B of ABC* are compared with immune responses to a separately administered licensed vaccine containing A and B; responses to new antigen C* are based on comparison with responses to a licensed product containing C*
replacement	One of the antigens in a combination is replaced by a novel (unlicensed) antigen (for the same vaccine component) (e.g. ABC → ABC*)	The aP component of a licensed DTaP combination is replaced by a new aP component containing a genetically modified aP antigen or antigens	Immune responses to antigens A and B of ABC* are compared with immune responses to the separately administered licensed vaccine, AB or ABC; responses to new antigen C* are based on criteria appropriate for C*; if a vaccine comparable to C* is already licensed, responses to C* should be compared with responses to the licensed product

Table 6.1 continued

Category	Scenario	Example	Suggested design ^b
	Increase or decrease in the amount of one or more of the antigens (e.g. ABC → ABc)	The diphtheria toxoid content of a combination vaccine is reduced	Immune responses to ABc are compared with immune responses to a licensed product with a matching composition or to the product with the most similar composition.
Change in manufacturing or formulation	Change in the nature or content of an adjuvant, preservative or another excipient	Introduction of a new adjuvant ^a	Immune responses to the investigational vaccine are compared with immune responses to the licensed product manufactured by the approved process
	Significant change in the manufacture of one or more of the individual vaccine components (e.g. ABC → ABC*)	Change from the use of lyophilized Hib-conjugate component to a fully liquid formulation	Immune responses to the investigational vaccine are compared with immune responses to the licensed product manufactured by the approved process
Antigen removal	Removal of one or more antigens (e.g. ABC → AB).	Removal of HepB antigen from a DTwP-Hib-HepB combination	Immune responses to antigens A and B of AB are compared with immune responses to the licensed vaccine ABC
New manufacturer	A combination comparable to another licensed product is produced by a new manufacturer (e.g. ABC \rightarrow A*B*C*)	Production by a new manufacturer of a DTwP- Hib-IPV combination	Immune responses to ABC are compared with immune responses to a licensed product with a similar composition ^e

IPV = inactivated poliomyelitis vaccine; DTwP = diphtheria-tetanus-whole-cell pertussis vaccine; HepB = hepatitis B virus vaccine; DTaP = diphtheria-tetanus-acellular pertussis vaccine; Hib = Haemophilus influenzae type b.

- a. This table does not specifically address the case in which a manufacturer formulates the final vaccine using one or more components purchased from another manufacturer. However, the source of the component is not expected to influence the overall design of clinical evaluations.
 - b Trial designs other than the suggested ones, including the choice of a comparator vaccine (or vaccines) may be used if justified, and if approved by the NRA.
- * When the antigen content is being reduced, clinical studies should be designed to verify that the reduction does not lead to a clinically important reduction in immunogenicity. A rationale for changing the adjuvant, preservative or another excipient should be provided. In particular, clinical studies evaluating a change in the adjuvant may need to take into consideration additional safety and immunogenicity parameters.
 - combination is an acellular pertussis vaccine. The WHO Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines (5) should be consulted for Due to the limitations of immunogenicity assessments, the selection of an appropriate comparator is particularly complex when one of the components of a new additional guidance.

C.2.3 Schedules and populations

In most cases, a new combined vaccine will be tested following the primary series schedule for vaccinations that have been approved for similar vaccines. However, a formal evaluation of the schedule may be necessary in certain circumstances, such as when a different schedule is required for programmatic reasons or if a candidate vaccine contains an antigen dose or an adjuvant that is considerably different from that used in licensed vaccines.

Safety and immunogenicity have been shown to vary for many vaccines according to the schedule used, the population studied, the antigen composition and the nature of the vaccines that are administered concomitantly. Whenever possible, the combined vaccine should be evaluated in the target population following the intended schedule. However, it may not be feasible to study new vaccines at every possible schedule in current use or in a wide range of geographical regions. For instance, within a specific population, immune responses or rates of some adverse events following immunization with a vaccine that has a 6-week, 10-week and 14-week schedule may differ from those following administration of the same vaccine on a 2-week, 4-week and 6-month schedule, or on a 3-month, 5-month and 12-month schedule. Manufacturers should justify the relevance of the clinical data provided to each country in which approval is sought, and should discuss the basis for extrapolating their findings. When it is anticipated that a vaccine will be used according to different schedules, the recommendation of WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (12) is that the primary evaluation should be conducted using the schedule expected to be the most restrictive (i.e. the schedule from which the least immune response is expected). However, there is still a need to collect some safety data using schedules that are proposed for approval because the local and systemic reactogenicity associated with a vaccine may vary when different schedules are used in a specific population due to the age-related prevalence of specific adverse events. For all clinical trials, the study population should be carefully defined and justified by the manufacturer, and the population studied should be approved by the NRA.

C.2.4 Co-administered vaccines

Vaccinees enrolled into the types of comparative studies described above will also receive other licensed vaccines according to the schedule of the country in which they reside, and experience has indicated that unexpected interactions can occur when vaccines are administered concomitantly. Due to the possible effects of these additional vaccines on the safety and immunogenicity of the test vaccine and control vaccine, as well as the possible effects of the test vaccine on other routinely administered vaccines, manufacturers should conduct studies that evaluate the effects of co-administration as described in guidance from

WHO (12). In some settings, multiple vaccines may be licensed for the same disease, and these may be given on the same schedule as the investigational vaccine. Whenever there is more than one licensed vaccine of a certain type that could be co-administered, the choice of the specific vaccine to be used in a clinical study should take into account the recommended routine immunization schedule as well as the likelihood of co-administration. The choice should be justified, and should be discussed with the NRA. If the results of the clinical trial indicate that immune responses to one or more of the antigens administered routinely are lower when they are co-administered with a new combined vaccine compared with the separately administered licensed vaccine, the NRA will need to consider the potential clinical consequences on a case by case basis. Any incremental increase in adverse reactions that is observed during co-administration will need to be weighed against the convenience of administering multiple vaccines at the time of a single health-care contact.

C.2.5 Studies in special populations

There may be underlying diseases and conditions that predispose an individual to a particular disease (e.g. conditions and diseases such as prematurity, immunodeficiency, or severe pulmonary disorders, including cystic fibrosis (mucoviscidosis) or that may be associated with a poor response to specific vaccines. Clinical studies may be conducted specifically to assess the safety and immunogenicity of new combined vaccines in populations that are at an increased risk for particular diseases. In many instances, these studies may be performed after initial licensure.

Assessment of immunogenicity in humans

C.3.1 Design and scope of immunogenicity studies

The specific questions to be addressed by immunogenicity studies depend on the nature of the new combined vaccine; however, the primary concerns usually relate to the potential for immunological interference among antigens. This document applies to a wide range of combined vaccines that potentially have a large number of antigens for which immunogenicity evaluation is required. The sections below provide guidance related to the selection of assays and end-points for these assessments. Many combined vaccines are developed for the purpose of primary immunization so this document discusses in detail the evaluation of vaccines used for primary immunization. However, booster immunizations for older children, adolescents and adults are also important for the control of several diseases. In some cases, the vaccine developed for primary immunization is also used for booster immunization, while in other cases vaccines have been developed solely for use as booster doses. Therefore, this section also includes information related to the evaluation of vaccines used for booster immunization.

C.3.2 Assays to assess antibody responses

For many of the antigens used in the DT-based combined vaccines that have been approved, WHO guidelines or recommendations are available that provide guidance on the most appropriate assays and end-points for the clinical evaluation of the component antigens (2–6, 8, 37). In addition, some NRAs or regional regulatory authorities have provided guidance that will assist in the selection and establishment of immunoassays. When available, all such guidance should be consulted. However, guidelines are not available for some of the individual antigens used in many combined vaccines. Table 6.2 lists antigens, commonly used assays, and suggested end-points. However, WHO guidelines should be considered the primary source of information.

For some antigens, the end-points used for primary immunization studies are not optimal for the evaluation of booster immunization. For example, this may occur if prior to immunization a significant proportion of a study population has a concentration of antibodies that exceeds a protective threshold. In such cases, an evaluation of the proportion of participants who show a significant increase in antibody concentration may provide a more sensitive assessment of the response to immunization. To reflect these differences, suggested end-points for studies of primary and booster immunizations are provided in separate columns in Table 6.2.

The assessment of the immune response should use a validated and standardized assay to measure the antibody concentration for each component antigen in serum (12). To improve the comparability and acceptability of serological data across trials, the results of immunogenicity outcomes should be expressed in IU/ml of human serum whenever an international reference is available. The selection of assays for evaluating the human immune response to the vaccine should be justified by the vaccine manufacturer. For many vaccines, suitable assays are unlikely to be commercially available. The use of validated quantitative assays is critical, and testing should be conducted by laboratories that implement quality assurance of testing procedures. Validation studies should be designed to demonstrate that the assay is suitable for the clinical study, and should consider the way in which the vaccines are to be compared with one another (e.g. whether the criteria for evaluation are based on percentages of post-primary series titres above a threshold, seroconversion rates, or geometric mean antibody concentrations). The validation report should include a detailed description of the calibration of any in-house references, and information on the processing and storage of samples, reference standards and reagents. The assay validation data should be reviewed and approved by the NRA.

When developing the clinical programme, emphasis should be given to the role of assays that measure the functional activity of antibodies induced by the individual vaccines. For some vaccine antigens, a functional assay is recommended for immunogenicity evaluation (Table 6.2). In other cases, a nonfunctional assay has been accepted for primary evaluations; however, in such cases, if a functional assay is available, it should be used in validation studies to verify that the nonfunctional assay provides a meaningful assessment of the immune response. It is important to note that no functional assay has been identified for some commonly used antigens included in some aP vaccines (5).

Cell-mediated immunity (CMI) responses may play a role in developing immunity to some infections. However, the standardization of immunological assays to evaluate CMI responses following immunization has been challenging, and such assays have not been used to support licensure. Nevertheless, when appropriate, an exploratory assessment of CMI should be encouraged in order to enlarge the body of knowledge regarding all aspects of the immune response to vaccine antigens.

C.3.3 Immunogenicity end-points for immunization studies

For antigens contained in licensed DT-based combined vaccines, Table 6.2 provides a summary of the recommended assays and suggested primary endpoints for the clinical evaluation of vaccines intended for primary or booster immunization. References to documents developed by WHO, to national or regional guidelines, or to other publications are provided. These should be consulted for more complete information.

C.3.4 Primary analyses

The primary analyses should be based on the antibody response following completion of the defined immunization series. In the case of vaccines used for booster indications, this typically will consist of only a single immunization. Responses to antigens shared between a new vaccine and the licensed comparator, and to antigens found only in a new vaccine, should be regarded as coprimary end-points.

The definition of the appropriate time intervals for assessing immune responses should take into account the study's objectives. In most cases, clinical studies for new vaccines are designed to determine the antibody response to the vaccine's components at approximately four weeks following the final dose. However, the timing of serum sampling should be justified, and should be approved by the NRA. In studies evaluating booster doses, blood samples are generally obtained at four weeks after the booster dose but in persons whose immune systems are already primed, the peak response may be achieved in a shorter time – i.e. within two weeks of the booster dose. Thus, some exploration of immune responses at less than four weeks after the booster dose in randomized subsets of the study population could be informative, and may provide insight into the rapidity of the response to antigen challenge.

WHO Technical Report Series No. 980, 2014

 Table 6.2

 Immunogenicity assays and end-points for studies of primary and booster immunizations

Antigen	Assay	References	Suggested prim	Suggested primary end-point(s)	Comments
			Primary immunization	Booster immunization	
Diphtheria toxoid	Microneutralization assay (Vero	2,31,38-41	Proportion with ≥ 0.01 IU/ml or ≥ 0.1 IU/ml (see comments)	Proportion with significant increase ^a	The toxin neutralization assay is generally preferred. An antigen-binding assay that has been shown to correlate with the neutralization assay may be acceptable; for primary immunization, a threshold of 0.01 IU/ml may be acceptable if the toxin neutralization assay is used and if a booster dose is administered during the second year of life; otherwise a threshold of 0.1 IU/ml should be used; for booster immunization, the decision to use a threshold level or significant increase should take into account the proportion of persons with antibody concentrations expected to exceed the threshold prior to vaccination
Tetanus toxoid	ELISA	3,30	Proportion with ≥ 0.1 IU/ml	Proportion with significant increase ^a	An antigen-binding assay that has been shown to correlate with the mouse neutralization assay is most commonly used

Table 6.2 continued

Antigen	Assay	References	Suggested prim	Suggested primary end-point(s)	Comments
			Primary immunization	Booster immunization	
Whole-cell pertussis (wP) ^b	Agglutination assay ELISA for pertussis toxin BELISA for other antigens	4, 42-45	a) GMT and/or GMC b) Proportion with four-fold rise	a) GMT and/or GMC b) Proportion with four-fold rise ^a	No value that correlates with protection has been defined; the goal is to compare the responses of the test and control groups for each assay; both a) and b) are recommended as coprimary end-points
Acellular pertussis (aP)	ELISA for all pertussis antigens in vaccine	5, 42–45	a) GMT and/or GMC b) Proportion with four-fold rise	a) GMT and/or GMC b) Proportion with four-fold rise ^a	No value that correlates with protection has been defined; the goal is to compare the responses of the test and control groups for each assay; both a) and b) are recommended as coprimary end-points
Inactivated poliovirus vaccine	Virus neutralization assay for each of the three serotypes	46	Proportion with neutralization titre ≥ 1:8	a) GMT and/or GMC b) Proportion with neutraliza- tion titre ≥ 1:8	The presence of neutralizing antibody (titre ≥ 1:8) is considered protective against poliovirus types 1, 2 and 3

Table 6.2 continued

Antigen	Assay	References	Suggested prim	Suggested primary end-point(s)	Comments
			Primary immunization	Booster immunization	
Haemophilus influenzae type b conjugate vaccine	ELISA (<i>Haemophilus</i> <i>influenzae</i> type b capsular polysaccharide; PRP	8, 47–49	a) Proportion with ≥ 0.15 µg/ml b) Proportion with ≥ 1.0 µg/ml	Proportion with ≥ 1.0 µg/ml	A post-vaccination anti-PRP level of 0.15 µg/ml is considered the minimum protective level; a post-vaccination level of 1.0 µg/ml indicates protection through a subsequent 1-year period; for primary immunization, both a) and b) are recommended as coprimary end-points
Hepatitis B vaccine	ELISA for hepatitis B surface antigen	9	Proportion with ≥ 10 mlU/ml	Proportion ≥ 10 mIU/mI	

IU = International Unit; ELISA = enzyme-linked immunosorbent assay; GMT = geometric mean titre; GMC = geometric mean concentration; PRP = polyribosylribitol phosphate.

antibodies

b. There is substantial heterogeneity in the antibody response to highly effective wP vaccines. However, the assays listed could be used in the evaluation of a magnitude of increase may be justified for subjects with a specified high antibody concentration prior to vaccination (2,3).

^a The magnitude of the increase (e.g. four-fold) in antibody concentration from pre-vaccination to post-vaccination should be predefined and justified. A lower

· The collection of additional supporting data using the pertussis toxin neutralization assay and the whole-cell agglutination assay is encouraged. comparative immunogenicity trial.

The selection of the primary parameters for the assessment of noninferiority, the predefined margins of noninferiority, and hence the total sample size for the comparative study will need careful justification. Factors to consider regarding the stringency of the noninferiority criteria include the clinical relevance of the end-point, the seriousness of the disease being prevented, and the vulnerability of the target population. More stringent margins may be justified for severe or debilitating diseases, for populations that are particularly vulnerable, or when the serological end-point is known to correlate well with protection against the disease. If a new vaccine is known to offer substantial benefits in terms of safety or improved coverage, less stringent margins may be considered. The noninferiority criteria will influence the study's sample size, and feasibility considerations may need to be taken into account. Thus, there may be situations in which different limits for the same antigen may be appropriate in different settings. In determining noninferiority margins, consideration also should be given to the potential for a downward drift in immunogenicity over time occurring with sequential comparative studies (50). The consequence of such drift, if it happens, is that a new vaccine could be considerably less immunogenic than the originally licensed vaccine. It should be noted, however, that there may be other explanations for a downward drift in immunogenicity, such as the absence of natural boosting following the reduction in pathogen circulation in the community.

Although studies that compare immune responses between a candidate and licensed vaccines are generally required, comparisons with historical data generated during previous protective efficacy studies using similar assays may, in some cases, provide supporting evidence.

For the majority of the antigens contained in currently approved DT-based combined vaccines, the primary assessment will be the proportion of participants who respond to the vaccine as defined in Table 6.2. Typically, this will be the proportion of participants reaching a prespecified threshold. However, for some vaccines and some indications, a response is defined as the proportion of vaccinees with a significant increase (e.g. greater than four-fold) in immune response above preimmunization levels. Alternative definitions for responders may be considered if they have been well justified. The groups should be compared using an appropriate predefined noninferiority limit; generally the upper bound of the two-sided 95% confidence interval of the observed difference (i.e. the comparator vaccine minus the new combined vaccine) should be less than the criterion agreed with the NRA, which is most commonly 0.05 or 0.10.

For some antigens and for some indications, coprimary analyses should compare the magnitude of the response to the vaccine antigens induced by the new vaccine and the licensed comparator. Such end-points are recommended, for instance, in the evaluation of whole-cell and acellular pertussis vaccines because no threshold of protective response has been widely accepted, and they are also recommended in the evaluation of the response to booster doses in situations in which a substantial proportion of the study population exceeds the protective threshold prior to immunization. When used, the magnitude of the response to each component vaccine is compared on the basis of the ratio of the geometric mean concentrations (GMCs) or geometric mean titres (GMTs) of the comparator vaccine to the new vaccine using a predefined margin of noninferiority. Specifically, the upper bound of the two-sided 95% confidence interval of the observed ratio of the GMC or GMT of the comparator vaccine relative to the new vaccine should be less than the criterion agreed with the NRA, which is most commonly 1.5 or 2.0.

Measurement of preimmunization and postimmunization antibody concentrations involves the collection of an extra blood sample, and may not be necessary in all studies. Preimmunization samples will be required when end-points are based on the proportion of participants having a rise in antibody concentration, but these samples may not be needed from all participants when the end-point is based on the proportion of participants that reaches a specified threshold. However, even when a preimmunization sample is not required to evaluate a study's end-point, it is recommended that at least some information on pre-vaccination antibody values should be generated during the clinical development programme in order to aid in interpreting post-vaccination antibody values.

Due to limitations on the volumes of serum that can be collected, it is commonly necessary to perform an additional randomization step to select serum samples for use in different antibody assays or to prioritize samples, or both, so that the most relevant questions for the combination vaccine can be addressed.

For complex combined vaccines, immunogenicity evaluations may include a substantial number of coprimary end-points. If any immune interference is observed with respect to any of the combined antigens, the possible clinical implications and the reasons for not meeting the predefined noninferiority criteria should be carefully considered before proceeding with clinical development or pursuing product approval. The NRA may take into consideration the results from the antibody responses to each of the antigens, any differences in composition between the test vaccine and the comparator vaccine, the severity of the disease, the likelihood that the measured immune parameters predict clinical protection, and the potential benefits of the combination in terms of improving coverage or safety.

C.3.5 Secondary analyses

For most studies, one or more secondary analyses should be defined to provide for a more complete assessment of immune responses. If not included among the primary end-points, comparisons of the magnitude of the response to the vaccine antigens induced by the new vaccine and the licensed comparator should be considered. As described above, the magnitude of the response for each vaccine component is compared by using the ratio of the GMC or GMT of the comparator vaccine to the test vaccine using a predefined margin of noninferiority. The noninferiority margins should be justified, and should be agreed with the NRA.

Assessment of functional antibody responses

When available, assays that measure the functional activity of antibodies against the individual vaccines used in the combined vaccine may play an important part in the evaluation, even when antigen-binding assays are used in the evaluation of the primary end-points. For example, the measurement of functional antibodies should be considered in at least a subpopulation of the comparator group and the test vaccine group, particularly when there is limited experience with an antigen or formulation. Additionally, as noted in section C.3.2, functional assays play an important role in validation studies by verifying that the nonfunctional assay provides a meaningful assessment of the immune response.

Additional information from reverse cumulative distribution curves C.3.7

The use of reverse cumulative distribution (RCD) curves, which display the accumulated proportion of individuals who have an antibody concentration greater than or equal to a given level, has been shown to be particularly useful when comparing the response to the test vaccine with the response to the licensed comparator vaccine, and when monitoring changes in antibody levels over time (51). As one example, the RCDs may reveal the proportion of the population that has values at or below the protective threshold, and provide data that can inform decisions on the timing of booster doses. When using RCDs, comparisons among the study groups are generally qualitative and exploratory in nature because RCD curves do not lend themselves readily to comparative statistical analyses.

Immune responses to carrier proteins C.3.8

The carrier proteins used in licensed polysaccharide conjugate vaccines have included a nontoxic genetically modified diphtheria toxin molecule (CRM197), diphtheria toxoid, tetanus toxoid, protein D from Haemophilus influenzae, and an outer membrane protein complex (OMPC) from Neisseria meningitidis serogroup B. Monitoring the immune response to these carrier proteins may be appropriate in some circumstances. Administration of a conjugate vaccine that employs diphtheria toxoid or tetanus toxoid or CRM197 as the carrier has been found to enhance the relevant antitoxin antibody levels. However, this has not been accepted yet as a replacement for routine immunization with

vaccines containing diphtheria toxoid or tetanus toxoid. The co-administration of a new conjugate vaccine with routine infant and toddler vaccines (i.e. vaccines containing diphtheria toxoid and tetanus toxoid) may result in high antitoxin levels (52). Careful attention should be paid to the reactogenicity observed under these circumstances since increased rates of some reactions could be associated with high antitoxin levels. As noted in section C.1.2, diminished responses to a combined vaccine antigen conjugated to a carrier protein may occur with concomitant administration of another conjugate vaccine that uses the same carrier protein.

C.3.9 Immune memory

For some antigens in a combined vaccine (e.g. polysaccharide conjugate vaccines) it may be appropriate for the clinical development programme to generate data to demonstrate that the vaccine induces an immune memory response during the infant immunization series. These data can be obtained as part of the assessment of immune responses to booster doses of the new vaccine.

C3.10 Persistence of antibody concentrations and timing of booster doses

The waning of antibody concentrations over time is inevitable, and longer-term follow-up to assess the persistence of immunity should occur at various time points following the primary vaccination series. The total duration of serological follow-up should be discussed and planned in advance with the NRA. In some situations, these data may be provided after first approval. The waning of antibody concentrations over time should not be interpreted per se as a loss of immunity or an indication of the need for a booster dose. Longer-term antibody concentrations should be viewed in conjunction with effectiveness data to assess the potential need for additional doses later in life in order to maintain protection. A determination of the need for, and timing of, booster doses should be based on epidemiological investigations and long-term surveillance (see section C.5). A detailed discussion of these issues is outside the scope of this document.

C.4 Safety evaluation

The prelicensure assessment of vaccine safety is a critically important part of the clinical programme, and should be developed to meet the general principles described in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (12). The assessment of safety, with appropriately defined objectives, should be part of the comparative studies mentioned in section C.2. Such studies should be designed to monitor actively for common adverse events as well as less common adverse events, including serious adverse events and specific adverse events that have been associated with vaccines of similar

composition (e.g. excessive limb swelling, hypotonic-hyporesponsive episodes and febrile seizures).

The minimum acceptable size of the safety database at the time of approval should take into account the vaccine's composition including all antigens and adjuvants, the presence of novel antigens, past experiences with vaccines having the same or similar composition, the severity of the diseases being prevented, and the size of the target population. For new vaccines, a total safety database comprising all trials in the targeted age group and approximately 3000–5000 participants who received the new vaccine is commonly expected because this allows for the detection of uncommon adverse events – i.e. those that occur at a rate of approximately 1 in 1000 (53). However, depending on the composition of the investigational vaccine and the relevant safety data about it, the NRA may accept a smaller number or may request a larger database prior to first approval.

Additionally, safety evaluations should include high-risk individuals (e.g. preterm infants, people with chronic illnesses, or people who are immunocompromised) who may benefit from vaccination. Safety in these groups is often assessed during post-marketing studies (see section C.5), but a prespecified plan for such studies may be requested at the time of application for marketing authorization.

C.5 **Post-marketing studies**

The manufacturer has a responsibility to assess the safety and effectiveness of the new vaccine following initial approval. At the time of first licensure, NRAs should ensure that adequate pharmacovigilance plans are in place regarding these activities. There should be specific commitments made by manufacturers to provide data to NRAs on a regular basis and in accordance with national regulations. The data that are collected and submitted to the responsible NRA should be assessed rapidly so that action can be taken if there are implications for the marketing authorization. The basic principles for the conduct of postlicensure studies and continued oversight of vaccines after licensure are provided in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (12).

Through active post-marketing surveillance, every effort should be made to improve the scientific understanding of the protection in humans afforded by vaccines. The effectiveness of the vaccine in the population should be reported whenever possible. However, reliable estimates of effectiveness can be obtained only in geographical locations where there is a suitable infrastructure in place to identify cases of disease. Ongoing surveillance programmes should be in place to monitor longer-term protection and to collect evidence of any changes in the vaccine's effectiveness. Post-marketing assessments designed to monitor effectiveness are particularly important in the specific case in which there has

been a transition to acellular pertussis vaccines that have not been evaluated in efficacy trials. The reason for this emphasis is that there are no immune responses that can be measured in preapproval studies that have been shown to predict clinical efficacy (5).

Because prelicensure studies may not be large enough to detect certain rare adverse events, safety should be monitored as part of post-marketing surveillance programmes. These programmes should specifically monitor any safety concerns identified in preapproval trials, as well as collect data on new and rare adverse events not detected prior to licensure.

The collection of reliable and comprehensive post-marketing data on safety and effectiveness requires close cooperation between manufacturers and public-health authorities. Preapproval and postapproval discussions between the vaccine manufacturers responsible for placing the product on the market and national and international public-health bodies are essential for ensuring that reliable data on safety and effectiveness are collected during the post-marketing period.

Part D. Recommendations for NRAs

D.1 **General**

The general recommendations for NRAs and NCLs given in the WHO Guidelines for national authorities on quality assurance for biological products (*54*) and the WHO Guidelines for independent lot release of vaccines by regulatory authorities (*35*) apply.

The details of production and quality control procedures, as well as any significant changes in them that may affect the quality, safety and efficacy of DT-based combined vaccines, should be discussed with and approved by the NRA. For control purposes, the international standards currently in use should be obtained to calibrate the national, regional and working standards (22). The NRA may obtain the product-specific or working references from the manufacturer to be used for lot release until an international or national standard preparation has been established.

Consistency in production has been recognized as an essential component in the quality assurance of DT-based combined vaccines. In particular, NRAs should carefully monitor production records and the results of quality-control tests on clinical lots as well as results from tests on a series of consecutive lots of the vaccine.

D.2 Release and certification by the NRA

A vaccine should be released only if it fulfils all national requirements or satisfies Part A of these Recommendations, or both (35).

A protocol based on the models given in Appendix 1 and Appendix 1a, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for the release of a vaccine for use.

A statement signed by the appropriate official of the NRA should be provided to the manufacturing establishment to certify that the lot of the vaccine in question meets all national requirements as well as Part A of the present Recommendations. The certificate should provide sufficient information about the vaccine lot. A model certificate is given in Appendix 2. The official national release certificate should be provided to importers of the vaccines. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

Authors and acknowledgements

The first draft of these Recommendations was prepared by Dr R. Dobbelaer and Dr B. Meade with contributions from other members of the drafting group: Dr M. Corbel, Dr K. Farizo, Dr M. Iwaki, Mrs T. Jivapaisarnpong, Dr H. Lechner, Dr D. Lei, Dr F. Mawas, Dr M. Powell, Dr S. Prieur, Dr M.P. Schmitt, Dr T. Sesardic, Dr P. Stickings, Dr M. Takahashi, Dr D. Xing, and Dr S. Zhang.

The second draft was prepared by Dr R. Dobbelaer, Dr B. Meade and Dr D. Lei after a consultation held on 7–11 November 2011 in Beijing, China, attended by: Dr M. Baca-Estrada, Health Canada, Canada; Ms H. Bai, State Food and Drug Administration, China; Mr M. Contorni, Novartis Vaccines and Diagnostics, Italy; Dr P. Desmons, GlaxoSmithKline Biologicals, China; Dr R. Dobbelaer, Consultant, Belgium; Dr R. Dominguez Morales, World Health Organization, Switzerland; Mrs L. Du, Beijing Luzhu Biopharmaceutical Company, China; Dr S. Gairola, Serum Institute of India, India; Mr S. Goel, Central Drugs Laboratory, India; Dr C. Hernandez, World Health Organization, Switzerland; Mrs Q. Hou, National Institutes for Food and Drug Control, China; Dr. K. Farizo, United States Food and Drug Administration, USA; Dr K. Friedrich, Instituto Nacional de Controle de Qualidade em Saude, Brazil; Mrs H. Han, Crucell Korea, Republic of Korea; Dr L. Hiep, Institute of Vaccines and Medical Biologicals, Viet Nam; Dr X. Hong, Chinese Pharmacopoeia Commission, China; Mr A. Horita, Chemo-Sero-Therapeutic Research Institute, Japan; Dr M. Iwaki, National Institute of Infectious Diseases, Japan; Mr Q. Jiang, Changchun Chang Sheng Life Sciences, China; Dr B. Kim, Korea Food and Drug Administration, Republic of Korea; Dr J. Kim, Korea Food and Drug Administration, Republic of Korea; Dr I. Knezevic, World Health Organization, Switzerland; Dr D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Mrs F. Lan, Chengdu Institute of Biological Products, China; Dr H. Langar, Regional Office for the Eastern Mediterranean, World Health Organization, Egypt; Dr H. Lechner, Paul-Ehrlich-Institut, Germany; Dr D. Lei, World Health Organization, Switzerland; Dr F. Li,

National Institutes for Food and Drug Control, China; Mr J. Liu, Beijing Minhai Biotechnology Company, China; Dr J. Luo, Center for Drug Evaluation, China; Dr K. Markey, National Institute for Biological Standards and Control, England; Dr F. Mawas-Kossaibati, National Institute for Biological Standards and Control, England; Dr B. Meade, Meade Biologics, USA; Mr P.V.V.S Murthy, Biological E. Limited, India; Dr S. Pakzad, Food and Drug Control Laboratory, Islamic Republic of Iran; Dr D. Pfeifer, Regional Office for Europe, World Health Organization, Denmark; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Mr M. Qin, Chengdu Institute of Biological Products, China; Mr M. Rahman, Panacea Biotec, India; Dr D. Sesardic, National Institute for Biological Standards and Control, England; Mrs J. Shi, Yuxi Walvax Biotechnological Company, China; Dr J. Shu, Sanofi Pasteur, China; Dr P. Stickings, National Institute for Biological Standards and Control, England; Mrs C. Tan, Chengdu Institute of Biological Products, China; Dr J. Tresnabudi, BioFarma, Indonesia; Dr J. Wang, National Institutes of Food and Drug Control, China; Mrs X. Wang, Chinese Pharmacopoeia Commission, China; Mr H. Wei, Changchun Institute of Biological Products, China; Mrs Z. Xiao, Beijing Tiantan Biological Products Company, China; Dr D. Xing, National Institute for Biological Standards and Control, England; Dr M. Xu, National Institutes for Food and Drug Control, China; Mr B. Yang, Wuhan Institute of Biological Products, China; Mr M. Yang, Lanzhou Institute of Biological Products, China; Mr. H. Yin, Center for Drug Evaluation, China; and Dr S. Zhang, State Food and Drug Administration, China.

The third and fourth drafts of the Recommendations were prepared by Dr R. Dobbelaer, Dr B. Meade and Dr D. Lei on the basis of comments received from participants in the consultation. Special acknowledgements are due to Dr M. Powell, Dr K. Farizo and Dr K. Goetz for their critical review and comments on the revised Recommendations.

The fifth draft was prepared on the basis of comments received from national regulators, the vaccine industry and the general public during a period of public consultation on the WHO web site.

References

- Requirements for diphtheria, tetanus, pertussis and combined vaccines (revised 1989). In: WHO Expert Committee on Biological Standardization. Fortieth report. Geneva, World Health Organization, 1990 (WHO Technical Report Series, No. 800), Annex 2.
- 2. Recommendations to assure the quality, safety and efficacy of diphtheria vaccines. In: WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2014 (WHO Technical Report Series, No. 980), Annex 4.
- Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed). In: WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2014 (WHO Technical Report Series, No. 980), Annex 5.

- 4. Recommendations for whole-cell pertussis vaccine. In: *WHO Expert Committee on Biological Standardization. Fifty-sixth report*. Geneva, World Health Organization, 2007 (WHO Technical Report Series, No. 941), Annex 6.
- Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines. In: WHO Expert Committee on Biological Standardization. Sixty-second report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 979), Annex 4.
- 6. Recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines. In: WHO Expert Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 4.
- 7. Recommendations for the production and control of poliomyelitis vaccine (inactivated). In: WHO Expert Committee on Biological Standardization. Fifty-first report. Geneva, World Health Organization, 2002 (WHO Technical Report Series, No. 910), Annex 2.
- 8. Recommendations for the production and control of *Haemophilus influenzae* type b conjugate vaccines. In: *WHO Expert Committee on Biological Standardization. Forty-ninth report*. Geneva, World Health Organization, 2000 (WHO Technical Report Series, No. 897), Annex 1.
- 9. Recommendations for diphtheria, tetanus, pertussis and combined vaccines (Amendments 2003). In: *WHO Expert Committee on Biological Standardization. Fifty-fourth report*. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 5.
- 10. Manual for quality control of diphtheria, tetanus and pertussis vaccines. Geneva, World Health Organization, 2013 (document WHO/IVB/11.11).
- 11. WHO guidelines on nonclinical evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-fourth report. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.
- 12. Guidelines on clinical evaluation of vaccines: regulatory expectations. In: *WHO Expert Committee on Biological Standardization. Fifty-second report*. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 924), Annex 1.
- 13. Procedure for assessing the acceptability, in principle, of vaccines for purchase by United Nations agencies. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 6.
- 14. Immunization Practices Advisory Committee (IPAC) web site (http://www.who.int/immunization_delivery/systems_policy/ipac/en/, accessed 12 July 2013).
- 15. Dagan R et al. Reduced response to multiple vaccines sharing common protein epitopes that are administered simultaneously to infants. *Infection and Immunity*, 1998, 66:2093–2098.
- Menactra. Comparative Safety and Immunogenicity Study in Adults 18–55 year old: Clinical Study MTA-09. Silver Spring, MD, United States Food and Drug Administration, 2005 (http://www.fda. gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM218550.pdf, accessed 12 July 2013).
- 17. Stickings P et al. Collaborative study for the calibration of a replacement international standard for diphtheria toxoid adsorbed. *Biologicals*, 2010, 38:529–538.
- 18. Stickings P et al. Animal refinement and reduction: alternative approaches for potency testing of diphtheria and tetanus vaccines. *Procedia in Vaccinology*, 2011, 5:200–212.
- 19. Winsnes R, Hendricksen C. Collaborative study for the validation of serological methods for potency testing of tetanus toxoid vaccines for human use (Part 1). *Pharmeuropa Special Issue Biologicals*, 2000, 1:82–124.

- 20. Winsnes R et al. Collaborative study for the validation of serological methods for potency testing of diphtheria toxoid vaccine (part 2). *Pharmeuropa Bio & Scientific Notes*, 2006, 2006(1):73–88.
- 21. Winsnes R et al. Collaborative study on a guinea pig serological method for the assay of acellular pertussis vaccines. *Pharmeuropa Bio & Scientific Notes*, 2009, 2009(1):27–40.
- WHO manual for the establishment of national and other secondary standards for vaccines. Geneva, World Health Organization, 2011 (WHO/IVB/11.03) (http://whqlibdoc.who.int/hq/2011/WHO_IVB_11.03_eng.pdf, accessed 12 July 2013).
- 23. Good manufacturing practices: main principles for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 3.
- 24. Good manufacturing practices for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 1.
- Guidelines on stability evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-seventh report. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 962), Annex 3.
- 26. Sawyer LA et al. Deleterious effect of thimerosal on the potency of inactivated poliovirus vaccine. *Vaccine*, 1994, 12:851–856.
- 27. WHO Policy Statement: the use of opened multi-dose vials of vaccine in subsequent immunization sessions. Geneva, World Health Organization, 2000 (WHO/V&B/00.09) (http://whqlibdoc.who.int/hq/2000/WHO_V&B_00.09.pdf, accessed 12 July 2013).
- Aluminium hydroxide, for adsorption. In: European Pharmacopoeia, volume 6.0, Strasbourg, European Directorate for Quality of Medicines & HealthCare, 2008 (01/2008:1664 p1150).
- Note for guidance on pharmaceutical and biological aspects of combined vaccines. London, European Medicines Agency, 1998 (CHMP/BWP/477/97).
- Borrow R, Balmer P, Roper MH. Module 3: tetanus update 2006. Geneva, World Health Organization, 2007 (The immunological basis for immunization series).
- Scheifele DW, Ochnio JJ. Module 2: diphtheria update 2009. Geneva, World Health Organization, 2009 (The immunological basis for immunization series).
- 32. Assay of tetanus vaccine (adsorbed). In: *European Pharmacopoeia*, 7th ed. Strasbourg, European Directorate for Quality of Medicines & HealthCare, 2011 (01/2011:20708).
- 33. Assay of pertussis vaccine. In: *European Pharmacopoeia*, volume 6.0, Strasbourg, European Directorate for Quality of Medicines & HealthCare 2008 (01/2008:20707) p222.
- 34. Assay of diphtheria vaccine (adsorbed). In: *European Pharmacopeia* volume 6.0, Strasbourg, European Directorate for Quality of Medicines & HealthCare 2008 (01/2008:20706) p217.
- 35. Guidelines for independent lot release of vaccines by regulatory authorities. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 2.
- 36. Guideline for Good Clinical Practice. E6(R1). ICH Harmonized Tripartite Guideline 1996. Geneva, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 1996 (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E6_R1/Step4/E6_R1__Guideline.pdf, accessed 13 July 2013).
- 37. Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines. In: WHO Expert Committee on Biological Standardization. Sixtieth report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 977), Annex 3.

- 38. Di Giovine P et al. External quality assessment for the determination of diphtheria antitoxin in human serum. *Clinical and Vaccine Immunology*, 2010, 17:1282–1290.
- 39. Efstratiou A, Maple PAC. *Diphtheria: Manual for the laboratory diagnosis of diphtheria.* Copenhagen, World Health Organization Regional Office for Europe, 1994 (http://whqlibdoc.who.int/euro/1994-97/ICP_EPI_038_%28C%29.pdf, accessed 13 July 2013).
- 40. Miyamura K et al. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. I. Studies on factors affecting the toxin and antitoxin titration. *Journal of Biological Standardization*, 1974, 2:189–201.
- 41. Walory J, Grzesiowski P, Hryniewicz W. Comparison of four serological methods for the detection of diphtheria anti-toxin antibody. *Journal of Immunological Methods*, 2000, 245:55–65.
- 42. Meade BD et al. Description and evaluation of serologic assays used in a multicenter trial of acellular pertussis vaccines. *Pediatrics*, 1995, 96:570–575.
- 43. Meade BD et al. Relationships between functional assays and enzyme immunoassays as measurements of responses to acellular and whole-cell pertussis vaccines. *Pediatrics*, 1995, 96:595–600.
- 44. Tondella ML et al. International Bordetella pertussis assay standardization and harmonization meeting report. Centers for Disease Control and Prevention, Atlanta, Georgia, United States, 19–20 July 2007. *Vaccine*, 2009, 27:803–814.
- 45. Xing D et al. Characterization of reference materials for human antiserum to pertussis antigens by an international collaborative study. *Clinical and Vaccine Immunology*, 2009, 16:303–311.
- 46. Sutter RW et al. Defining surrogate serologic tests with respect to predicting protective vaccine efficacy: poliovirus vaccination. *Annals of the New York Academy of Sciences*, 1995, 754:289–299.
- 47. Robbins JB et al. Quantitative measurement of "natural" and immunization-induced *Haemophilus influenzae* type b capsular polysaccharide antibodies. *Pediatric Research*, 1973, 7:103–110.
- 48. Anderson P. The protective level of serum antibodies to the capsular polysaccharide of *Haemophilus influenzae* type b. *Journal of Infectious Diseases*, 1984, 149:1034–1035.
- 49. Peltola H et al. *Haemophilus influenzae* type b capsular polysaccharide vaccine in children: a double-blind field study of 100,000 vaccinees 3 months to 5 years of age in Finland. *Pediatrics*, 1977, 60:730–737.
- 50. Horne AD et al. Analysis of studies to evaluate immune response to combination vaccines. *Clinical Infectious Diseases*, 2001, 33(Suppl. 4):S306–S311.
- 51. Reed GF, Meade BD, Steinhoff MC. The reverse cumulative distribution plot: a graphic method for exploratory analysis of antibody data. *Pediatrics*, 1995, 96:600–603.
- 52. Granstrom M et al. Scientific panel on childhood immunisation schedule: Diphtheria-tetanus-pertussis (DTP) vaccination. In: *ECDC guidance*. Stockholm, European Centre for Disease Prevention and Control, 2009 (http://www.ecdc.europa.eu/en/publications/Publications/0911_GUI_Scientific_Panel_on_Childhood_Immunisation_DTP.pdf, accessed 13 July 2013).
- 53. Hanley JA, Lippman-Hand A. If nothing goes wrong, is everything all right? Interpreting zero numerators. *Journal of the American Medical Association*, 1983, 249:1743–1745.
- 54. Guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 2.

Appendix 1

Model protocol for the manufacturing and control of DT-based combined vaccines

The following protocol is intended for guidance. It indicates the information that should be provided as a minimum by a manufacturer to the NRA. Information and tests may be added or omitted as necessary, with the authorization of the NRA.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO Recommendations for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that will accompany the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot-release certificate from the NRA or from the NCL in the country where the vaccine was produced or released stating that the product meets national requirements as well as the recommendations in Part A of this annex.

The following summary protocol is given as an example for a combined vaccine that consists of a freeze-dried Hib component to be reconstituted with a liquid D, T, aP or wP, IPV and HepB component.

A summary protocol for the Hib component has also been provided below as a separate appendix (Appendix 1a). This is done solely for the purpose of simplifying the layout of the guideline. The information provided by the manufacturer in individual protocols should not use cross-references between different products.

1. Summary information on finished product (final lot)

International name:
Trade name/commercial name:
Product licence (marketing authorization) number:
Country:
Name and address of manufacturer:
Name and address of licence holder,
if different:
Final packaging lot number:
Type of container:

Number of containers in this packaging: Final container lot number: Number of filled containers in this final lot: Date of manufacture: Description of final product (adsorbed): Preservative, and nominal concentration: Volume of each single human dose:
Number of doses per final container:
Summary of the composition (include a summary of the qualitative and quantitative composition of the vaccine in each human dose, including any adjuvant used and other excipients):
Shelf-life approved (months):
Expiry date:
Storage conditions:
The following sections are intended for recording the results of the tests performed during the production of the vaccine so that the complete document will provide evidence of consistency in production. If any test has to be repeated, this must be indicated. Any abnormal result must be recorded on a separate sheet.
Production information
Site(s) of manufacture for each production stage
of each component:
Date(s) of manufacture:

2. Detailed information on manufacture and control Starting materials or source materials, and bulk antigens

production stages, identification numbers and blending scheme.

• For a D component: refer to the sections on "Production strain and seed lots", "Single harvests" and "Bulk purified toxoid" in the corresponding WHO Recommendations (1).

Summary information on lot-specific production data, including dates of different

• For a T component: refer to the sections on "Production strain and seed lots", "Single harvests" and "Bulk purified toxoid" in the corresponding WHO Recommendations (2).

- For an aP component: refer to the sections on "Strain", "Culture media for production", "Control of antigen purification", "Test on purified antigens", "Detoxification" and "Control of bulk materials" in the corresponding WHO Recommendations (3).
- For a wP component: refer to the sections on "Strains" and "Control of single harvests" in WHO Recommendations for whole-cell pertussis vaccine (4).
- For an IPV component: for information on virus-seed lots, cell cultures and serum for cell cultures refer to the section on "Control of source materials"; and for information on single harvests and monovalent pools refer to the section on "Control of vaccine production" in the corresponding WHO Recommendations (5).
- For a HepB component: refer to the sections on "Cell substrate for antigen production", "Fermentation", "Single harvests (or pools)" and "Control of aqueous bulk (purified antigen)" in the corresponding WHO Recommendations (6).
- For a Hib component: refer to the relevant sections in Appendix 1a below on the Hib component of the generic summary protocol for the production and testing of a combined vaccine.

Adsorbed bulk concentrates (individual or combined components as applicable)

Lot number(s):
Date(s) of adsorption:
Volume(s), storage temperature, storage time and
duration of approved storage period:
Report results of tests for each adsorbed bulk concentrate
Completeness of adsorption
Method:
Specification:
Date:
Result:
Final bulk vaccine (D, T, aP, IPV, HepB)
Lot number:
Date(s) of manufacture:
Volume(s), storage temperature, storage time and
duration of approved storage period:

Information on composition of the final bulk: specify the relevant (adsorption, blending) production dates, reference number(s), volume(s) and concentrations (in Lf/ml for each of diphtheria and tetanus; in $\mu g/ml$ for the aP component; in DU/ml of D antigen of the IPV component; and in $\mu g/ml$ of HBsAg for the HepB component).

Blending	Identification	Prescription (SHD)	Added
Tetanus toxoid (Lf):			
Diphtheria toxoid (Lf):			
aP (μg/ml):			
IPV D antigen (DU/ml):			
HBsAg (μg/ml):			
Adjuvant (mg):			
Preservative (specify):			
Others (salt):		· 	
Final volume (ml):			
, ,			
Appearance			
Method:			
Specification:			
Date of test:			
Result:			
pН			
Method:			
Specification:			
Date of test:			
Result:			
icourt.			
Aluminium content			
Method:			
Specification: Date of test:			
Result:			
Result:			
Osmolality			
Method:			
Specification:			
Date of test:			
Result:			

Antimicrobial preservative
Method:
Specification:
Date of test:
Result:
Residual bovine serum albumin
Method:
Specification:
Date of test:
Result:
Free formaldehyde
Method:
Specification:
Date of test:
Result:
Test for sterility
Method:
Media:
Volume inoculated:
Date of start of test:
Date of end of test:
Result:
Absence of residual activity of pertussis toxin
Specify the number, strain and sex of animals used – this test is not necessary for
a product obtained by genetic modification:
Method:
Dose:
Specification:
Date of start of test:
Date of end of test:
Result:



Reversion to toxicity of pertussis toxin Specify the dates of the beginning and end of incubation, and the numbe and sex of animals used – this test is not necessary for a product obta genetic modification:	
Method:	
Dose:	
Specification:	
Date of start of test:	
Date of end of test:	
Result:	
for IPV and HepB components Specify the strain, sex, weight range and number of animals used; the volumes, route and doses used for immunization and challenge or bleed nature, lot number and potency of the reference vaccine; and the respectable dose. Express results in International Units (IUs) where applicate specify the confidence interval, slope of the parallel line model and the of tests for the absence of linearity and parallelism:	ing; the onses at ole; and
Method:	
Specification:	
Date of start of test:	
Date of end of test:	
Result:	
For the IPV in vivo assay (where applicable)	
Species, strain, sex, and weight range:	
Date of vaccination:	
Lot number of reference vaccine:	
Vaccine doses:	
Date of bleeding:	
Date of assay:	
Number of animals responding at each dose:	
ED ₅₀ of reference and test vaccines:	
Potency of test vaccine:	
Validity criteria (linearity, parallelism, precision,	
ED ₅₀ between highest and lowest responses):	

Results:

In vitro assay, depending on the licence dossier, for IPV and HepB components
Method:
Reference preparation:
Specification:
Validity criteria (linearity, parallelism):
Date of start of test:
Date of end of test:
Result:
Final bulk vaccine (Hib)
Refer to the section on "Final bulk" in Appendix 1a below for the Hib component
of this model protocol for the production and testing of a combined vaccine.
Final lot
For the D, T, aP, IPV, HepB vaccine
Lot number:
Date of filling:
Type of container:
Number of containers remaining after inspection:
Filling volume:
Appagagas
Appearance Method:
Specification: Date of test:
Result:
icsuit.
Identity of each component
Method:
Specification:
Date of test:
Result:
Degree of adsorption for each component
Method:
Specification:
Date of test:
Result:
Extractable volume
Method:
Specification:

Date of test:	
Result:	
рН	
Method:	
Specification:	
Date of test:	
Result:	_
Aluminium content	
Method:	_
Specification:	_
Date of test:	_
Result:	_
Test for sterility	
Method:	_
Media:	_
Volume inoculated:	_
Date of start of test:	_
Date of end of test:	_
Result:	_
Bacterial endotoxins	
Method:	_
Specification:	_
Date of test:	_
Result:	_
Preservative content	
Method:	_
Specification:	
Date of test:	
Result:	_
Osmolality	
Method:	
Specification:	_
Date of test:	_
Result:	

If performed at the final lot stage: in vivo assay for D, T, aP and, depending on marketing authorization, for IPV and HepB components Specify the strain, sex, weight range and number of animals used; the da volumes, route and doses used for immunization and challenge or bleeding; nature, lot number and potency of the reference vaccine; and the responses each dose. Express results in International Units (IUs) where applicable; a specify the confidence interval, slope of the parallel line model and the outco of tests for the absence of linearity and parallelism:
In vivo assay for IPV (where applicable) (if not performed on the final bulk)
Species, strain, sex, and weight range:
Date of vaccination:
Lot number of reference vaccine:
Vaccine doses:
Date of bleeding:
Date of assay:
Number of animals responding at each dose:
ED ₅₀ of reference and test vaccines:
Potency of test vaccine:
Validity criteria (linearity, parallelism, precision,
ED ₅₀ between highest and lowest responses):
Results:
In vitro assay, depending on the marketing authorization, for IPV
and HepB components
Method:
Reference preparation:
Specification:
Validity criteria (linearity, parallelism):
Date of start of test:
Date of end of test:
Result:
Date of start of period of validity:
For Hib component
Lot number:
Date of filling:

Appearance Method: Specification: Date of test: Result:
Identity
Method:
Specification:
Date of test:
Result:
Extractable volume
Method:
Specification:
Date of test:
Result:
100010
рН
Method:
Specification:
Date of test:
Result:
PRP content
Method:
Specification:
Date of test:
Result:
Aluminium
Method:
Specification:
Date of test:
Result:
Antimicrobial preservative
Method:
Specification:
Date of test:
Result:

Residual moisture (for freeze-dried preparations)	
Method:	
Specification:	
Date of test:	
Result:	
Test for sterility	
Method:	
Media:	
Volume inoculated:	
Date of start of test:	
Date of end of test:	
Result:	
Result.	
Pyrogenicity or bacterial endotoxins	
Method:	
Specification:	
Date of test:	
Result:	
Free purified polysaccharide	
Method:	
Specification:	
Date of test:	
Result:	
Stabilizer	
Method:	
Specification:	
Date of test:	
Result:	
Date of start of period of validity:	
Additional tests that may be performed on the final mixture	
(D, T, aP, IPV, HepB and Hib) if applicable	
Bacterial endotoxins	
Method:	
Specification:	
Date of test:	
Daylt.	

Osmolality
Method:
Specification:
Date of test:
Result:
рН
Method:
Specification:
Date of test:
Result:
Appearance
Method:
Specification:
Date of test:
Result:
Inspection of final containers
Date of inspection:
Organoleptic characteristics:
Number of containers inspected:
% of containers rejected:
3. Certification by the manufacturer
Name of the manufacturer
Name of head of production (typed)
Certification by the person from the control laboratory of the manufacturing company taking responsibility for the production and control of the vaccine
I certify that lot no of DT-based combined vaccine, whose number appears on the label of the final container, meets all national requirements and/or satisfies Part A¹ of the WHO Recommendations to assure the quality, safety and efficacy of DT-based combined vaccines (2014).²
SignatureName (typed)Date

¹ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

² WHO Technical Report Series, No. 980, Annex 6.

4. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 2), a label from a final container and an instruction leaflet for users.

Appendix 1a

Model protocol for the manufacturing and control of the Hib component of DT-based combined vaccines

Identification and source of starting materials (particularly any materials of human or animal origin – e.g. strain of bacteria; master and working seeds; excipients and preservatives).
Preparation date and reference number of seed lot(s). Date of approval of protocol indicating compliance with national requirements and with the marketing authorization.
Tests on starting materials:
Production details, in-process controls and dates of tests
Intermediate stages
Purified polysaccharide (PRP)
Lot number(s):
Date(s) of manufacture:
Quantities, storage temperature, storage time and duration of approved storage period:
Identity
Method:
Specification:
Date of test:
Result:
Moisture content
Method:
Specification:
Date of test:

•	7	
(
(7	,
	_	
(١
(X	
-	X	
	_	
	C	•
	Ì	
4	<	
	u	
	ğ	
٠	2	
	7	
	Ų	Ļ
	9	
٠,	٠.	
	C	
	Ś	۰
	Q	ì
-	٦,	,
	_	۰
-	7	
	ζ	
	5	
	Š	
•	-	•
	0	
1	V	Ļ
ľ	_	۰
(_	,
	=	
1	1	
-	-	

Molecular size distribution Method: Specification: Date of test: Result: Degree of polymerization Method: Specification: Date of test: Result: Result: Result: Result: Result: Result: Ribose content Method: Specification: Date of test: Result: Result: Result: Result: Result:
Specification: Date of test: Result: Degree of polymerization Method: Specification: Date of test: Result: Result: Result: Date of test:
Date of test: Result: Degree of polymerization Method: Specification: Date of test: Result: Ribose content Method: Specification: Date of test:
Result:
Degree of polymerization Method: Specification: Date of test: Result: Ribose content Method: Specification: Date of test:
Method: Specification: Date of test: Result: Ribose content Method: Specification: Date of test:
Method: Specification: Date of test: Result: Ribose content Method: Specification: Date of test:
Specification: Date of test: Result: Ribose content Method: Specification: Date of test:
Date of test: Result: Ribose content Method: Specification: Date of test:
Result:
Method: Specification: Date of test:
Method:Specification:
Specification: Date of test:
Date of test:
Recult
Result:
Phosphorus content
Method:
Specification:
Date of test:
Result:
Protein content
Method:
Specification:
Date of test:
Result:
Nucleic acid content
Method:
Specification:
Date of test:
Result:
Pyrogenicity or bacterial endotoxins
Method:
Specification:

Date of test:Result:
Residual reagents
Method:
Specification:
Date of test:
Result:
Carrier protein
Lot number(s):
Date(s) of manufacture:
Quantities, storage temperature, storage time and
duration of approved storage period:
For diphtheria toxoid or tetanus toxoid used as a carrier protein
Certification of production in compliance with corresponding WHO Recommendations for diphtheria vaccines (1) and for tetanus vaccines (2) – unless different requirements are approved for the antigenic purity for tetanus toxoid for use as a carrier protein.
Identity
Method:
Specification:
Date of test:
Result:
Test for sterility
Method:
Media:
Volume inoculated:
Date of start of test:
Date of end of test:
Result:
Specific toxicity of diphtheria toxin or tetanus toxin Method (specify Lf injected): Specification: Date of test:
Result.

Reversion to toxicity

Specify the dates of the beginning and end of incubation, the dates of the beginning and end of the test, the number of animals used, the volume inoculated into cell culture (for diphtheria only) or injected into animals, the number of animals used (if relevant), and the test results.

animals used (if relevant), and the test results.
Method (specify Lf injected):
Specification:
Date of test:
Result:
Antigenic purity
Method:
Specification:
Date of test:
Result (Lf/mg protein (nondialysable) nitrogen):
For diabeth aris protein CDM107
For diphtheria protein CRM197
Identity
Method:
Specification:
Date of test:
Result:
Test for sterility
Method:
Media:
Volume inoculated:
Date of start of test:
Date of end of test:
Result:
TOO GIVE
Purity
Method:
Specification:
Date of test:
Result:
Mount,
Toxicity
Method:
Specification

Date of test:
Result (Lf/mg protein (nondialysable) nitrogen):
For meningococcal group B outer membrane protein complex
Identity
Method:
Specification:
Date of test:
Result:
result.
Test for sterility
Method:
Media:
Volume inoculated:
Date of start of test:
Date of end of test:
Result:
100000
Lipopolysaccharide content
Method:
Specification:
Date of test:
Result:
Pyrogenicity
Method:
Specification:
Date of test:
Result:
Bulk conjugate
Lot number(s):
Date(s) of manufacture:
Volume(s), storage temperature, storage time and
duration of approved storage period:
PRP content
Method:
Specification:
Date of test:
Result:

Protein content
Method:
Specification:
Date of test:
Result:
100000
PRP to protein ratio
Method:
Specification:
Date of test:
Result:
Molecular size distribution
Method:
Specification:
Date of test:
Result:
Free PRP
Method:
Specification:
Date of test:
Result:
Free carrier protein
Method:
Specification:
Date of test:
Result:
Totali.
Unreacted functional groups
Method:
Specification:
Date of test:
Result:
Residual reagents
Method:
Specification:
Date of test:
Result:

Test for sterility
Method:
Media:
Volume inoculated:
Date of start of test:
Date of end of test:
Result:
Final bulk vaccine
Lot number:
Date of manufacture:
Volume, storage temperature, storage time and
duration of approved storage period:
Test for sterility
Method:
Media:
Volume inoculated:
Date of start of test:
Date of end of test:
Result:
Antimicrobial preservative Method:
Specification:
Date of test:
Result:

References

- 1. Recommendations to assure the quality, safety and efficacy of diphtheria vaccines. In: WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2014 (WHO Technical Report Series, No. 980), Annex 4.
- 2. Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed). In: WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2014 (WHO Technical Report Series, No. 980), Annex 5.
- 3. Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines. In: WHO Expert Committee on Biological Standardization. Sixty-second report. Geneva, World Health Organization, 2014 (WHO Technical Report Series, No. 979), Annex 4.
- 4. Recommendations for whole-cell pertussis vaccine. In: WHO Expert Committee on Biological Standardization. Fifty-sixth report. Geneva, World Health Organization, 2007 (WHO Technical Report Series, No. 941), Annex 6.

WHO Technical Report Series No. 980, 2014

- 5. Recommendations for the production and control of poliomyelitis vaccine (inactivated). In: WHO Expert Committee on Biological Standardization. Fifty-first report. Geneva, World Health Organization, 2002 (WHO Technical Report Series, No. 910), Annex 2.
- 6. Recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines. In: WHO Expert Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 4.

Appendix 2

Model certificate for the release of DT-based combined vaccines by NRAs

Lot-release certificate
Certificate no
The following lot(s) of combined vaccine produced b
labels of the final containers, complies with the relevant national specification and provisions for the release of biological products ³ and Part A ⁴ of the WHO Recommendations to assure the quality, safety and efficacy of DT-base combined vaccines (2014), ⁵ and with corresponding WHO recommendation for each of the vaccine's individual components, as well as with WHO goo manufacturing practices: main principles for pharmaceutical products; ⁶ Goo manufacturing practices for biological products; ⁷ and Guidelines for independent lot release of vaccines by regulatory authorities. ⁸
The release decision is based on

The certificate may include the following information:

- name and address of manufacturer
- site(s) of manufacturing
- trade name and common name of product
- marketing authorization number

¹ Name of manufacturer.

² Country of origin.

³ If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.

With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

⁵ WHO Technical Report Series, No. 980, Annex 6.

⁶ WHO Technical Report Series, No. 961, Annex 3.

⁷ WHO Technical Report Series, No. 822, Annex 1.

⁸ WHO Technical Report Series, No. 978, Annex 2.

⁹ Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.

WHO Technical Report Series No. 980, 2014

- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)
- type of container used
- number of doses per container
- number of containers or lot size
- date of start of period of validity (e.g. manufacturing date) and/or expiry date
- storage conditions
- signature and function of the person authorized to issue the certificate
- date of issue of certificate
- certificate number.

The Director o	f the NRA (or other appi	ropriate aut	hority)
----------------	-------------	---------------	--------------	---------

Name (typed)			
Signature			
Date			

Appendix 3

Examples of international names, proper names and abbreviations of combined vaccines

DT-based combined vaccines¹

- diphtheria and tetanus vaccine (adsorbed) abbreviation: DT;
- diphtheria and tetanus vaccine (adsorbed, reduced diphtheria antigen content) – abbreviation: dT;
- diphtheria and tetanus vaccine (adsorbed, reduced diphtheria and tetanus antigen content) – abbreviation: dt;
- diphtheria, tetanus and whole cell pertussis vaccine (adsorbed) abbreviation: DTwP;
- diphtheria, tetanus and acellular pertussis vaccine (adsorbed) abbreviation: DTaP;
- diphtheria, tetanus and acellular pertussis vaccine (adsorbed, reduced diphtheria, tetanus and acellular pertussis antigen content) – abbreviation: dtap.

DTwP-based combined vaccines

- diphtheria, tetanus, whole-cell pertussis and poliomyelitis (inactivated) vaccine (adsorbed) – abbreviation: DTwP-IPV;
- diphtheria, tetanus, whole-cell pertussis and recombinant hepatitis B vaccine (adsorbed) – abbreviation: DTwP-HepB;
- diphtheria, tetanus, whole-cell pertussis, recombinant hepatitis B vaccine and poliomyelitis (inactivated) vaccine (adsorbed) – abbreviation: DTwP-HepB-IPV.

DTwP-based combined vaccines with Hib

diphtheria, tetanus, pertussis and *Haemophilus influenzae* type b
 (X-) conjugate vaccine (adsorbed); fully liquid or lyo-liquid –
 abbreviation: DTwP-Hib_x or DTwP+Hib_x;²

¹ The acellular pertussis vaccine component of the combination vaccine may be produced by purification or co-purification of the acellular pertussis components. In accordance with section A.1.1 of WHO Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines, the international name for this component is "acellular pertussis vaccine" in both cases.

² Subscript "X" denotes the carrier protein – e.g. tetanus toxoid or CRM197.

- diphtheria, tetanus, pertussis, poliomyelitis (inactivated) and
 Haemophilus influenzae type b (X-) conjugate vaccine (adsorbed)
 (all-in-one or with separate freeze-dried Hib) abbreviation:
 DTwP-IPV-Hib_x or DTwP-IPV+Hib_x;
- diphtheria, tetanus, pertussis, hepatitis B and Haemophilus influenzae type b (X-) conjugate vaccine (adsorbed) (all-in-one or with separate freeze-dried Hib) – abbreviation: DTwP-HepB-Hib_X or DTwP-HepB+Hib_X;
- diphtheria, tetanus, pertussis, hepatitis b, poliomyelitis (inactivated) and *Haemophilus influenzae* type b (X-) conjugate vaccine (adsorbed) (all-in-one or with separate freeze-dried Hib) abbreviation:
 DTwP-HepB-IPV-Hibx or DTwP-HepB-IPV+Hibx.

DTaP-based combined vaccines

- diphtheria, tetanus, acellular pertussis and poliomyelitis (inactivated)
 vaccine (adsorbed) abbreviation: DTaP-IPV;
- diphtheria, tetanus, acellular pertussis and recombinant hepatitis B vaccine (adsorbed) – abbreviation: DTaP-HepB;
- diphtheria, tetanus, acellular pertussis, recombinant hepatitis b and poliomyelitis (inactivated) vaccine (adsorbed) – abbreviation: DTaP-HepB-IPV.

DTaP-based combined vaccines with Hib

- diphtheria, tetanus, acellular pertussis and *Haemophilus influenzae* type b (X-) conjugate vaccine (adsorbed) (all-in-one or with separate freeze-dried Hib) abbreviation: DTaP-Hib_x or DTaP+Hib_x;
- diphtheria, tetanus, acellular pertussis, poliomyelitis (inactivated) and *Haemophilus influenzae* type b (X-) conjugate vaccine (adsorbed) (all-in-one or with separate freeze-dried Hib) – abbreviation: DTaP-IPV-Hib_x or DTaP-IPV+Hib_x;
- diphtheria, tetanus, acellular pertussis, recombinant hepatitis B and Haemophilus influenzae type b (X-) conjugate vaccine (adsorbed) (all-in-one or with separate freeze-dried Hib) abbreviation:
 DTaP-HepB-Hib_x or DTaP-HepB+Hib_x;
- diphtheria, tetanus, acellular pertussis, hepatitis b, poliomyelitis (inactivated) and *Haemophilus influenzae* type b (X-) conjugate vaccine (adsorbed) (all-in-one or with separate freeze-dried Hib) – abbreviation: DTaP-HepB-IPV-Hib_x or DTaP-HepB-IPV+Hib_x.

Annex 7

Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use

Replacement of Annex 3 of WHO Technical Report Series, 910

Introdu	ıction	409
Genera	l considerations	410
Part A.	Manufacturing recommendations	413
A.1	Definitions	413
	General manufacturing recommendations	416
	Control of source materials	416
A.4	Control of vaccine production	427
A.5	Filling and containers	433
A.6	Control of final lot	434
A.7	Records	436
A.8	Retained samples	436
	Labelling	436
	0 Distribution and transport	437
A.1	1 Stability, storage and expiry date	437
Part B.	Nonclinical evaluation of Japanese encephalitis vaccines	
(live, at	tenuated) for human use	438
B.1	General principles	438
B.2	Product characterization and process development	439
	Nonclinical immunogenicity and protection	439
B.4	Nonclinical toxicity and safety	440
B.5	Characterization of vaccine virus in vector mosquitoes	443
Part C.	Clinical evaluation of Japanese encephalitis vaccines	
(live, at	tenuated) for human use	444
C.1	General considerations for clinical studies	444
C.2	Assessment of immunogenicity in humans	445
	Safety	449
	Post-licensure investigations	450
Part D.	Environmental risk assessment of Japanese encephalitis	
vaccine	es (live, attenuated) for human use derived by recombinant DNA	
techno	logy	451
D.1	Introduction	451
D.2		452

Part E. Recommendations for NRAs			456
	E.1 General E.2 Release and certification by the NRA		456 456
Autl	thors		456
Ack	cnowledgements		457
Refe	erences		458
Арр	pendix 1		
	Passage history of Japanese encephalitis SA14-14-2 virus		463
Арр	pendix 2		
	Derivation of Japanese encephalitis–yellow fever chimeric	virus (CV) vaccine	464
Арр	pendix 3		
	Production and passage level of live-attenuated Japanese e	encephalitis vaccine	465
Арр	pendix 4		
	Model protocol for the manufacturing and control of Japan vaccines (live, attenuated) for human use	ese encephalitis	466
Арр	pendix 5		
	Model certificate for the release of Japanese encephalitis va (live, attenuated) for human use by NRAs	accines	485

Recommendations published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes recommendations for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments or examples intended to provide additional guidance to manufacturers and NRAs.

Introduction

These WHO Recommendations are intended to provide national regulatory authorities (NRAs) and vaccine manufacturers with guidance on evaluating the quality, safety and efficacy of live-attenuated Japanese encephalitis (JE) vaccines for use in humans to facilitate their international licensure and use.

The Recommendations replace the WHO Guidelines for the production and control of Japanese encephalitis vaccine (live) for human use (1), and should be read in conjunction with the other WHO Recommendations and Guidelines referred to in each part.

The scope of these Recommendations is limited to live-attenuated JE vaccines for human use. Other types of JE vaccines are outside the scope of these Recommendations. Revised WHO Recommendations for inactivated JE vaccines (2) are available separately.

Given the advances made in the immunization of humans with liveattenuated vaccines to prevent JE, there is increased interest in defining quality standards for the vaccines' manufacture, developing guidelines for nonclinical studies, and in conducting clinical studies that will assess the vaccines' efficacy and safety in humans as well as the risks the vaccine poses to public health and the environment. To this end, a group of international experts met in February 2012 to review the latest advances in this field, and to propose a revision of the 2002 Guidelines (1). The main changes introduced in this revision include:

- updating information on monitoring the use of animals from which primary hamster kidney cells are prepared;
- updating information on methods for testing and specifications for primary hamster kidney cell cultures used in vaccine production, according to the revised recommendations on cell substrates;
- updating information on testing for the attenuation of SA14-14-2 vaccine;
- the addition of recommendations on evaluating lot-release potency specifications, and information on the need for monitoring the upper limit of potency in addition to the existing minimum potency specification for the immunizing dose;
- the addition of information on the relationship between passage levels of vaccine seeds and production in the current production scheme for live JE vaccines;
- the addition of new specifications for the manufacture and quality control of a live JE vaccine based on an attenuated strain of yellow fever virus used as a viral vector and grown in Vero cell cultures;

- the addition of guidelines for the nonclinical and clinical evaluation of new, live JE vaccines;
- the addition of guidelines for assessing the environmental risk of live JE vaccines derived using recombinant DNA technology;
- the addition of a model protocol for lot release, and a model certificate for vaccine release by NRAs.

These Recommendations are based on experience gained with liveattenuated JE vaccines that have been licensed through the procedures described below. The Recommendations will need to be updated as new data become available.

Part A sets out recommendations for manufacturing and quality control. Parts B, C and D provide guidelines specific to the nonclinical evaluation, clinical evaluation and environmental risk assessment of the vaccines, respectively. Part E provides recommendations for NRAs.

In the following section on general considerations, brief overviews of JE disease and vaccine development provide the scientific basis for formulating detailed technical recommendations (Parts A and E) and guidelines (Parts B, C and D).

General considerations

JE is caused by mosquito-borne Japanese encephalitis virus (JEV) infection, and is the most important viral encephalitis in Asia, accounting for at least 50 000 clinical cases, with 25–30% case-fatality rates annually (3). A study in 2011 estimated that approximately 67 900 JE cases typically occur each year in the 24 JE-endemic countries, giving an incidence of 1.8 cases per 100 000 overall population. Approximately 51 000 (75%) of these cases occur in children aged 0–14 years, which gives an estimated overall annual incidence of 5.4 per 100 000 in this age group (4). The high fatality rate and frequent residual neuropsychiatric sequelae in survivors make JE a considerable health problem. For example, a study from China demonstrated that significant neurological and overall functional disability were evident in a high proportion of JE survivors many years after infection, with 22% of JE patients having objective neurological deficits, and 28% having subnormal intelligence quotients (5).

Since the 1980s, JEV transmission has intensified in certain countries, and the disease has extended its geographical range to areas of Asia where it had not been previously recognized, as well as to northern Australia. Two epidemiological patterns of JEV infection are recognized. In northern temperate areas, JE occurs in summer epidemics, whereas in tropical areas, JE may occur all year round. In temperate zones and in the northern part of the tropical zone,

outbreaks have a marked seasonal incidence, occurring during the rainy season. In tropical areas, there is an endemic pattern of infection, with the occurrence of sporadic cases throughout the year. The incidence is highest in rural agricultural areas, and within that population it is highest in males because of their increased exposure to areas of rice cultivation.

JEV is a member of the genus Flavivirus, family Flaviviridae. JE disease was first reported in Japan in 1924, and subsequently reported in other Asian countries; it was first reported in Australia in 1955. JEV is the prototype of the JE antigenic complex (which includes the West Nile, Usutu, Murray Valley encephalitis and St Louis encephalitis viruses), and cross-reactions in neutralization antibody tests, and cross-protection in animals, have been demonstrated with other flaviviruses that are members of the complex. In the mouse model, considerable variation has been demonstrated in neurovirulence and peripheral pathogenicity. JEV has a genome comprising a positive-sense, single-stranded RNA molecule of approximately 11 kb that is capped at the 5'-end and is not polyadenylated at the 3'-end. It carries a single open-reading frame encoding a polyprotein that is processed into three structural proteins – core (C), membrane (M) and envelope (E) – and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), flanked by 5'- and 3'-nontranslated regions. Based on nucleotide sequencing of the C/PrM and E genes, five genotypes have been identified.

- Genotype I includes isolates from Australia, Cambodia, China and the Province of Taiwan, India, Japan, the Lao People's Democratic Republic, Malaysia, the Republic of Korea, Thailand and Viet Nam (1967–present).
- Genotype II includes isolates from Australia, Indonesia, Malaysia,
 Papua New Guinea, the Republic of Korea and Thailand (1951–1999).
- Genotype III includes isolates from mostly temperate regions in Asia – i.e. China and the Province of Taiwan, India, Indonesia, Japan, Malaysia, Myanmar, Nepal, the Philippines, the Republic of Korea, the Russian Federation, Sri Lanka, Thailand and Viet Nam (1935–present).
- Genotype IV includes isolates only from Indonesia (1980–1981).
- Genotype V virus was originally isolated from a human infected with JE in Malaysia and subsequently from mosquitoes in China and the Republic of Korea.

Until the later part of the 20th century, genotype III was the predominant JEV genotype involved in human infection. The genotypes of JEV have diverged in the order IV, III, II and I. Since the 1980s there has been a genotype replacement,

whereby genotype I is replacing genotype III as the dominant genotype. Although current JE vaccines are based on genotype III strains, all evidence indicates that these vaccines induce protective immunity to all genotypes of JEV.

JEV is maintained in an enzootic cycle, which typically involves culicine mosquitoes (primarily *Culex tritaeniorhynchus*) that breed in rice paddies, and domesticated swine or ardeidae (principally egrets and herons), which serve as virus-amplifying vertebrate hosts. Humans and other nonavian vertebrates are dead-end hosts because they fail to produce viraemias of sufficient titres to infect mosquitoes. However, infection of certain nonavian vertebrates, such as horses, can lead to clinical disease and encephalitis.

Humans of all ages are susceptible unless immunized by natural infection or vaccination. Evidence shows that effective vaccines will protect both animals and humans against clinical signs and disease. Although the control of mosquitoes and the vaccination of pigs are effective in certain circumstances, these measures are not practical means of preventing human illness. It is also important to recognize that humans are incidental hosts and for vaccination to be effective, coverage should be maintained indefinitely in all persons who may be exposed to the virus.

The virus replicates in a variety of cultured cells of vertebrate and nonvertebrate origin. Since the 1960s, both live and inactivated vaccines have been developed that provide active immunity against JEV. The development of these vaccines represented a major advance in the ability to control JEV infection and reduce the burden of disease. Viruses isolated from human patients in Japan in 1935 and in China in 1949 provided the prototype Nakayama and Beijing (Beijing-1) and P3 (Beijing-3) strains, respectively; these are the principal strains used in the production of inactivated JE vaccine. The SA14-14-2 strain, which was derived from a mosquito isolate, is widely used in the production of a liveattenuated JE vaccine, and is used as donor strain in a replicating recombinant vaccine. Systematic vaccination programmes – such as those in China (Province of Taiwan), Japan and the Republic of Korea - using an inactivated JE vaccine that meets international requirements, have controlled the disease to the point of elimination. However, in other countries the expense and complexity of producing the vaccine, and the need for repeated doses, have limited the use of this vaccine. In addition to the problems posed by multiple doses, use of the vaccine has been associated with hypersensitivity. A number of vaccine manufacturers have developed second-generation inactivated vaccines using African green monkey kidney-derived Vero cells.

As an alternative to inactivated vaccines, there are two different live-attenuated JE vaccines, namely the SA14-14-2-strain vaccine produced in primary hamster kidney (PHK) cells and the JE chimeric virus (JE-CV) vaccine produced in Vero cells. The SA14-14-2 vaccine was developed in China by empirical passage of a naturally occuring mosquito isolate (i.e. SA14) in mice,

hamster, chicken embryo and PHK cells; it is manufactured in PHK cells. Since its licensure in China in 1988, more than 300 000 000 doses of the live SA14-14-2 vaccine have been produced for administration to children during annual vaccination programmes. The vaccine is of considerable interest to countries where JEV is endemic and, as of 2012, had been licensed in Cambodia, the Democratic People's Republic of Korea, India, the Lao People's Democratic Republic, Myanmar, Nepal, the Republic of Korea, Sri Lanka and Thailand. The JE-CV vaccine is based on yellow fever (YF) vaccine strain 17D (YF-17D) that has been genetically modified to contain premembrane (prM) and E structural genes from JE vaccine strain SA14-14-2. The JE-CV vaccine is manufactured in Vero cells, and has been licensed in Australia and Thailand since 2010. JE-CV vaccine was previously known as ChimeriVax-JE and is now available under the tradenames IMOJEV and THAIJEV.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name should be Japanese encephalitis vaccine (live, attenuated) for human use. The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 Descriptive definition

A live-attenuated JE vaccine defined in section A.1.1 should contain live-attenuated JEVs or replication-competent vector viruses that encode genes to express the major structural antigen of JEVs. The vaccine may be presented as a sterile, aqueous suspension or as freeze-dried material. The preparation should satisfy all the specifications given below.

A.1.3 International reference materials

No international reference materials commonly applicable for already licensed live, attenuated JE vaccines are available either for potency testing or for neurovirulence testing.

A.1.4 Expression of dose related to vaccine potency

The potency of a live-virus vaccine is typically expressed in terms of the number of infectious units of virus contained in a human dose, using a specified tissue culture substrate and based on the results of phase I and phase II clinical trials.

In the case of live-attenuated JE vaccines, potency will have to be assessed in terms of virus infectivity titres. When product-specific international reference standards for different types of vaccines become available, the dose related to vaccine potency should be calculated against a product-specific standard, and should be expressed in product-specific International Units (IUs) if this results in a reduction in variation among laboratories. Until then, alternatives are to use plaque-forming units (PFUs) or the median cell culture infectious dose (CCID $_{50}$) to express the potency and dose of the vaccine. The dose should also serve as the basis for establishing parameters for stability and for the expiry date.

A.1.5 Terminology

The definitions given below apply to the terms as used in these Recommendations. They may have different meanings in other contexts.

Adventitious agents: contaminating microorganisms of the cell culture or source materials, including bacteria, fungi, mollicutes (mycoplasmas or spiroplasmas), mycobacteria, rickettsia, protozoa, parasites, agents causing transmissible spongiform encephalopathies (TSEs), and viruses that have been unintentionally introduced into the manufacturing process of a biological product.

Bulk material: one or more single harvests after clarification or purification, or both, from which the final bulk is prepared.

Cell bank: a collection of appropriate containers whose contents are of uniform composition and that are stored under defined conditions. Each container represents an aliquot of a single pool of cells.

Cell culture infectious dose 50%: the amount of a virus sufficient to cause a cytopathic effect in 50% of inoculated replicate cell cultures, as determined in an end-point dilution assay in monolayer cell cultures.

Cell seed: quantity of vials containing well characterized cells derived from a single tissue or cell of human or animal origin, stored frozen in liquid nitrogen in aliquots of uniform composition, one or more of which may be used for the production of a master cell bank.

Cell substrates: cells used for the production of a vaccine.

Final bulk: the finished vaccine prepared from virus harvest pools or bulk, or both, held in a single vessel from which the final containers are filled.

Final lot: a collection of sealed final containers of finished vaccine that is homogeneous with respect to the risk of contamination during filling and, if applicable, freeze-drying. Therefore, all of the final containers should have been filled from one vessel of final bulk and, if applicable, freeze-dried under standardized conditions in a common chamber during one working session.

Genetically modified organism: an organism in which the genetic material has been altered using recombinant DNA techniques (or genetic

engineering techniques) in a way that does not occur naturally by mating or natural recombination and selection.

Master cell bank: a quantity of well characterized cells of animal or other origin that have been derived from a cell seed at a specific population doubling level or passage level, dispensed into multiple containers, cryopreserved and stored frozen under defined conditions, such as the vapour or liquid phase of liquid nitrogen, in aliquots of uniform composition. The master cell bank is prepared from a single homogeneously mixed pool of cells. It is considered best practice for the master cell bank to be used to derive working cell banks.

Neuroinvasiveness: the ability of a virus to replicate in peripheral tissues, induce viraemia and invade the central nervous system (CNS) (6).

Neurovirulence: the ability of a virus to initiate cytopathic infection in the CNS and to cause encephalitis (6). In animal experimental settings, clinical, virological and histopathological evaluations are often carried out after intracerebral inoculation of a virus.

Plaque-forming unit (PFU): the amount of a virus sufficient to cause a single visible focus of infection due to cytopathic effect in a cell culture monolayer after cells have been properly stained.

Primary culture: a culture started from cells, tissues or organs taken directly from one or more organisms. A primary culture may be regarded as such until it is successfully subcultured for the first time. It then becomes a cell line if it can continue to be subcultured at least several times.

Production cell culture: a collection of cell cultures used for biological production that have been prepared together from one or more containers from the working cell bank, or in the case of primary cell cultures, from the tissues of one or more animals.

Single harvest: a quantity of virus suspension harvested from production cell cultures and inoculated with the same virus working seed, and processed together in a single production run.

Virus master seed: A suspension of vaccine virus that has been aliquoted into identical vials and stored at a temperature and under conditions deemed to stabilize the virus in each container. The virus master seed is used as a source of infectious virus for the generation of each virus working seed lot.

Virus pool: a suspension of two or more single harvests of the virus collected into a single vessel.

Virus working seed: A quantity of virus of uniform composition, well characterized and derived from a virus master seed lot (see above) in a production cell. The working seed lot is used for the production of a single harvest.

Working cell bank: a quantity of well characterized cells of animal or other origin that have been derived from the master cell bank at a specific population doubling level or passage level, dispensed into multiple containers,

cryopreserved and stored frozen under defined conditions, such as in the vapour or liquid phase of liquid nitrogen, in aliquots of uniform composition. The working cell bank is prepared from a single homogeneously mixed pool of cells. One or more of the working cell bank containers is used for each production culture.

A.2 General manufacturing recommendations

The general manufacturing recommendations contained in the WHO Good manufacturing practices for biological products (7) should be applied with the addition of the following recommendations.

- All staff directly involved in the production and testing of live JE vaccine should be shown to be immune to JEV by appropriate neutralizing antibody tests.
- Written descriptions of the standard operating procedures used for the preparation and testing of live JE vaccine, together with evidence of appropriate validation for each production step, should be submitted for approval to the NRA as part of the licensing application. Proposals for any modifications to the manufacturing or control methods should be submitted for approval to the NRA before they are implemented.
- Production steps and quality control operations involving manipulations of live virus should be conducted under the appropriate biosafety level agreed with the NRA, and in accordance with national biosafety laws.

A3 Control of source materials

A.3.1 Cell cultures for virus propagation

A.3.1.1 Conformity with WHO recommendations on cell substrates

Live JE viruses for vaccine production should be propagated in cell substrates that meet the WHO Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (8), and should be approved by the NRA. All information on the source and method of preparation of the cell culture system used should be made available to the NRA.

A.3.1.2 Types of cell cultures

Live JE vaccines have been produced in either PHK cells or a continuous cell line – i.e. Vero cells. Sections A.3.1.3 and A.3.1.4 apply, respectively, to each type of cell.

A.3.1.3 PHK cells

A.3.1.3.1 Animals

Syrian hamsters aged between 10 days and 14 days may be used as the source of kidneys for cell culture. Only hamster stock derived from a healthy colony that is free from specific pathogens should be used as the source of tissue. The colony forms a group of animals that shares a common environment and has its own caretakers, who have no contact with other animal colonies. The animals should be tested according to a defined programme to ensure freedom from specified pathogens and from the antibodies to those pathogens. At the time the colony is established, all animals should be tested and show no detectable antibodies to Hantaan virus, Kilham rat virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse hepatitis virus, mouse poliovirus, pneumonia virus of mice, respiratory enteric orphan virus type 3, Sendai virus (murine parainfluenza virus type 1), simian virus type 5 and Toolans H-a virus. In addition, lysates of primary kidney cells should be tested for the presence of specific pathogens by inoculating hamsters, mice and rats; this should be followed by tests to detect antibodies in the animals' serum samples. No antibodies should be detected. Detailed lists of pathogens to be screened for in hamster, mouse and rat antibody production tests are summarized elsewhere (9). A test for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse transcriptase (RTase) assay should also be included. The results of such assays may need to be interpreted with caution because RTase activity is not unique to retroviruses, and may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (10). Nucleic acid amplification tests for retroviruses may also be used. A PCR test for hamster polyomavirus should be used on a selected number of hamster tissues – especially kidney – to qualify the colony, and should be repeated at intervals thereafter. When new animals are introduced into the colony, they should be maintained in quarantine in vermin-proof quarters for a minimum of two months, and should be shown to be free from these specified pathogens.

The parents of animals to be used as a source of tissue should be maintained in vermin-proof quarters. Neither the parent hamsters nor their progeny should previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored at regular intervals for zoonotic viruses and markers of contamination by following a defined programme.

Once the colony has been established, it should be monitored by testing for antibodies to the relevant pathogens in a representative group of animals – consisting of at least 5% of the animals – that are bled at intervals acceptable to the NRA.

For example, birds used in the production of chick embryo fibroblast cells for measles vaccine (11) are bled at monthly intervals.

In addition, the colony should be screened for pathogenic bacteria (including mycobacteria), fungi and mycoplasmas, as agreed with the NRA. The screening tests should be carried out on a regular basis over a defined period. The sample size, tests, method and testing intervals should be agreed with the NRA.

Any animal that dies should be investigated to determine the cause of death. If the presence of an infectious agent is demonstrated in the colony, the NRA should be informed, and the manufacture of live JE vaccine may be discontinued. Manufacturing should not be resumed until a thorough investigation has been completed and precautions have been taken to prevent the presence of the infectious agent in both the colony and the product; manufacturing should be resumed only with the approval of the NRA.

At the time of harvesting the kidneys, the animals should be visually examined for any gross abnormalities. If any kidney abnormalities or other evidence of pathology (e.g. abnormal size, protein ascites) is found, animals with the abnormalities should not be used for vaccine production.

Each group of control cultures derived from a single group of animals used to produce a single virus harvest should remain identifiable as such until all testing, especially for adventitious agents, has been completed.

A.3.1.3.2 Primary cell cultures

Kidneys derived from animals that fulfil the requirements in section A.3.1.3.1 should be dissected and homogenized under conditions approved by the NRA. A primary cell suspension is obtained after digestion with trypsin, and this is distributed, together with growth medium, into culture vessels. Penicillin and other beta-lactam antibiotics should not be used during any stage of manufacturing.

Minimal concentrations of suitable antibiotics, such as kanamycin, may be used if approved by the NRA.

A.3.1.4 Vero cells

The use of Vero cells for the manufacture of live JE vaccines should be based on the cell bank system. The cell seed should be approved by the NRA. The maximum number of passages or population doublings allowable between the cell seed, the master bank, the working cell bank and the production passage levels should be established by the manufacturer, and approved by the NRA.

Additional tests may include but are not limited to propagation of the master cell bank or working cell bank to or beyond the maximum in vitro age for production, and examination for the presence of retroviruses and tumorigenicity in an animal test system (8).

WHO has established a bank of Vero cells, designated as WHO Vero reference cell bank 10-87, which has been characterized as suitable in accordance with the Requirements for continuous cell lines used for biologicals production (12). The cell bank is available to manufacturers as a well characterized starting material for preparation of their own master cell bank and working cell bank.¹

In normal practice, a master cell bank is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer and approved by the NRA, at which point the cells are combined to give a single pool that is distributed into ampoules and preserved cryogenically to form the working cell bank.

The manufacturer's working cell bank is used for the preparation of production cell culture, and thus for the production of vaccine batches.

The cell seed (if applicable), the master cell bank and working cell bank, and the end of production cells or extended cell bank, should be characterized according to the WHO Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (8).

A.3.1.5 Cell-culture medium

When serum is used for propagating cells for JE vaccine production, it should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas according to the requirements given in Part A, section 5.2 of the General requirements for the sterility of biological substances no. 6 (1973), and section 5.3 of the General requirements for the sterility of biological substances no. 6 (amended 1995) (13, 14), and freedom from infectious viruses should also be shown.

Detailed guidelines for detecting bovine viruses in serum used to establish master cell banks and working cell banks are given in Appendix 1 of the WHO Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (8). The principles outlined in the cell-substrate Recommendations should be applied as appropriate, and guidelines for detecting bovine viruses in serum used to establish the cell banks may be applicable to production cell cultures as well. In particular, validated molecular tests for bovine viruses may replace the cell culture tests on bovine serum if the NRA agrees. As an additional indicator of quality, serum samples may be examined to ensure they are free from

¹ Contact the Coordinator, Technologies Standards and Norms, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).

phages and endotoxins. Gamma radiation may be used to inactivate potentially contaminating viruses, while recognizing that some viruses are relatively resistant to gamma radiation.

The sources of animal components used in culture medium should be approved by the NRA. These components should comply with guidelines relating to animal-transmissible spongiform encephalopathies (15).

Human serum should not be used. If human albumin is used, it should meet the revised Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (16), as well as guidelines relating to human-transmissible spongiform encephalopathies (15).

The use of human albumin as a component of a cell culture medium requires careful consideration due to potential difficulties with the validity period of albumin (which is based on the length of time during which it is suitable for use in clinical practice) in relation to the potential long-term storage of vaccine intermediates. In addition, if human albumin is used, it should be tested according to the WHO Recommendations for cell substrates (8).

Penicillin and other beta-lactam antibiotics should not be used at any stage of manufacturing. Other antibiotics may be used during any stage, provided that the quantity present in the final product is acceptable to the NRA.

Any other substances added should be approved by the NRA.

Nontoxic pH indicators may be added (e.g. phenol red at a concentration of 0.002%).

If porcine or bovine trypsin is used for preparing cell cultures, it should be prepared, tested and found free from cultivable bacteria, fungi, mycoplasmas and infectious viruses as described in WHO Recommendations for cell substrates (8). The methods used to ensure this should be approved by the NRA.

If used, the source or sources of trypsin of bovine origin should be approved by the NRA, and should comply with guidelines relating to animal-transmissible spongiform encephalopathies (15).

A.3.2 Virus seeds

A.3.2.1 Vaccine virus strain certification

Only a designated strain of virus for live JE vaccine that has been approved by the NRA should be used in the production of vaccine. The strain should be identified by historical records, which should include information on the strain's origin, its method of attenuation, whether the strain has been biologically or molecularly cloned prior to generation of the master seed, and the passage level (or levels) at which attenuation, immunogenicity, safety and efficacy were demonstrated by clinical studies. The entire genome sequence of both the master seed and working seed viruses should be determined.

A.3.2.2 Strains derived by recombinant DNA techniques

In some countries, if a vaccine seed derived by recombinant DNA technology is used, and because it is a live-attenuated vaccine, the candidate vaccine is considered to be a genetically modified organism (GMO) and should comply with the regulations of the producing and recipient countries regarding GMOs (see Part D).

The entire nucleotide sequence of any complementary DNA (cDNA) clone used to generate vaccine-virus stocks should be determined prior to any further nonclinical or clinical trial. The cell substrate used for transfection to generate the virus should be appropriate for human vaccine production, and should be approved by the NRA.

Entire genomes of preseed lot virus stocks derived from passaging of the primary virus stock should also be sequenced as part of a nonclinical evaluation.

A.3.2.3 Virus-seed lot system

The production of vaccine should be based on the virus-seed lot system, which includes a master seed and a working seed. The virus working seed lot used for the production of vaccine batches should be prepared from a qualified virus master seed lot by means of a method approved by the NRA.

Seed lots should be prepared in the same type of cells and using the same conditions for virus growth (other than scale) as those used for production of the final vaccine. The virus working seed should have a well defined relationship to the virus master seed, with respect to passage level and method of preparation, so that the virus working seed retains all of the in vitro and in vivo phenotypes and the genetic character of the virus master seed.

The maximum passage level of master seed and working seed lots should be approved by the NRA. The inoculum for infecting cells used in the production of vaccine should be from a virus working seed lot without intervening passage in order to ensure that no vaccine is manufactured that is more than one passage level from the working seed.

The virus master seed and working seed lots used to produce live-attenuated JE vaccines should be demonstrated to be safe and immunogenic using appropriate laboratory tests. All virus seed lots should be stored in a suitable manner to ensure their stability over prolonged periods. The tests specified in sections A.3.2.4 and A.3.2.5 should be performed on both the master seed and working seed. However, the master seed is a crucial feature of the production of a safe vaccine, and should ideally be made in large amounts to avoid the need to remake it. The use of additional tests to provide further assurance of quality should be considered; these are detailed in Part B.

A.3.2.4 Control cell cultures for virus seeds

In agreement with NRAs, tests on control cell cultures may be required and should be performed as described in section A.4.1.

A.3.2.5 Tests on virus master seed and working seed lots

A.3.2.5.1 Identity test

Each virus master seed and working seed lot should be identified as JE vaccine seed virus by serological methods approved by the NRA.

Appropriate serological methods include enzyme immunoassays that use a vaccine seed virus-specific monoclonal antibody, immunofluoresence or neutralization assays that use a reference serum or monoclonal antibody specific to JEV.

A test for genetic identity should also be performed.

A.3.2.5.2 Virus titration for infectivity

The infectivity of each virus master seed and working seed lot should be established using an assay that is acceptable to the NRA. Manufacturers should determine the appropriate titre necessary to produce vaccine reliably.

Depending on the type of vaccines and available data, plaque assays, $CCID_{50}$ assays or $CCID_{50}$ with a molecular read-out, such as quantitative PCR, may be used.

All assays should be validated.

For intra-assay validation, titration should be carried out in parallel with titration of a reference vaccine, as approved by the NRA.

A.3.2.5.3 *Genetic and phenotypic characterization*

In addition to the infectivity titration, it will be necessary to examine genetic and phenotypic stability relevant to the consistency of production. The applicable tests will be identified in the course of the nonclinical evaluation of the strains. Each seed should be characterized by full-length consensus nucleotide sequence determination and by other relevant laboratory and animal tests, which will provide information on the consistency of each virus seed.

Mutations introduced during the derivation of each vaccine strain should be maintained in the consensus sequence unless spontaneous mutations induced during tissue culture passage are shown to be innocuous in nonclinical and small-scale clinical trials. Some variations in the nucleotide sequence of the virus population on passage are to be expected, but the determination of what is acceptable should be based on experience in production and clinical use.

For any new virus master seed and working seed, it is recommended that the first three consecutive consistency bulk-vaccine lots should be analysed for consensus genome sequence changes from the virus master seed. The nucleotide sequence results should be used to demonstrate the consistency of the production process.

A.3.2.5.4 Tests for bacteria, fungi, mycoplasmas and mycobacteria

Each virus master seed and working seed lot should be shown to be free from bacterial, fungal and mycoplasmal contamination using the appropriate tests specified in Part A, section 5.2 of the General requirements for the sterility of biological substances no. 6 (1973), and section 5.3 of the General requirements for the sterility of biological substances no. 6 (amended 1995) (13, 14).

Nucleic acid amplification techniques (NATs) – used alone or in combination with cell culture and an appropriate detection method – may be used as alternatives to one or both of the compendial mycoplasma detection methods, if they have been validated and the NRA is in agreement (8).

Seed lots should be shown to be free from mycobacteria by a method approved by the NRA.

NATs may be used as an alternative to microbiological methods for culturing mycobacteria or to the in vivo guinea-pig test for the detection of mycobacteria after they have been validated and approved by the NRA (8).

A.3.2.5.5 Tests for adventitious agents

Each virus master seed and working seed lot should be tested in cell cultures and in animals for adventitious viruses relevant to the passage history of the seed virus.

Where antiserum is used to neutralize JEV or the recombinant JEV, the antigen used to generate the antiserum should be produced in a cell culture from a species different from that used for the production of the vaccine, and it should be free from extraneous agents.

Depending on the derivation of the seed lot, a volume of each virus master seed and working seed lot of at least 10 ml should be tested for adventitious agents as described below.

• For virus grown in hamster or its cultured cells, the neutralized virus should be tested for adventitious agents by inoculating it on to cultures of human cells, mouse cells, simian cells, mosquito cells (e.g. C6/36), baby hamster kidney (BHK)-21 cells and PHK cells.

The cell culture should not be from the same batch as that used in the preparation of the virus seed. Uninoculated control cell cultures should be included in the tests. All cell cultures (except mosquito cells) should be observed for at least 14 days. At least one subculture of one cell culture fluid should be made, and should be observed for 14 days in order to enhance the opportunity to detect adventitious agents.

For virus grown in simian or human cells, the neutralized virus should be tested on separate cultures of simian and human cells. If other cell systems are used, cells of that species, but from a separate batch, are also inoculated. At the end of the observation period, the cells should be tested for haemadsorbing viruses. If the virus master seed was prepared in primary cell cultures, at least one subculture of one cell culture fluid should be made, and should be observed for 14 days in order to enhance the opportunity to detect adventitious agents.

The cells should be observed microscopically for cytopathic changes. At the end of the observation period, the cells or fluids should be tested for haemadsorbing viruses. For the test to be valid, 20% or fewer of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test. For the seed virus to be satisfactory, no cytopathic changes or adventitious agents should be detected. Tests for a number of agents should be conducted on the virus master seed lot to rule out the presence of adventitious agents associated with any primary cell cultures that were used in the adaptation of the JEV. For rodent primary cell substrates, such agents include mouse viruses identified using the mouse antibody production assay; hamster viruses identified using the hamster antibody production assay; human, porcine and bovine viruses identified by PCR; and endogenous retroviruses identified using a sensitive assay such as product-enhanced RTase (see section A.3.2.5.7 for details). Consideration should be given to using electron microscopy with the negative-stain technique in order to obtain additional information on extraneous agents that may not be detected by other methods.

Each virus master seed and working seed lot should also be tested in animals; these animals may include guinea-pigs and mice, as appropriate (8).

Additional testing for adventitious viruses may be performed using a validated NAT-based assay. New molecular methods with broad capabilities are being developed to detect adventitious agents. These methods include: (i) degenerate NAT for whole virus families that analyses the amplicons by hybridization, sequencing or mass spectrometry; (ii) NAT with random primers that is followed by analysis

of the amplicons on large oligonucleotide microarrays of conserved viral sequencing or digital subtraction of expressed sequences; and (iii) high-throughput sequencing. These methods may be used in the future to supplement existing methods, or as alternatives to both in vivo and in vitro tests after appropriate NRA validation and agreement.

A.3.2.5.6 Tests for attenuation

The tests applied to the SA14-14-2 and JE-CV live vaccines differ slightly because of the nature of the viruses concerned. Reference preparations to be included in each test should be identified as a high priority, and could take the form of working seeds shown to produce satisfactory vaccines.

Section A.3.2.5.6.1 applies to SA14-14-2 and section A.3.2.5.6.2 applies to JE-CV.

A.3.2.5.6.1 Tests for neurovirulence of SA14-14-2 seeds

Test for neurovirulence in weanling mice

Both master seed and working seed should be tested for neurovirulence in weanling mice.

Ten animals of the Kunming strain of Swiss mice aged 17–19 days should be inoculated by the intracerebral route with 0.03 ml of the master seed or working seed at a titre of not less than 5.7 log₁₀ PFU per ml. Mice are observed daily for 14 days after inoculation. Mice that die within three days of inoculation are considered to have died from brain trauma, and are not included in the evaluation of the test. If more than 20% of mice die within three days, the test is considered invalid. If any mice die or show clinical signs of JE infection, the preparation is unacceptable.

Test for reversion in suckling mice

Both master seed and working seed should be tested for reversion to virulence in suckling mice.

Each of 10 animals of the Kunming strain of Swiss mice aged 3–5 days should be inoculated by the intracerebral route with 0.02 ml of the master seed or working seed at a titre of not less than 5.7 log₁₀ PFU per ml. Mice showing clinical signs or dying within three days of inoculation are not included in the evaluation. All mice are expected to develop clinical signs of encephalitis over a period of 6–8 days. The first three animals showing clinical signs of encephalitis should be euthanized, their brains aseptically removed, and a pooled 10% brain homogenate prepared. Dilutions of 1:10, 1:100, 1:1000 and 1:10 000 of the homogenate should be made, and 0.03 ml of each dilution should be inoculated intracerebrally into each of 4 Kunming strain Swiss mice

aged 17–19 days. The mice are observed daily for 14 days. Mice that die within three days of injection are considered to have died from injection trauma, and are not included in the evaluation. If the end-point titre of the 10% brain homogenate is greater than $3\log_{10} \mathrm{LD}_{50}$, the virus is considered to have undergone unacceptable reversion on passage in the young mice, and the preparation is unacceptable for use.

Test for neuroinvasiveness in weanling mice

Master seed should be tested for neuroinvasiveness in weanling mice.

Ten mice of the Kunming strain of Swiss mice aged 17–19 days should be conditioned by injecting a sterile needle intracerebrally to locally destroy the blood–brain barrier; the mice are then inoculated subcutaneously (between the leg and the abdomen) with 0.1 ml of the master-virus seed. The mice are then observed for 14 days. If any mice show clinical signs of encephalitis with JEV (such as convulsions) during the observation period, the preparation is considered unsuitable. Omission of this test on a new virus master seed lot may be considered with the approval of the NRA.

Test for neurovirulence in monkeys

New virus master seed lots of SA14-14-2 should be tested for neurovirulence in monkeys. To avoid the unnecessary use of monkeys, virus master seed lots should be prepared in large quantities. A reference preparation should be included in each test.

An alternative test may be used, with the agreement of the NRA, if equal or greater sensitivity has been demonstrated. Testing of the working seed of SA14-14-2 in monkeys is not required.

A.3.2.5.6.2 Tests for neurovirulence of JE-CV

Test in mice

Both master seed and working seed should be tested for neurovirulence in mice.

Groups consisting of eight HSD:ICR (CD-1) outbred infant mice (eight days of age) should be inoculated by the intracerebral route with 0.02 ml of either the test article (2.0×10^2 PFU, 2.0×10^3 PFU and 2.0×10^4 PFU), a negative control, or YF-17D vaccine as a control. The animals are observed for 21 days for clinical signs, and euthanized if necessary. The numbers of dead or severely diseased animals and their survival times are recorded. The test material is acceptable if it is statistically less virulent than the YF-17D control. The assay is considered valid if 80% of the mice in the negative control group survive, and no more than two mice per litter die within the first 48 hours.

Test in monkeys

Because JE-CV vaccine is based on YF vaccine, both master seed and working seed should be tested in monkeys by following the WHO Recommendations for YF vaccine (17).

A.3.2.5.7 Test for retroviruses

Seeds should be examined for retroviruses using an assay for RTase that is acceptable to the NRA.

Highly sensitive PCR-based assays for RTase may be considered as tests for retroviruses, but the results need to be interpreted with caution because RTase activity is not unique to retroviruses and it may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (10). NAT assays for retroviruses may also be used.

A.4 Control of vaccine production

A.4.1 Control of production cell cultures

A fraction equivalent to at least 5% of the total volume of the cell suspension (or 500 ml, or 100 000 000 cells) should be used to prepare control cultures.

The control cells should be maintained under conditions similar to those of the infected cells in terms of time, temperature and media. The control cultures should be observed microscopically at regular intervals for cytopathic and morphological changes attributable to the presence of adventitious agents at a temperature of 35–37 °C for at least 14 days after the day of inoculation of the production cultures, or until the time of virus harvest, whichever is later. At the end of the observation period, at least one fourth of the cell-culture flasks should be checked for haemadsorbing viruses as described in section A.4.1.1.

Samples that are not tested immediately should be stored at $-60\,^{\circ}\text{C}$ or below. If any tests in control cultures show evidence of any adventitious agents, the harvest of virus should not be used for vaccine production. For the test to be valid, 20% or fewer of the control culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test.

A.4.1.1 Test for haemadsorbing viruses

At the end of the observation period, 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea-pig red blood cells. If the red cells have been stored, the duration of storage should not have exceeded seven days, and the temperature of storage should have been in the range of 2-8 °C.

In some countries, the NRA requires that the control cell cultures described above be tested for the presence of haemadsorbing viruses at the end of the production culture incubation period instead of 28 days of observations following subpassage. If this is the case, the test for haemadsorbing viruses described here may be omitted. In some countries, the NRA requires that other types of red cells, including cells from humans (blood group O), monkeys and chickens (or other avian species) be used in addition to guinea-pig cells. In all tests, readings should be taken after cells have been incubated for 30 minutes at 2–8 °C, and after a further incubation for 30 minutes at 20–25 °C. For tests using monkey red blood cells, readings should also be taken after a final incubation for 30 minutes at 34–37 °C. For the tests to be valid, 20% or fewer of the control culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test.

A.4.1.2 Tests for adventitious agents in control cell-culture fluids

At the time of harvest, a sample of 10 ml of the pooled fluid from each group of control cultures should be tested in the same type of cell culture, but not the same batch, as that used for virus production. The test should also be performed in both human and cercopithecid cell cultures.

Each sample should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 part in 4. The area of the cell sheet should be at least 3 cm² per ml of pooled fluid. At least one bottle of each kind of cell culture should remain uninoculated to serve as a control.

The cultures should be incubated at 35–37 °C, and should be observed for at least 14 days. During this observation period, the cultures should be examined at intervals for cytopathic changes.

The tests are satisfactory if no cytopathic changes attributable to adventitious agents are detected in the test sample. For the tests to be valid, 20% or fewer of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test.

A.4.1.3 **Identity of cells**

Depending on the type of cells used at the production level, the cells – especially those propagated from the working cell bank – should be identified by means of tests approved by the NRA.

Suitable methods include but are not limited to biochemical tests (e.g. isoenzyme analyses), immunological tests (e.g. major histocompatibility complex assays), cytogenetic tests (e.g. for chromosomal markers) or tests for genetic markers (e.g. DNA fingerprinting).

A.4.2 Production and control of a single virus harvest

A.4.2.1 Cells used for virus inoculation

On the day of inoculation with the working seed virus, each production cell culture flask (or bottle) or control cell culture flask should be examined for cytopathic effects potentially caused by infectious agents. If the examination shows evidence of an adventitious agent, all cell cultures should be discarded.

If animal serum is used in the growth medium, the medium should be removed from the cell culture either before or after inoculation with the virus working seed. The cell cultures should be rinsed, and the growth medium replaced with serum-free maintenance medium.

Penicillin and other beta-lactam antibiotics should not be used during any stage of manufacturing.

Minimal concentrations of other suitable antibiotics may be used if approved by the NRA.

A.4.2.2 Virus inoculation and incubation

Cell cultures are inoculated with virus working seed at a defined optimal multiplicity of infection. After viral adsorption, cell cultures are fed with maintenance medium, and are incubated at a temperature within a defined range and for a defined period.

The multiplicity of infection, temperature range and duration of incubation depends on the vaccine strain and the production method, and specifications should be defined by each manufacturer.

A.4.2.3 Harvest of vaccine virus

Vaccine virus fluid should be harvested when the cytopathic effect becomes obvious. Vaccine virus is harvested within a defined period after inoculation or for a defined level of cytopathic effect (CPE).

Samples of single virus harvests should be taken for testing. If they are not processed immediately, they should be stored at $-60\,^{\circ}\text{C}$ or below. The manufacturer should submit data to support the conditions chosen for these procedures.

Harvests derived from cultures of continuous cell lines should be subjected either to further purification to minimize the amount of cellular DNA or treatment with DNase to reduce the size of the DNA, or both.

Alternatively, this step may be performed on a virus pool.

If the harvests are not processed immediately, they should be stored at a temperature agreed with the NRA.

A.4.2.4 Tests on a single virus harvest

A.4.2.4.1 Tests for bacteria, fungi, mycoplasmas and mycobacteria

A sample of each single-harvest or virus-culture supernatant should be tested for bacterial, fungal and mycoplasmal sterility as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances no. 6 (1973), and section 5.3 of the General requirements for the sterility of biological substances no. 6 (amended 1995) (13, 14). If contamination is detected, the harvest should be discarded.

NAT-based assays used alone or in combination with cell culture and an appropriate detection method, may be used as alternatives to one or both of the compendial mycoplasma detection methods if they have been validated and the NRA agrees.

The harvest should be shown to be free from mycobacteria by a method approved by the NRA.

NAT-based assays may be used as an alternative to microbiological methods for culturing mycobacteria or to the in vivo guinea-pig test for detection if they have been validated and the NRA agrees (8).

A.4.2.4.2 Virus titration for infectivity

In the case of pooling of viral harvests, the virus content of each single harvest should be tested with an infectivity assay that is acceptable to the NRA to determine the acceptability of the material for further processing, and to confirm the consistency of production. For information on selecting and validating a test method see section A.3.2.5.2.

A.4.2.4.3 *Test for identity*

A test for identity should be performed if this has not been done on the virus pool or on the bulk material.

A.4.2.4.4 Test for adventitious agents

If the single harvests are not pooled on the same day, a test for adventitious agents should be performed on each single harvest.

A.4.3 Preparation and control of virus pool or bulk material

A.4.3.1 Preparation of virus pool or bulk material

Only virus harvests meeting the recommendations for sterility and virus content should be pooled.

The vaccine virus pool should be clarified or filtered by a method that maximizes the removal of cells and cell debris. Samples of the clarified bulk

suspension should be taken immediately after clarification in order to ensure that no microscopically observable cells or cell particles remain. Samples should also be taken to confirm the identity and determine the content of infectious virus in the pool. If not tested immediately for virus content, the samples should be stored below -60 °C until testing is done.

A.4.3.2 Tests on virus pool or bulk material

A.4.3.2.1 Virus titration for infectivity

The virus content of the pool should be assayed by titrating in cell culture against a reference preparation of live JE vaccine as described in section A.3.2.5.2 of these Recommendations, and should be approved by the NRA.

A.4.3.2.2 *Test for identity*

A test for identity should be performed if it was not carried out on the single harvest. However, it is not necessary to perform the genetic identity test on the virus pool.

A.4.3.2.3 Tests for bacteria and fungi

After clarification, the virus pool should be tested for bacterial and fungal sterility in accordance with Annex 4, Part A, section 5.2 of the WHO General requirements for the sterility of biological substances (13), and associated amendments (14), or by a method approved by the NRA.

A.4.3.2.4 *Test for adventitious agents*

A test for adventitious agents should be performed if it was not carried out on the single virus harvests (see section A.3.2.5.5).

A.4.3.2.5 Test for residual materials

Each manufacturer should demonstrate, by testing each virus pool or by validating the manufacturing process, that any residual materials used in manufacturing – such as animal serum, antibiotics, residual cellular DNA and DNase – are consistently reduced to a level acceptable to the NRA.

The host-cell protein profile should be examined as part of characterization studies (8).

For viruses grown in continuous cell-line cells, purified bulk material should be tested for the amount of residual cellular DNA, and the total amount of cell DNA per dose of vaccine should be not more than the upper limit agreed by the NRA. If this is technically feasible, the size distribution of the DNA should be examined as a characterization test, taking into account the amount of DNA detectable using state of the art methods approved by the NRA.

A.4.3.2.6 *Test for retroviruses for vaccine prepared on primary cells*

Samples from the filtered virus pool should be examined for the presence of retroviruses using an RTase assay that is acceptable to the NRA. Confirmation that the assays used will detect retroviruses potentially present in PHK cells should be presented.

Highly sensitive PCR-based assays for RTase may be considered as tests for retroviruses, but the results need to be interpreted with caution because RTase activity is not unique to retroviruses and it may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (10). NAT assays for retroviruses may also be used.

A.4.3.2.7 Test for consistency of virus characteristics

The virus pool or bulk material should be tested to compare it with virus working seed or a suitable comparator to ensure that the vaccine virus has not undergone critical changes during multiplication in the production culture system.

Relevant assays should be identified in nonclinical studies and may include, for example, virus yield in tissue culture, plaque phenotype or temperature sensitivity.

Other identifying characteristics may also be applicable, such as consensus nucleotide sequencing, to ensure the integrity of attenuating mutations.

The test may be omitted as a routine test once the consistency of the production process has been demonstrated on a significant number of batches, and if the NRA agrees. When there is a significant change in the manufacturing process, the test should be reintroduced.

A.4.4 Preparation and control of final bulk

A.4.4.1 **Preparation of final bulk**

A.4.4.1.1 Pooling of virus pool or bulk material

More than one virus pool or bulk material satisfying the control tests of these Recommendations may be pooled and diluted to form the final bulk.

A.4.4.1.2 Added substances

In the preparation of the final bulk, only substances approved by the NRA may be added, such as diluents or stabilizers. The concentration of the substances should be approved by the NRA. The substances should have been shown by appropriate tests not to impair the safety or effectiveness of the vaccine.

A.4.4.2 Tests on final bulk

A.4.4.2.1 Virus titration for infectivity

If the live-virus content of the final bulk was pooled, it should be assayed by titration in cell culture of live JE vaccine as described in section A.3.2.5.2 of these Recommendations, and should be approved by the NRA.

A.4.4.2.2 Tests for bacteria and fungi

Each final bulk should be tested for bacterial and fungal sterility in accordance with Annex 4, Part A, section 5.2 of the WHO General requirements for the sterility of biological substances (13), and associated amendments (14), or by a method approved by the NRA.

A.4.4.2.3 Test for neurovirulence in mice

Each final bulk should be tested for neurovirulence in mice. This test should be validated if it has not been tested previously with final bulk.

The test may be performed on the final product or the bulk material, whichever is acceptable to the NRA, and as described in sections A.3.2.5.6.1 and A.3.2.5.6.2.

The test for neuroinvasiveness is not required for single harvests, the pooled harvest or the final product.

A.4.4.3 Storage

Prior to filling, the final bulk suspension should be stored under conditions shown by the manufacturer to allow the final bulk to retain the desired viral potency.

A.5 Filling and containers

The requirements concerning good manufacturing practices for biological products appropriate to a vaccine apply (7).

Care should be taken to ensure that the materials from which the container and, if applicable, the closure are made do not adversely affect the infectivity (potency) of the vaccine under the recommended conditions of storage.

A final filtration stage may be included during the filling operations to assure sterility.

The manufacturer should provide the NRA with adequate data to prove that the product is stable under appropriate conditions of storage and shipping.

A.6 Control of final lot

A.6.1 Inspection of final containers

Each container in each final lot should be inspected visually, and those showing abnormalities should be discarded.

The appearance of the freeze-dried or liquid vaccine should be described with respect to form and colour. In the case of freeze-dried vaccines, a visual inspection should be performed of the vaccine, the diluent and a sample of the reconstituted vaccine.

A.6.2 Tests on the final lot

A.6.2.1 **Identity test**

An identity test, as described in section A.3.2.5.1, should be performed on at least one final, labelled container from each filling lot after the vaccine has been reconstituted according to the manufacturer's instructions for preparing the vaccine for human administration. However, it is not necessary to perform the genetic identity test on the final lot.

A.6.2.2 Potency test

The potency (virus content) in each of at least three containers randomly selected from the final lot should be determined individually. The virus content should be determined after the freeze-dried product has been reconstituted with the approved diluent. Limits for determining the accuracy and precision of virus titration should be agreed with the NRA. Since no international reference materials have been established for live JE vaccine, no recommendations for potency based on such materials can be formulated. The NRA should provide or approve a reference preparation of live JE vaccine for use in tests to determine virus concentration. The NRA should specify the minimum amount of vaccine virus that one human dose should contain. Consideration should be given to establishing the upper limit of the lot-release specification (see section C.2.3).

A.6.2.3 Thermal stability test

The purpose of the thermal stability test is to demonstrate the consistency of production. Additional guidance on the evaluation of vaccine stability is provided in the Guidelines on stability evaluation of vaccines (18). At least three containers of the final vaccine lot should be incubated at the appropriate elevated temperature for the appropriate time (e.g. 37 °C for seven days). The geometric mean titre (GMT) of infectious virus in the containers should not have decreased during the period of exposure by more than a specified amount (e.g. $1\log_{10}$) that has been justified by the production data and approved by the NRA. Titration of non-exposed and exposed containers should be carried out in parallel. A reagent for intra-assay validity control should be included in each assay.

A.6.2.4 Sterility test for bacteria and fungi

Each final lot should be tested for bacterial and fungal sterility as specified in Part A, section 5.2, of the General requirements for the sterility of biological substances no. 6 (1973) (13).

A.6.2.5 **General safety tests**

Each final lot should be tested for unexpected toxicity (i.e. abnormal toxicity) using a general safety test approved by the NRA.

This test may be omitted for routine lot release once the consistency of production has been established to the satisfaction of the NRA and when good manufacturing practices are in place.

Each lot, if tested, should pass a general safety test.

A.6.2.6 Test for pH values

The pH of the final lot should be tested in a pool of final containers. A pH value approved by the NRA should be maintained when freeze-dried vaccine is dissolved using the approved diluent, and this value should be within the range of values found in vaccine lots shown to be clinically safe and effective.

A.6.2.7 Test for residual moisture

The residual moisture in a representative sample of each freeze-dried lot should be determined by a method approved by the NRA. The upper limit for moisture content should be approved by the NRA using results from stability testing.

Moisture levels of 3% or lower are generally considered acceptable.

A.6.2.8 **Test for residual animal serum protein**

If applicable – i.e. when animal serum has been used during production – a sample of the final lot should be tested to verify that the level of serum albumin in the final reconstituted vaccine is less than 50 ng per human dose.

Alternatively, this test may be performed on the clarified virus pool or on the final bulk.

A.6.2.9 Test for residual antibiotics

If any antibiotics were added during production, the content of the residual antibiotics should be determined, and this should be within the limits approved by the NRA.

Alternatively, this test may be performed on the clarified virus pool or on the final bulk.

A.6.2.10 Test for endotoxins

In some countries, determination of endotoxin content may be required, and specifications will be approved by the NRA.

A.6.3 Control of diluents

The recommendations given in WHO good manufacturing practices for pharmaceutical products: main principles (19) should apply to the manufacturing and control of the diluents used to reconstitute live-attenuated JE vaccines. An expiry date should be established for the diluent using the stability data. For lot release of the diluent, tests should be carried out to assess the appearance, identity (if applicable), volume, sterility and content of key components.

A.7 Records

The recommendations in Good manufacturing practices for biological products (7) apply, as appropriate to the level of development of the candidate vaccine.

A.8 Retained samples

A sufficient number of samples should be retained for future studies and needs. Vaccine lots that are to be used for clinical trials may serve as reference materials in the future, and a sufficient number of vials should be reserved and stored appropriately for that purpose.

A.9 Labelling

The recommendations provided in section 7 of Good manufacturing practices for biological products (7) that are appropriate to a candidate vaccine apply.

In addition, the label on the carton enclosing one or more final containers, or the leaflet accompanying the container, should include:

- a statement that the candidate vaccine complies with Part A of these WHO Recommendations;
- a statement on the nature of the preparation, specifying the designation of the strain of JE or recombinant virus contained in the live-attenuated JE vaccine;
- the minimum number of infective units per human dose, the nature of any cellular systems used for the production of the vaccine, and whether the vaccine strains were derived by molecular methods;
- a statement of the nature and quantity, or upper limit, of any antibiotics present in the vaccine;
- a statement that contact with disinfectants should be avoided;

- a statement concerning the photosensitivity of the vaccine, based on photostability data;
- a statement indicating the volume and nature of diluent to be added to reconstitute the vaccine, and specifying that the diluent to be used is that supplied by the manufacturer;
- a statement advising that after the vaccine has been reconstituted, it should be used without delay or if not used immediately, it should be stored at 2–8 °C and protected from light for the maximum period defined by the stability studies.

A.10 **Distribution and transport**

The recommendations given in Good manufacturing practices for biological products (7) that are appropriate to a candidate vaccine apply.

Shipments should be maintained within specified temperature ranges, and packages should contain cold-chain monitors (20).

A.11 Stability, storage and expiry date

The recommendations given in Good manufacturing practices for biological products (7) and the Guidelines on stability evaluation of vaccines (18) that are appropriate to a candidate vaccine apply. The statements concerning storage temperature and expiry date that appear on the primary or secondary packaging should be based on experimental evidence, and should be submitted to the NRA for approval.

A.11.1 Stability testing

Stability testing should be performed at different stages of production, namely on stored intermediates (including single harvests, purified bulk and final bulk) and the final lot. Stability-indicating parameters should be defined or selected according to the stage of production. It is advisable to assign a shelf-life to all in-process materials during vaccine production, particularly to stored intermediates such as single harvests, purified bulk and final bulk.

The stability of the vaccine in its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRA on at least three lots of final product. Accelerated thermal-stability studies may be undertaken on each final lot to provide additional information on the overall stability of a vaccine (see section A.6.2.3).

The vaccine should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with the NRA.

A.11.2 Storage conditions

Before being distributed by the manufacturer or before being issued from a storage site, the vaccine should be stored at a temperature shown by the manufacturer to be compatible with a minimal loss of titre. The maximum duration of storage should be fixed and approved by the NRA, and should ensure that all quality specifications for the final product, including the minimum titre specified on the label of the container (or package), will be maintained until the end of the shelf-life.

A.11.3 Expiry date

The expiry date should be approved by the NRA and should be based on the shelf-life, as also approved by the NRA. If the vaccine is stored at a temperature lower than that used for stability studies and is intended to be released without re-assay, the expiry date should be calculated from the date of removal from cold storage. The expiry dates for the vaccine and the diluent may be different unless they are in the same package.

A.11.4 Expiry of reconstituted vaccine

For single-dose containers, the reconstituted vaccine should be used immediately. For multidose containers, the container should be kept in the dark at 2–8 °C unless photostability studies have shown that this not required; the expiry time for the use of an opened container should be defined by stability studies and approved by the NRA but it should not exceed 6 hours.

Part B. Nonclinical evaluation of Japanese encephalitis vaccines (live, attenuated) for human use

B.1 General principles

The nonclinical evaluation of new JE vaccines should be based on the WHO guidelines on nonclinical evaluation of vaccines (21). Testing should confirm the safety and efficacy of the candidate vaccine. Tests should include product characterization at each stage of manufacturing (including quantification of contaminants, such as cellular proteins and DNA), proof of concept immunogenicity studies (including dose-ranging studies in animals), toxicity (if required by the NRA), a test for vaccine potency to ensure efficacy throughout the anticipated shelf-life, and safety testing in animals. The following specific issues should be considered in the context of developing new live-attenuated JE vaccines. The nonclinical studies conducted during the development of the vaccine should be discussed with the NRA prior to and during the process of licensure.

Product characterization and process development

It is vital that vaccine-production processes are standardized and controlled to ensure consistency in manufacturing, and that the safety and potential efficacy of a vaccine to be used in humans are indicated by nonclinical data. Such standardization and control are prerequisites for entering the clinical-trial phase.

New, live JE vaccine formulations should be characterized to define, as far as is practical, the critical genetic and phenotypic markers of attenuation that indicate that the vaccine virus genome is stable following tissue culture passage. Each vaccine virus should be evaluated using in vitro and in vivo approaches to determine whether the genetic basis of attenuation is stable enough to demonstrate a lack of risk of reversion to virulence during either manufacturing or replication in a vaccinee. To this end, laboratory and animal studies should define genetic changes that have occurred in the virus genome during attenuation. Phenotypic markers may be useful for detecting reversion events and differentiating vaccine strains from wild-type virus strains during epidemiological surveillance following human immunization.

Qualification of a vaccine-seed strain should include obtaining the consensus nucleotide sequence of the entire genome of the candidate vaccine by using the consensus nucleotide sequence of the genome of the parent virus as a comparator. This is essential for documenting mutations in the phenotype of the vaccine virus genome that may correlate with the attenuated phenotype. It is also good practice to document any in vitro studies that might serve as indicators of the stability of the mutations that differentiate the vaccine virus from its virulent parent. Such markers include but are not limited to plaque size, replication efficiency in mosquito vectors, induction of viraemia in nonhuman primates, neurovirulence, neuroinvasion, virulence in any other animal model, and temperature sensitivity. Identifying changes in virus phenotype markers may facilitate the detection of minor or quasispecies genomes present in the master-virus seed that have emerged during vaccine production, and which are different and may have a wild-type virus phenotype. Developers should bear in mind that consensus genome sequencing is unsuitable for identifying minor or quasispecies genomes in a vaccine seed or batch (22).

The investigative use of next-generation sequencing or microarray technology to establish polymorphism is encouraged. These methods must be validated if they are to be used for regulatory purposes.

B.3 Nonclinical immunogenicity and protection

Assessing the innate and adaptive immune responses to the JE vaccine in animals provides evidence that the virus has replicated in the host, and has stimulated the production of antibodies and a virus-specific T cell immune response. Animals, especially mice and nonhuman primates, have been appropriate

hosts in which to assess the various elements of the immune response to liveattenuated IE vaccines. Specific correlates of protection have been interpreted from clinical studies conducted with inactivated IE vaccines (23-25). It is generally accepted that individuals with a serum neutralizing antibody titre of at least 1:10 are protected from JEV disease. Immunization of 21-day-old mice and nonhuman primates with live-attenuated JE vaccines stimulates neutralizing antibodies that protect against virulent virus challenge (26–33). In neutralization assays and passive protection studies, antibodies stimulated by JEV genotype III SA14-14-2 virus or JE-CV show protection against JEV of genotypes I, II, III and IV (31). Immunization of nonhuman primates with the live-attenuated JE vaccines stimulates high titre neutralizing antibodies that are protective against intracerebral virulent JEV challenge (32, 33). The ability of vaccine to stimulate virus-specific CD4+ and CD8+ T cells, as well as levels of proinflammatory cytokines, and an increase in immunomodulatory interleukin 4 and interleukin 5 cytokines that may enhance the survival of the animals, may be considered. Prior to the initiation of clinical studies with new, live-attenuated IE vaccines, immunogenicity should be determined at least in nonhuman primates and a second species, as should protection from JEV disease arising from active virus challenge (26, 27, 32, 33). It is recommended that antibodies stimulated by new vaccines in animal models should be tested for neutralization of JEV isolates representing all genotypes of the virus (34–38).

B.4 Nonclinical toxicity and safety

B.4.1 **Toxicity and safety testing**

General guidance on the nonclinical assessment of toxicity and the design of nonclinical studies that apply to vaccines is provided in the WHO guidelines on nonclinical evaluation of vaccines (21). The term toxicity is generally associated with the untoward consequences of the administration of a nonreplicating medicine or biological that is directly related to the dose-dependent effect in the test animal. Thus, toxicity studies entail the careful analysis of all major organs, as well as tissues proximal to and distal from the site of administration, in order to detect any unanticipated, direct toxic effects over a range of doses of a biological that is replicating; these studies should include doses that sufficiently exceed the intended clinically relevant dose or amount. It is generally expected that if a live, attenuated vaccine does not replicate in the test animal, then direct toxic effects are unlikely to be detected. For live-attenuated vaccines the emphasis is on the demonstration of nonclinical safety as a consequence of vaccine virus replication in an animal that is susceptible to infection with the vaccine virus.

Single-dose toxicity or repeat-dose toxicity, an assessment of viraemia and vaccine virus excretion, the tissue distribution of the vaccine virus, and

local tolerance may be considered on a case by case basis, according to WHO guidelines (21).

Genotoxicity and carcinogenicity studies may not be necessary. If the live JE vaccine is intended to be used to immunize women of childbearing age, studies of developmental and reproductive toxicity should be performed, according to WHO guidelines (21).

Nonclinical safety studies of live vaccines are required for live-attenuated vaccines during the early stages of development and testing. These studies are designed for the primary purpose of demonstrating that the vaccines are less virulent in animal models than comparable wild-type viruses, and that the vaccine does not exhibit any unexpected harmful tissue tropism and damage, and does not have the capacity to elicit a harmful immune response. For instance, the viraemia and tissue-distribution profile may be used as a marker for tropism; viraemia and invasion of the central nervous system may be used as correlates for neurovirulence. Nonhuman primates and mice are good animal models for evaluating the neurovirulence of JE and YF viruses, respectively (29–32). To support clinical trials, the design of nonclinical safety studies should reflect the proposed route and frequency of vaccine administration (21).

B.4.2 Neurovirulence in mice and monkeys

JEV infection has been studied in many different mouse models (39–41). When appropriate, a mouse model may be selected to evaluate the attenuation of a candidate vaccine virus relative to the parent wild-type strain. In mouse experiments, the titre of virus in the blood, brain and other tissue at various times after infection may be evaluated to determine the pathogenesis of the viral encephalitis.

If viral replication is not detected in the mouse, tests in monkeys should be considered.

The tests for attenuation described in Part A, section A.3.2.5.6, may be applicable. These include: (i) neurovirulence tests in mice and nonhuman primates; (ii) reversion to neurovirulence in susceptible mice with either the vaccine seed beyond the level of passages in production or the seed passaged in the brains of suckling mice or vaccine viruses recovered from viraemic patients in clinical studies; and (iii) tests for neuroinvasiveness in mice.

The use of additional tests should be considered. A reference preparation should be prepared and included as a positive control to validate each test. The selection of one or more reference preparations is a matter of high priority, and should be made in consultation with experts in neurovirulence testing who should advise also on the development and implementation of a collaborative study to validate the ability of the test system to reliably distinguish suitable vaccine preparations from those that are unsuitable.

Modifications to these neurovirulence tests in mice may be used to evaluate a new virus master seed lot if approved by the NRA. Female ICR mice aged 28–32 days provide a neurovirulence test system for laboratory strains of SA14-14-2 virus, and may be further evaluated and considered as an alternative to Kunming Swiss mice (33). Outbred NIH mice have also been used as an alternative to Kunming Swiss mice.

8.4.3 New, live JE vaccines derived by recombinant DNA technology

The established model for vaccine neurovirulence is the nonhuman primate, which has historically been used to evaluate new seeds of YF vaccines (17D substrains 17D-204-derived or 17DD-derived) and live poliovirus vaccines. New, live JE vaccines derived by recombinant DNA technology or by serial passage in cell culture should be tested once for neurovirulence in nonhuman primates. If any vaccine-virus strain is determined to be neurovirulent in nonhuman primates on the basis of neurovirulence testing, neuroinvasiveness in nonhuman primates should also be evaluated via the clinical or peripheral inoculation route as part of the nonclinical safety study.

In the case of a recombinant JE vaccine that uses YF vaccines as viral vector, testing for neurovirulence in nonhuman primates via the intracerebral inoculation route should follow WHO recommendations for the neurovirulence testing of YF vaccines (40–42), as appropriate (see the brief description of the procedure below).

Groups of at least 10 monkeys that have been determined to be nonimmune to JEV, YF virus and other flaviviruses prior to inoculation with the JE vaccine master seed, should be inoculated intracerebrally into the frontal lobe. An active comparator group of 10 monkeys that also have been demonstrated to be nonimmune to JEV, YF virus and other flaviviruses should receive WHO yellow fever reference virus 168-73 or an appropriate YF-17D vaccine. All monkeys should be observed for 30 days for signs of encephalitis prior to necropsy. If the number of monkeys, the observation period or the timepoints for necropsy for histopathological examination are different from these recommendations, they should be justified and agreed with the NRA. Clinical scores and scores of the histological lesions in the central nervous system should be recorded (43). Advanced methods of histopathological examination and automated image analysis (44) may be implemented to provide a quantitative assessment of virus-induced histopathology in the central nervous systems of nonhuman primates if the methods have been properly validated by and are acceptable to the NRA. The overall mean clinical and histological scores of the test group should not exceed the scores of the YF-17D vaccine control group. The method of statistical analysis and the significance level of the statistical difference between the test group and the control group should be agreed with the NRA.

B.4.4 Growth characteristics in vector mosquitoes

Flaviviruses exhibit a high degree of specificity in their ability to infect and be transmitted by arthropod vectors. Vector competence is under genetic control, with the susceptibility of the midgut epithelium being the primary determinant (45–48). Biological transmission of a flavivirus depends on the ingestion by the vector of a blood meal that contains virus that can infect the epithelial cells lining the midgut; this allows the virus to escape and disseminate into the haemocoele to infect the salivary glands, from where the virus is secreted into saliva during refeeding on a susceptible host.

JE-CV vaccine has been evaluated for its ability to replicate and to be transmitted by vector mosquitoes. In studies, *Culex tritaeniorhynchus*, *Aedes albopictus* and *Aedes aegypti* mosquitoes ingested a virus-laden blood meal or were inoculated intrathoracically. JE-CV did not replicate following oral feeding in any of the three mosquito species. In *Cx. tritaeniorhynchus*, replication was not detected after intrathoracic inoculation with the attenuated JE-CV vaccine (45, 47). None of three additional mosquitoes (*Cx. annulirostris*, *Cx. gelidus* and *Ae. vigilax*) became infected after being fed orally with 6.1 log₁₀ PFU/ml of JE-CV vaccine (46). Viraemias in individuals immunized with the JE-CV vaccine were of short duration and of low titre; 64% of subjects receiving the vaccine developed detectable viraemia on at least one day after inoculation (49).

Studies on the replication of the attenuated SA-14-14-2 JE vaccine virus in *Cx. tritaeniorhynchus* mosquitoes indicate that the virus does not replicate in mosquitoes through oral feeding on virus-infected blood solutions, and replicates very poorly in mosquitoes inoculated by the intrathoracic route. The SA14-14-2 attenuated virus was not transmitted to suckling mice bitten by infected *Cx. tritaeniorhyncus* mosquitoes (41, 50–52).

Even though vector mosquitoes can be infected with JE vaccine virus, if the virus does not replicate effectively in the mosquitoes and does not spread to the salivary glands to facilitate transmission, it cannot infect vertebrate hosts. For these reasons, mosquitoes that are able to transmit wild-type JEV from infected birds or pigs to humans are unable to transmit the attenuated vaccine viruses to wildlife, domestic animals and humans. As a measure of attenuation and safety, all live-attenuated JE vaccines should be shown in a laboratory setting to replicate poorly in cells of the mosquito midgut, and should fail to disseminate to the mosquito salivary glands (41, 45–48, 50–56).

Characterization of vaccine virus in vector mosquitoes

For live JE vaccines, the primary environmental risks relate to their capacity to spread from human to human by vector mosquitoes, and to the potential for prolonged or repeated cycles of multiplication in the mosquito, facilitating reversion of the virus to virulence. It has been suggested that the currently licensed live-attenuated JE vaccine viruses replicate poorly in human vaccinees, as has been demonstrated by viraemia studies. It has been suggested that they do not replicate in mosquitoes, so that the risk of transmission by mosquitoes is very low or non-existent (37, 45, 46). These factors markedly reduce the chance that JE vaccines will revert in mosquitoes to a virulent phenotype. In addition, genetic stability after passage in mosquitoes has been reported for both SA14-14-2 (41) and JE-CV (45, 46). Similar studies will need to be performed for future candidate vaccines.

Some investigators have raised a concern that live flavivirus vaccines could revert to virulence in mosquitoes via intragenic recombination with wild-type flaviviruses. Such a phenomenon would seem to be highly unlikely because of the factors noted above, and it is questionable whether flaviviruses are able to undergo recombination at all, even under ideal conditions in vitro (48).

Guidelines for assessing the environmental risk of live JE vaccines derived by recombinant DNA technology are described in Part D of these Recommendations.

Part C. Clinical evaluation of Japanese encephalitis vaccines (live, attenuated) for human use

General considerations for clinical studies

Clinical trials should adhere to the principles described in the Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (57) and to the Guidelines on clinical evaluation of vaccines: regulatory expectations (58). All clinical trials should be approved by the relevant local ethics authorities and the NRA.

Some issues that are specific to the clinical evaluation of new, live-attenuated JE vaccines are discussed in the following sections. These sections should be read in conjunction with the general guidance mentioned above. It is also recommended that manufacturers consult with the relevant NRA regarding their overall clinical development programme and their plans for assessing immune responses.

The availability and widespread deployment of effective vaccines in areas where JE is endemic makes it unethical to conduct protective efficacy studies (i.e. those that have as their end-point the prevention of clinically apparent illness) that compare a group given a new JE vaccine with an unvaccinated group. In addition, the use of available JE vaccines has reduced the incidence of clinically apparent infections to the extent that a study with sufficient power to estimate the relative protective efficacy of a new vaccine compared with a licensed JE vaccine would require such large sample sizes that it would not be feasible.

As a result, the evaluation of the likely protective efficacy of new, live JE vaccines should be based on evidence derived from active and passive protection in animal models (see Part B), and on the vaccine's noninferiority to a licensed vaccine as assessed by using an immunological parameter that is a suitable correlate for clinical protection in humans.

C.2 Assessment of immunogenicity in humans

C.2.1 Assessment of immune response

It is recommended that the primary assessment of the immunogenicity of a new, live JE vaccine should be based on measurement of serum neutralizing antibody in pre-vaccination and post-vaccination serum samples. The plaque-reduction neutralization test (PRNT) is the most commonly used method for measuring neutralizing antibody. However, the PRNT is technically demanding, and test methods vary among laboratories, especially regarding the choice of cell substrate, incubation conditions, exogenous complement, the size of wells and the definition of end-points. Therefore, it is essential that the methods employed for determining PRNT titres in clinical studies are fully validated. It is also preferable to use a single laboratory to perform these assays throughout the duration of a clinical development programme. If this is not possible, cross-validation data should be exchanged between different laboratories.

Expressing neutralizing antibody titres in terms of the highest dilutions of serum that accomplish at least a 50% reduction in viral plaques after mixing with virus preparation (i.e. $PRNT_{50}$) is preferable to the use of a 90% reduction in plaques (i.e. $PRNT_{90}$). The $PRNT_{90}$ end-point has been claimed to provide better differentiation among antibody specific to JE as opposed to antibody directed against closely related co-circulating flaviviruses. However, when assessing immune responses to vaccination, the $PRNT_{50}$ is generally considered to provide more accurate results from the linear portion of the titration curve (59).

Initial studies should seek to establish whether vaccination elicits adequate immune responses to the vaccine strain (i.e. whether it is a homologous virus), and should evaluate antibody kinetics. Further studies should evaluate post-vaccination $PRNT_{50}$ titres against other (i.e. heterologous) strains of JEV in randomly chosen subsets of serum samples. Heterologous strains representing the five JEV genotypes should be tested using PRNTs under conditions agreed with the NRA.

Consideration may also be given to the assessment of vaccine-induced cell-mediated immunity. Studies in mice have shown that the adoptive transfer of T lymphocytes can confer passive protection against viral challenge. Also, peripheral blood mononuclear cells harvested from vaccinated persons can be stimulated to demonstrate CD4 or CD8 responses. However, uncertainties regarding the interpretation of assays of cell-mediated immunity mean that they would be considered secondary immunogenicity parameters.

C.2.2 End-points and analyses

The primary assessment of immune responses should be based on the proportions of previously seronegative subjects who reach a PRNT $_{50}$ titre against homologous virus of at least 1:10 after vaccination.

The primary population should be predefined in the protocol and should be selected in accordance with the study's objectives. The population to be used in the primary analysis of immune responses should usually be confined to those subjects who are seronegative for JEV before vaccination (i.e. they have PRNT $_{50}$ titres < 1:10). Therefore, before commencement of a study in a particular geographical area, an estimate should be made of the likely percentage of subjects who will have pre-vaccination PRNT $_{50}$ titres \geq 1:10. In some instances, it may be appropriate to actively exclude those with a history of prior vaccination against JE in order to reduce the likelihood that they will already be seropositive. Alternatively, or additionally, studies could include a screening visit so that a subject's pre-vaccination serostatus can be determined before they are enrolled in the trial and the vaccine is administered.

For people who are seronegative before vaccination, the most appropriate primary parameter for assessing the immune response will be the proportion reaching PRNT $_{50}$ titres $\geq 1:10$ after vaccination; this will equal the seroconversion rate. Other parameters to be examined should include increases in titres after sequential doses, GMTs and the reverse cumulative distributions of titres. Variability among subjects' immune responses should also be reported.

In endemic areas it will be important to obtain data on the safety and immunogenicity of the new, live JE vaccine in subjects who are seropositive owing to previous administration of other JE vaccines or to naturally acquired infection with JEV, or both. This is critical because routine or emergency (i.e. outbreak control) vaccination programmes do not determine the serostatus of individuals before vaccinating them. Therefore, some studies should enrol and vaccinate subjects who are already seropositive for JEV. Analyses that include data from all vaccinated persons regardless of baseline serostatus, and that compare responses between previously seronegative and previously seropositive cohorts, should be planned. Depending on the study design and its objectives, immune responses may also be compared among subjects of various ages or with certain demographic characteristics, or some combination of these.

In people who are seropositive at baseline (i.e. who have PRNT₅₀ titres \geq 1:10), the primary assessment of immune responses to vaccination would usually be based on the proportions that achieve substantial increases (e.g. at least a four-fold rise) in titre after one or more doses of JE vaccine.

After completion of what is considered to constitute a primary course of vaccination, it is essential that studies of vaccinees are conducted to determine the persistence of antibodies specific to JEV. Protocols should

include appropriate long-term serological follow-up in a significant number of subjects in each cohort. It would generally be expected that subjects would be followed for a minimum of two years, and ideally for up to five years, after completion of the primary series. In endemic areas, antibody persistence may reflect past vaccination as well as natural boosting due to exposure to JEV or other flaviviruses, or both. Therefore, data on antibody persistence should not be extrapolated to non-endemic areas or to other endemic areas with much lower or higher risks of exposure to flaviviruses.

Data on antibody persistence should be used to guide the need for and response to booster doses. However, it may also be useful to plan in advance to administer a booster dose to selected cohorts at specified times after the primary series. The timing of the booster doses may be based on currently approved vaccines. It is important to assess antibody responses before and after the booster dose and to follow up after the booster since doing so will provide evidence of past priming with the new JE vaccine.

C.2.3 Dose and schedule

It is essential that sufficient immunogenicity data are generated to support the use of the dose of the chosen vaccine antigen, the number of doses and the dose intervals. However, it is recognized that there are limitations to the number of regimens that can realistically be explored. Therefore, it is essential to justify the choice of regimen by using preliminary data from animal or human studies, and the potency of the vaccine available. As a minimum, an appropriate schedule should be identified for children in endemic areas, taking into account the recommended age at which vaccination to prevent JEV infection should commence.

Across the entire clinical programme, sufficient safety and immunogenicity data should be generated to support the range of viral titres expected to be administered so that the clinical data will help to provide evidence for setting upper and lower specifications for the vaccine virus titre at lot release and at the end of the shelf-life.

If the vaccine is proposed to be used in travellers from non-endemic areas, who are likely to be nonimmune, different primary vaccination schedules may need to be explored. For example, it may be appropriate to investigate the efficacy of an accelerated immunization schedule for people who have to travel at very short notice.

An assessment of the need and optimal timing for booster doses should be built into the overall clinical development plan. However, as with other vaccines, it may be possible to gain an initial marketing authorization without having specific data on antibody persistence and responses to booster doses; when sufficient data are available the prescribing information may need to be modified.

C.2.4 Comparative immunogenicity studies

The clinical development programme for a novel, live-attenuated JE vaccine should include at least one study in which the immune response of a candidate vaccine is compared with that of a licensed and widely used JE vaccine, which may be an inactivated JE vaccine. The comparisons should preferably be made in seronegative persons since such studies would be more sensitive and thus would be better able to detect differences between the vaccines.

In some instances it may be useful or necessary to perform studies to compare a new, live JE vaccine against more than one licensed product, depending on the regions where subjects are enrolled and on the JE vaccines available. If more than one comparative vaccine is used in the same study, the protocol should predetermine whether the primary analysis will compare the new vaccine with pooled comparative vaccines or with individual comparative vaccines. Each of these study designs raises some potentially complex statistical issues, and expert advice should be sought before finalizing the plans for the protocol and analysis.

The comparison of immune responses to the candidate and licensed vaccines should be assessed against their respective vaccine strains if the vaccine strains are different. In such a case, if a common virus strain is used to assess immune responses, the relevance and validity of its selection should be justified because antibody measurements vary widely depending on the virus strain used in neutralization assays, and strains closely related in phylogeny tend to produce higher neutralizing antibody measurements. Immune responses against virus strains that are heterologous to both vaccine strains or to circulating virus isolates of epidemiological significance should also be assessed, using a subset of serum samples from study subjects. The selection of the primary immune parameter should take into consideration the points made in section C.2.2. Whatever is chosen as the primary parameter, the margin of noninferiority will need careful justification; published guidance should be consulted, and expert statistical input should be obtained. In addition, protocols should plan for secondary analyses based on the examination of a full range of immune-response parameters.

Although provision of at least one comparative study during the process of vaccine licensure would be expected, it is recognized that in some countries there is no licensed JE vaccine and in others the comparative vaccine or vaccines that are chosen for study may not be licensed. In these countries the regulatory approach to the data from comparative studies may not be the same as in countries in which at least one of the selected comparative vaccines is licensed. As a result, regulators may place less emphasis on the demonstration of noninferiority and relatively more reliance on immune responses to the new vaccine (especially PRNT $_{50}$ titres).

C.2.5 Concomitant vaccinations

As with all vaccines, a specific endorsement in the prescribing information for co-administration with another vaccine should be supported by clinical data (see the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations) (58).

Additional considerations will arise if it is proposed that a new, live JE vaccine could be co-administered with a vaccine against another flavivirus. YF vaccines are widely available and widely used, and vaccines against dengue are in development. Data on the co-administration of flavivirus vaccines could be especially useful in regions where JE co-circulates with either YF or dengue, and for vaccinating travellers during a single clinic visit. However, the effects of co-administering vaccines of closely related flaviviruses on safety and immunogenicity cannot be predicted. It is suggested that if co-administration studies are planned, they should be initiated with some degree of caution in seronegative adults (for instance, perhaps by delivering the injections a few days apart before proceeding to same-day co-administration).

C.3 Safety

The general approach to the assessment of safety of an experimental live JE vaccine during preapproval clinical studies should be in accordance with the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (58). The routine monitoring of safety during all prelicensure clinical studies should follow the usual principles, taking into account issues relevant to live-attenuated vaccines.

In the specific case of live-attenuated JE vaccines, adverse events of special interest (AESIs) primarily pertain to neurological disorders that have first onset following vaccination. These AESIs could include episodes of acute encephalitis that cannot be attributed to any other cause, as well as the first appearance of disorders that could represent sequelae of vaccination (e.g. movement disorders, seizures). It is recommended that subjects should be followed up for the occurrence of AESIs for at least six months after vaccination or for a duration that has been agreed between the manufacturer and the NRA. However, establishing whether vaccination had a role in an adverse event is difficult since there are many possible causative factors.

For a novel type of vaccine it is usually expected that a minimum of approximately 3000 subjects will be exposed during prelicensure studies, but this number provides only an indication of adverse events that occur at least uncommonly (i.e. in < 1/100 but > 1/1000 vaccinated persons). If no AESIs are observed in a dataset of this size it becomes more likely that if they do occur as a result of vaccination, the rate is < 1/1000 vaccinated persons, but it cannot be ruled out that the actual rate may be higher.

Many more than 3000 vaccinated subjects would have to be evaluated for safety pre-approval to obtain a more precise estimate of the frequency of AESIs. In addition, rates of AESIs observed following administration of the live-attenuated JE vaccine would have to be compared with rates observed in a setting where no JE vaccines are being used (i.e. they would be compared with background rates) or in which inactivated JE vaccines considered to have an acceptable safety profile are already in use (i.e. the relative risk would be compared with a licensed vaccine).

Individual NRAs may have different opinions regarding the precision of the estimated rates of AESIs that they require before initially approving a live-attenuated JE vaccine. These considerations will direct the possible need to conduct prelicensure studies with predefined safety end-points as opposed to agreeing on a protocol for a post-approval safety study.

c.4 Post-licensure investigations

C.4.1 Effectiveness

Because it is not feasible to study the protective efficacy of a new, live JE vaccine, it is highly desirable that plans should be made to assess its effectiveness using disease surveillance after its introduction into a vaccination programme. However, the issues described below need to be considered.

- Unless a specific, live JE vaccine is to be the only such product used in a country or region, the overall effectiveness measured will not be product-specific but campaign-specific.
- The effectiveness of JE vaccines in a country or region may be heavily influenced by pre-existing immunity in the population, whether this occurred as the result of natural exposure or previous vaccination. Therefore it may not be possible to extrapolate findings from one area to another.
- It is not likely to be possible or appropriate for manufacturers to conduct studies to estimate vaccine effectiveness since coordinated national or regional public health networks and infrastructures are necessary to ensure that cases are reliably detected. However, in countries that have reliable disease surveillance systems, manufacturers should discuss with the NRA arrangements for continual disease surveillance and the potential for estimating effectiveness.
- Effectiveness data should be used in conjunction with data on antibody persistence to identify the need for and timing of booster doses.

Because JE vaccine may be administered during periods when the virus is actively transmitted, it may be challenging to differentiate cases of vaccine failure (i.e. illness caused by wild-type JE infection) from cases resulting from possible loss of attenuation of the vaccine virus. Every attempt should be made to isolate and fully characterize viruses from any suspected case of vaccine failure in order to differentiate wild-type from vaccine-derived viruses, and hence determine the etiology of the illness.

C.4.2 Post-licensure safety

The general considerations for safety surveillance and for development of a pharmacovigilance plan are the same as for all other types of vaccines (58).

See section C.3 for more information about preapproval and post-approval safety studies that aim at estimating the risk of vaccine-associated AESIs.

Part D. Environmental risk assessment of Japanese encephalitis vaccines (live, attenuated) for human use derived by recombinant DNA technology

D.1 Introduction

D.1.1 Scope

Some countries have legislation covering environmental and other issues related to the use of live vaccines derived by recombinant DNA technology because those countries consider that the vaccines use genetically modified organisms (GMOs).

This section of the Guidelines considers the environmental risk assessment (ERA) that may be performed during the development of a JE vaccine. An ERA assesses the risk to public health and the environment. It does not assess the risk to the intended recipient of the vaccine because that is assessed through clinical studies of the vaccine. Nor does it assess the risk to laboratory workers.

The NRA is not usually responsible for assessing the environmental impact of a vaccine. Nonetheless, the NRA should receive a copy of the ERA and of any associated decisions taken to ensure that the appropriate procedures have been followed.

D.1.2 Principles and objectives

Live JE vaccines in which the genome has been genetically modified by recombinant DNA technology may be considered GMOs. The manufacture, use and transboundary shipping of such live, recombinant vaccines, for research or commercial use, should comply with any relevant legislation or regulations regarding GMOs in the producing and recipient countries. In some regulatory

regimens, in order to comply with environmental regulations, an ERA should be undertaken if the live vaccine is being tested in a clinical trial or if it is placed on the market. It should be noted that this guidance on ERAs for live, recombinant JE vaccines is not intended to replace existing GMO legislation that countries already have in place.

As explained in detail in WHO Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated) (60), the objective of an ERA is to identify and evaluate, on a case by case basis, the potential adverse effects of a GMO on public health and the environment, regardless of whether these effects might be direct or indirect, immediate or delayed. This means that for each different live, recombinant JE vaccine, a specific ERA should be performed. The data needed for an ERA do not have to be derived solely from experiments performed by the applicant; data available in the scientific literature can also be used in the assessment. An ERA can be based on data from experiments performed previously for other purposes, such as product characterization tests, and nonclinical safety and toxicity studies. Regardless of the source, data should be both relevant and of an acceptable scientific quality.

D.1.3 Procedure for an environmental risk assessment

The principles and methods of an ERA should be applicable irrespective of the geographical location of the intended release of the GMO. An ERA should take into account the specificities associated with the mosquito vector, and whether virus-amplifying hosts are enzootic or non-enzootic in the region in which the vaccine trials will be carried out or where licensure is being requested. Depending on local regulatory requirements, an ERA may be undertaken by the applicant or by the appropriate local authority. In all cases, the appropriate local authority should use an ERA as a basis for deciding whether any of the identified environmental risks are acceptable. However, this decision may vary from country to country. Several national and international documents address issues associated with ERAs (61–63).

D.2 Example taken from live-recombinant Japanese encephalitis vaccines

ERAs for live JE vaccines should be conducted according to the general principles described above, particularly taking into consideration the vector responsible for disease transmission. Important issues to consider include the genetic stability of the live-recombinant virus, including reversion and recombination; the potential for transmission of the vaccine virus among hosts by the vector; and the immune status of the population. These issues are further outlined below.

D.2.1 Genetic stability

A live-attenuated recombinant JE vaccine based on strain SA14-14-2 has been licensed, as has a recombinant virus vaccine based on the YF-17D vaccine strain. In this live, recombinant vaccine, the prM/E structural genes of the JEV are cloned into the backbone of the YF-17D vaccine to replace the corresponding structural YF-17D genes (64) (see also Introduction, General considerations above).

D211 Reversion

After vaccination, there is the potential for live-attenuated JE vaccine viruses to revert to a virulent form of JEV, although this has not been seen in clinical trials. The potential for reversion is based on the number, stability and nature of the attenuating mutation(s). An attenuating mutation that depends on a single base change may be more susceptible to reversion than a mutation that is stabilized by multiple base substitutions. In addition, attenuating mutations that have been derived by deletions of segments of RNA are generally more stable against reversion. Changes in virus genotype have the potential to influence disease transmission, the tropism of vector vaccine, virulence, or patterns of disease, or some combination of these, resulting in a virus with a previously unknown combination of properties. However, the likelihood of such a reversion depends on the number of attenuation mutations present and the genes involved in the vaccine virus (65).

The likelihood of reversions should be taken into account in an ERA.

Reversion to virulence in the case of JE-CV is unlikely because the attenuation of the YF-17D genome is multigenic, and the genome is known to be relatively stable since analyses of different vaccine lots over the years have revealed identical genome sequences (37, 66). In the case of JE-CV, it has been shown that at least three simultaneous reversions are necessary to increase virulence (39).

The attenuated SA14-14-2 strain of JE differs in 45 nucleotides from its virulent parent SA14 (67, 68). In the E protein of the SA14-14-2 strain are four conserved amino acid changes that are thought to be important to the attenuation of this strain (69). Although the likelihood of reversion is considered to be low, it should be taken into account in an ERA.

D.2.1.2 **Recombination**

Whether recombination takes place among flaviviruses is debatable. Theoretically, recombination between a live JE vaccine virus and a wild-type flavivirus could produce a virus with an altered phenotype, but there is no evidence to support this for flaviviruses (37, 55, 56, 70, 71).

The potential for recombination within and between flaviviruses has been widely discussed and challenged in the literature (37, 53–55, 70, 72, 73) and as a result of data obtained from specific experiments. In particular, a "recombination trap" has been designed to allow the products of rare recombination events to be selected and amplified in the case of West Nile virus, tickborne encephalitis and JEV (55). Intergenomic but aberrant recombination was observed only in the case of JEV, and not in the West Nile or tickborne encephalitis viruses. Moreover, its frequency appeared to be low, and it generated viruses with impaired growth properties (68). Similarly, no homologous recombination was seen using YF-17D replicons (56).

Nevertheless, while the likelihood of a recombined JEV is low, the potential adverse effects of these viruses should be evaluated in an ERA. In this respect, a worst-case scenario for chimeras has been constructed to address this risk (72, 73).

Different studies have shown that recombinants constructed artificially from a wild-type flavivirus and a recombinant vaccine (70), or from two wild-type viruses – including JEV, Kunjin and the highly virulent YF Asibi virus (37, 73) – were largely attenuated when compared with their parental viruses. The constructed viruses were nonpathogenic in cell culture, mosquito vectors and animal models, including monkeys. These data provide experimental evidence that the ability of these particular recombinant viruses to cause disease or to spread, should they ever emerge, would probably be low.

D.2.2 Vector transmission

Pigs and various wild birds represent the natural reservoir of JEV, which could be transmitted to new animal hosts and occasionally to humans by mosquitoes. The mosquito vectors play a key role in the transmission of flaviviruses and potentially the transmission of live JE vaccines from a vaccinated subject to other individuals. JE does not spread directly from person to person, except via blood transfusion in rare instances in which a donor was JE viraemic. Therefore, transmission of the JE vaccine virus in regions where the vector is absent is highly unlikely. JE is present in almost all Asian countries. As a result of climate change, there is the possibility of a geographical shift in mosquito populations. Conceivably, this could lead to the spread of JE to areas that are currently non-endemic.

Recombination between a live JE vaccine virus and a wild-type flavivirus could theoretically occur in a vaccinee (see section D.2.1.2) and possibly also within an infected mosquito or natural reservoir of JEV. A recombined JEV could potentially – in combination with climate change – use new vectors for transmission, leading to previously unknown transmission characteristics. Therefore, the presence of a relevant mosquito vector and a climate that

favours JEV in the vaccination area should be taken into account in ERAs for live JE vaccines.

To assess the likelihood of effective transmission of the vaccine virus from a vaccinated individual, one has to take three parameters into consideration – namely, the level of viraemia in the vaccinated hosts; the ability of the mosquito vectors to transmit the live JE vaccine virus to new hosts; and, for transmission to be sustained, the capacity and presence of amplifying hosts to be fed upon, to be infected, and to sustain viraemia that is adequate to allow other feeding mosquitoes to become infected.

The ability of JE-CV to replicate and be transmitted to mosquitoes has been studied (37, 45, 68). Compared with wild-type JEV, the JE-CV virus cannot infect by the oral route and cannot replicate in different mosquito vectors known to transmit members of Flaviviridae. The combination of the low-level replication of the vaccine virus, and the absence of virus replication and dissemination in the mosquito vector, make it unlikely that JE-CV would be transmitted. As a result, it is also unlikely that other amplifying hosts, such as pigs and birds, will become infected. It has been shown that pig infection with JE-CV does not result in any detectable viraemia (37, 68). Thus, it is highly unlikely that vaccinated subjects would ever spread the vaccine virus via mosquito transmission.

The outcome of an ERA for clinical trials in regions where the vector is absent will be that the environmental risk is negligible. The mosquito vector is not present and therefore the vaccine, or theoretical de novo recombinant viruses, cannot be transmitted to other people. However, in endemic areas, NRAs need to decide whether to perform an ERA.

D.2.3 Immune status

Live JE vaccines are able to replicate in vaccinated individuals. The immune status of a vaccinee in relation to the vaccine antigens, the viral vectors or the cross-reacting flaviviruses – or to a combination of these – may be confounding factors in assessing the environmental risk of a live JE vaccine. In general, the presence of pre-existing immunity resulting from earlier exposure to JEV will reduce the extent and duration of vaccine virus replication and dissemination within a vaccinee. The potential for transmission of the vaccine virus is therefore considered to be greater in naive or immunocompromised individuals. Results from clinical studies of individuals who were naive for YF virus might also inform a risk assessment (68). However, the potential for transmission into the environment would still be limited by the virus' lack of ability to replicate in mosquitoes (37, 45, 68).

An unvaccinated population with no pre-existing immunity will respond differently upon exposure to the vaccine when compared with a population in an area where JE is endemic. Moreover, it has been reported that in a case where a person is infected with dengue virus prior to JEV, high antibody titres are present due to the cross-reactivity of JEV with other flaviviruses (74). The immune status of a population should therefore be taken into account in an ERA because it can influence the environmental impact of vaccines (75).

Part E. Recommendations for NRAs

F1 General

The general recommendations for NRAs and national control laboratories given in the Guidelines for national authorities on quality assurance for biological products (76) and Guidelines for independent lot release of vaccines by regulatory authorities (77) apply.

The detailed procedures for the production and control of live-attenuated JE vaccine and any significant changes in them that may affect the quality, safety and efficacy of the vaccine should be discussed with and approved by the NRA. The NRA may obtain the product-specific working reference from the manufacturer and use this for lot release until an international or national standard preparation has been established.

Consistency in production has been recognized as an essential component in the quality assurance of vaccines. In particular, NRAs should carefully monitor production records and results of quality control tests on clinical lots, as well as a series of consecutive lots of the final bulk.

E.2 Release and certification by the NRA

A vaccine should be released only if it fulfils all national requirements or satisfies Part A of these Recommendations, or both (77).

A protocol based on the model given in Appendix 4, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for release of the vaccine for use.

A certificate signed by the appropriate official of the NRA should be provided to the manufacturing establishment, and should certify that the lot of vaccine in question meets all national requirements as well as Part A of these Recommendations. The certificate should provide sufficient information about the product. A model certificate is given in Appendix 5. The official national release certificate should be provided to importers of the vaccines. The purpose of the certificate is to facilitate exchange of vaccines between countries.

Authors

These Recommendations were prepared by a drafting group consisting of Dr P. Minor, National Institute of Biological Standards and Control, England; Dr A. Barrett and Dr D. Trent, University of Texas Medical Branch, USA; Dr M. Powell,

Medicines and Healthcare Regulatory Agency, England; Dr D. Bleijs, National Institute for Public Health and the Environment, the Netherlands; and Dr J. Shin, World Health Organization, Switzerland. The drafting group was assisted by Dr I. Knezevic and Dr D. Wood, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland.

The first draft was prepared and consultations took place at a meeting of a working group held 21–23 February 2012 in Bangkok, Thailand, and attended by the members of the drafting group listed above and by the following participants: Mr P. Akapanon, Thai Food and Drug Administration, Thailand; Dr M. Alali, Therapeutic Goods Administration, Australia; Dr G. Dong, National Institutes of Food and Drug Control China, China; Dr M. Dornbusch, Government of Australia, Australia; Dr S. Hossain, Regional Office for the Western Pacific, World Health Organization, the Philippines; Mrs T. Jivapaisarnpong, Ministry of Public Health, Thailand; Mrs J. Kim, National Center for Lot Release, Republic of Korea; Ms K. Kullabutr, Institute of Biological Products, Thailand; Mrs D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Dr N. Linh, National Institute for the Control of Vaccines and Biologicals, Viet Nam; Dr C. Logvinoff, Sanofi Pasteur, Canada (representing the International Federation of Pharmaceutical Manufacturers and Associations); Dr K. Min, Korea Food and Drug Administration, Republic of Korea; Mr A. Rao, Central Drugs Standard Control Organization, India; Dr S. Sahu, Central Drugs Laboratory, India; Ms N. Somdach, Ministry of Public Health, Thailand; Dr T. Takasaki, National Institute of Infectious Diseases, Japan; Mrs P. Thanaphollert, Ministry of Public Health, Thailand; Dr T. Tsai, Novartis Vaccines, USA (representing the International Federation of Pharmaceutical Manufacturers and Associations); Dr O. Wimalaratne, Medical Research Institute, Sri Lanka; Ms L. Yang and Dr Y. Yao, Chengdu Institute of Biological Products Company, China (representing the Developing Countries Vaccine Manufacturers Network); Dr H. Yin, Center for Drug Evaluation, China; and Dr Y. Yu, National Institutes of Food and Drug Control, China.

An improved version of the draft was prepared, taking into consideration proposed changes and comments provided by the working group, and was posted for further consultation on the WHO web site during May and June 2012, before being submitted for consideration by the WHO Expert Committee on Biological Standardization.

Acknowledgements

Acknowledgements are due to the following individuals and institutions for providing written comments on the draft of these Recommendations: Dr C. Logvinoff, Sanofi Pasteur, Canada; Dr A. Rinfret, Health Canada, Canada; Dr S. Morgeaux and Dr J. Korimbocus, Agence Nationale de Sécurité du Médicament

et des Produits de Santé, France; Dr O. Maximova, Dr S. Whitehead and Dr A. Pletnev, National Institute of Allergy and Infectious Diseases, USA; Dr K. Min, Korea Food and Drug Administration, Republic of Korea; Dr T. Tsai, Novartis Vaccines, USA; Ms L. Yang, Chengdu Institute of Biological Products Company, China; Dr Y. Yu, National Institutes of Food and Drug Control, China; Central Drug Evaluation and National Institutes of Food and Drug Control, China; United States Food and Drug Administration Center for Biologics Evaluation and Research, USA.

References

- Guidelines for the production and control of Japanese encephalitis vaccine (live) for human use.
 In: WHO Expert Committee on Biological Standardization. Fifty-first report. Geneva, World Health Organization, 2002 (WHO Technical Report Series, No. 910), Annex 3.
- Recommendations for inactivated Japanese encephalitis vaccine for human use (Revised 2007).
 In: WHO Expert Committee on Biological Standardization. Fifty-eighth report. Geneva, World Health Organization, 2011 (WHO Technical Report Series No. 963), Annex 1.
- 3. Japanese encephalitis vaccines: WHO Position Paper. *Weekly Epidemiological Record*, 2006, 81:331–340 (http://www.who.int/wer/2006/wer8134_35.pdf, accessed 15 July 2013).
- 4. Campbell GL et al. Estimated global incidence of Japanese encephalitis: a systematic review. *Bulletin of the World Health Organization*, 2011, 89:766–774E (http://www.who.int/bulletin/volumes/89/10/10-085233/en/, accessed 15 July 2013).
- 5. Ding D et al. Long-term disability from acute childhood Japanese encephalitis in Shanghai, China. *American Journal of Tropical Medicine and Hygiene*, 2007, 77:528–533.
- 6. McMinn PC. The molecular basis of virulence of the encephalitogenic flaviviruses. *Journal of General Virology*, 1997, 78 (Pt. 11):2711–2722.
- 7. Good manufacturing practices for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 1.
- 8. Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. In: WHO Expert Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 3.
- 9. Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin. Document Q5A(R1). Geneva, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 1999 (http://www.ich.org/products/guidelines/quality/quality-single/article/viral-safety-evaluation-of-biotechnology-products-derived-from-cell-lines-of-human-or-animal-origin.html, accessed 15 July 2013).
- 10. Robertson JS et al. Assessing the significance of reverse transcriptase activity in chick cell-derived vaccines. *Biologicals*, 1997, 25:403–414.
- Requirements for measles, mumps and rubella vaccines and combined vaccine (live) (Requirements for biological substances No. 47). In: WHO Expert Committee on Biological Standardization. Forty-third report. Geneva, World Health Organization, 1994 (WHO Technical Report Series, No. 840), Annex 3.

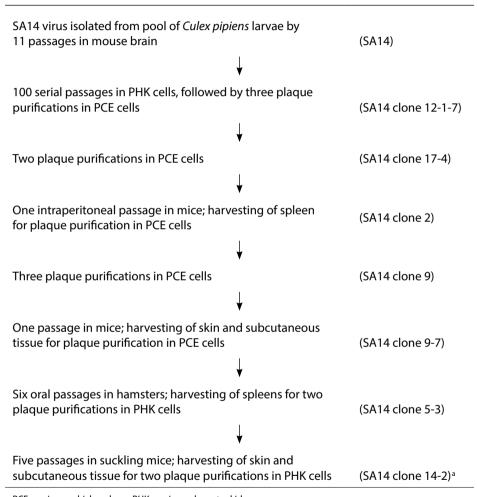
- Requirements for continuous cell lines used for biologicals production (Requirements for biological substances No. 37). In: WHO Expert Committee on Biological Standardization. Thirtysixth report. Geneva, World Health Organization, 1987 (WHO Technical Report Series, No. 745), Annex 3.
- General requirements for the sterility of biological substances (Requirements for biological substances No. 6) (Revised 1973). In: WHO Expert Committee on Biological Standardization. Twenty-fifth report. Geneva, World Health Organization, 1973 (WHO Technical Report Series, No. 530), Annex 4.
- General requirements for the sterility of biological substances. (Requirements for Biological Substances No. 6, revised 1973, amendment 1995). In: WHO Expert Committee on Biological Standardization. Forty-sixth report. Geneva, World Health Organization, 1998 (WHO Technical Report Series, No. 872), Annex 3.
- 15. WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products. Geneva, World Health Organization, 2003 (WHO/BCT/QSD/03.01) (http://www.who.int/biologicals/publications/en/whotse2003.pdf, accessed 22 June 2013).
- Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (revised 1992). In: WHO Expert Committee on Biological Standardization. Forty-third report. Geneva, World Health Organization, 1994 (WHO Technical Report Series, No. 840), Annex 2.
- 17. Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines. In: WHO Expert Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 5.
- 18. Guidelines on stability evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-seventh report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 962). Annex 3.
- 19. Good manufacturing practices: main principles for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 3.
- Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical
 products (jointly with the Expert Committee on Biological Standardization). In: WHO Expert
 Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report. Geneva, World
 Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 9.
- WHO guidelines on nonclinical evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-fourth report. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.
- 22. Mantel N et al. Genetic stability of a dengue vaccine based on chimeric yellow fever/dengue viruses. *Vaccine*, 2011, 29:6629–6635.
- 23. Hombach J et al. Report on a WHO consultation on immunological endpoints for evaluation of new Japanese encephalitis vaccines, WHO, Geneva, 2–3 September, 2004. *Vaccine*, 2005, 23:5205–5211.
- 24. Van Gessel Y et al. Correlation of protection against Japanese encephalitis virus and JE vaccine (Ixiaro(R)) induced neutralizing antibody titers. *Vaccine*, 2011, 29:5925–5931.
- Plotkin SA. Correlates of protection induced by vaccination. Clinical and Vaccine Immunology, 2010, 17:1055–1065.
- Guirakhoo F et al. Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever-Japanese encephalitis virus (ChimeriVax-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. Virology, 1999, 257:363–372.

- 27. Larena M et al. Pivotal role of antibody and subsidiary contribution of CD8+ T cells to recovery from infection in a murine model of Japanese encephalitis. *Journal of Virology*, 2011, 85:5446–5455.
- 28. Sil BK et al. Immunogenicity of experimental live attenuated Japanese encephalitis vaccine viruses and comparison with wild-type strains using monoclonal and polyclonal antibodies. *Vaccine*, 1992, 10:329–333.
- 29. Lee T et al. Immune response in mice infected with the attenuated Japanese encephalitis vaccine strain SA14-14-2. *Acta Virologica*, 1995, 39:161–164.
- 30. Jia L et al. Protection of SA14-14-2 live attenuated Japanese encephalitis vaccine against the wild-type JE viruses. *Chinese Medical Journal*, 2003, 116:941–943.
- 31. Beasley DW et al. Protection against Japanese encephalitis virus strains representing four genotypes by passive transfer of sera raised against ChimeriVax-JE experimental vaccine. *Vaccine*, 2004, 22:3722–3726.
- 32. Monath TP et al. Recombinant, chimaeric live, attenuated vaccine (ChimeriVax) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yellow fever (17D) virus is safe, immunogenic and protective in non-human primates. *Vaccine*, 1999, 17:1869–1882.
- Monath TP et al. Chimeric yellow fever virus 17D-Japanese encephalitis virus vaccine: doseresponse effectiveness and extended safety testing in rhesus monkeys. *Journal of Virology*, 2000, 74:1742–1751.
- 34. Solomon T et al. Origin and evolution of Japanese encephalitis virus in southeast Asia. *Journal of Virology*, 2003, 77:3091–3098.
- 35. Wang HY et al. Molecular epidemiological analysis of Japanese encephalitis virus in China. *Journal of General Virology*, 2007, 88:885–894.
- 36. Chen SP. Molecular phylogenetic and evolutionary analysis of Japanese encephalitis virus in China. *Epidemiology and Infection*, 2012, 140:1637–1643.
- 37. Guy B et al. Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. *Vaccine*, 2010, 28:632–649.
- 38. Takhampunya R et al. Emergence of Japanese encephalitis virus genotype V in the Republic of Korea. *Virology Journal*, 2011, 8:449.
- 39. Arroyo J et al. Molecular basis for attenuation of neurovirulence of a yellow fever virus/Japanese encephalitis virus chimera vaccine (ChimeriVax-JE). *Journal of Virology*, 2001, 75:934–942.
- 40. Chambers TJ et al. JE Nakayama/JE SA14-14-2 virus structural region intertypic viruses: biological properties in the mouse model of neuroinvasive disease. *Virology*, 2007, 366:51–61.
- 41. Yu Y. Phenotypic and genotypic characteristics of Japanese encephalitis attenuated live vaccine virus SA14-14-2 and their stabilities. *Vaccine*, 2010, 28:3635–3641.
- 42. Monath TP et al. Safety testing for neurovirulence of novel live, attenuated flavivirus vaccines: infant mice provide an accurate surrogate for the test in monkeys. *Biologicals*, 2005, 33:131–144.
- 43. Levenbook IS et al. The monkey safety test for neurovirulence of yellow fever vaccines: the utility of quantitative clinical evaluation and histological examination. *Journal of Biological Standardization*, 1987, 15:305–313.
- Maximova OA et al. High-throughput automated image analysis of neuroinflammation and neurodegeneration enables quantitative assessment of virus neurovirulence. *Vaccine*, 2010, 28:8315–8326.

- 45. Bhatt TR et al. Growth characteristics of the chimeric Japanese encephalitis virus vaccine candidate, ChimeriVax-JE (YF/JE SA14--14--2), in Culex tritaeniorhynchus, Aedes albopictus, and Aedes aegypti mosquitoes. *American Journal of Tropical Medicine and Hygiene*, 2000, 62:480–484.
- 46. Reid M et al. Experimental infection of Culex annulirostris, Culex gelidus, and Aedes vigilax with a yellow fever/Japanese encephalitis virus vaccine chimera (ChimeriVax-JE). *American Journal of Tropical Medicine and Hygiene*, 2006, 75:659–663.
- 47. Chen BQ, Beaty BJ. Japanese encephalitis vaccine (2-8 strain) and parent (SA 14 strain) viruses in Culex tritaeniorhynchus mosquitoes. *American Journal of Tropical Medicine and Hygiene*, 1982, 31:403–407.
- 48. Guy B et al. Safety of flavivirus chimeric vaccines: answer to Ishikawa et al. [*Vaccine*, 2008, 26:2772–2781]. *Vaccine*, 2008, 26:4107–4108.
- 49. Monath TP et al. Chimeric live, attenuated vaccine against Japanese encephalitis (ChimeriVax-JE): phase 2 clinical trials for safety and immunogenicity, effect of vaccine dose and schedule, and memory response to challenge with inactivated Japanese encephalitis antigen. *Journal of Infectious Diseases*, 2003, 188:1213–1230.
- 50. Liu Z-E et al. Biological and molecular characteristics of live attenuated Japanese encephalitis vaccine virus strain SA14-14-2 inoculated intrathoracically to *Culex tritaeniorhynchus*. *Chinese Journal of Biologicals*, 2007, 20:419–421.
- 51. Feng Y et al. Observations on the replication of virus and stability of virulence in *Culex tritaeniorhynchus*. *Chinese Journal of Zoonoses*, 2007, 23:279–281.
- 52. Zhang YZ et al. [Research on Culex tritaeniorhynchus and Culex pipiens quinquefasciatus intrathoracically infected with attenuated Japanese encephalitis virus SA14-14-2 vaccine strain]. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*, 2005, 19:344–346.
- 53. Hombach J et al. Arguments for live flavivirus vaccines. *Lancet*, 2004, 364:498–499.
- Monath TP et al. Recombination and flavivirus vaccines: a commentary. Vaccine, 2005, 23:2956– 2958.
- 55. Taucher C et al. A trans-complementing recombination trap demonstrates a low propensity of flaviviruses for intermolecular recombination. *Journal of Virology*, 2010, 84:599–611.
- 56. McGee CE et al. Stability of yellow fever virus under recombinatory pressure as compared with chikungunya virus. PLoS One, 2011, 6(8):e23247 (doi:10.1371/journal.pone.0023247).
- 57. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products. In: *The Use of Essential Drugs. Sixth report of the WHO Expert Committee.* Geneva, World Health Organization, 1995 (WHO Technical Report Series, No. 850), Annex 3.
- 58. Guidelines on clinical evaluation of vaccines: regulatory expectations. In: *WHO Expert Committee on Biological Standardization. Fifty-second report*. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 924), Annex 1.
- 59. Guidelines for plaque reduction neutralization testing of human antibodies to dengue viruses. Geneva, World Health Organization, 2007 (WHO/IVB/07.07) (http://whqlibdoc.who.int/hq/2007/WHO_IVB_07.07_eng.pdf, accessed 15 July 2013).
- 60. Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated). In: WHO Expert Committee on Biological Standardization. Sixty-second report. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 979), Annex 2.
- Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities, 2001, 17.4.2001:L106/1–L106/38.

- 62. Commission Decision of 24 July 2002 establishing guidance notes supplementing Annex II to Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities, 2002, 30.7.2002:L200/22–L200/24.
- 63. Risk Analysis Framework April 2009. Canberra, Office of the Gene Technology Regulator, 2009 (http://www.health.gov.au/internet/ogtr/publishing.nsf/Content/riskassessments-1, accessed 15 July 2013).
- 64. Wilder-Smith A, Halstead SB. Japanese encephalitis: update on vaccines and vaccine recommendations. *Current Opinion in Infectious Diseases*, 2010, 23:426–431.
- 65. dos Santos CN et al. Complete nucleotide sequence of yellow fever virus vaccine strains 17DD and 17D-213. *Virus Research*, 1995, 35:35–41.
- 66. Barban V et al. High stability of yellow fever 17D-204 vaccine: a 12-year restrospective analysis of large-scale production. *Vaccine*, 2007, 25:2941–2950.
- 67. Nitayaphan S et al. Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14-14-2. *Virology*, 1990, 177:541–552.
- 68. Appaiahgari MB, Vrati S. Imojev ((R)): a yellow fever virus-based novel Japanese encephalitis vaccine. *Expert Review of Vaccines*, 2010, 9:1371–1384.
- 69. Ni H et al. Molecular basis of attenuation of neurovirulence of wild-type Japanese encephalitis virus strain SA14. *Journal of General Virology*, 1995, 76 (Part. 2):409–413.
- 70. Pugachev KV et al. Construction and biological characterization of artificial recombinants between a wild type flavivirus (Kunjin) and a live chimeric flavivirus vaccine (ChimeriVax-JE). *Vaccine*, 2007, 25:6661–6671.
- 71. Seligman SJ, Gould EA. Safety concerns with regard to live attenuated flavivirus vaccines. *Journal of Infectious Diseases*, 2008, 198:794–795.
- 72. McGee CE et al. Recombinant chimeric virus with wild-type dengue 4 virus premembrane and envelope and virulent yellow fever virus Asibi backbone sequences is dramatically attenuated in nonhuman primates. *Journal of Infectious Diseases*, 2008, 197:693–697.
- 73. McGee CE et al. Substitution of wild-type yellow fever Asibi sequences for 17D vaccine sequences in ChimeriVax-dengue 4 does not enhance infection of Aedes aegypti mosquitoes. *Journal of Infectious Diseases*, 2008, 197:686–692.
- 74. Innis BL et al. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *American Journal of Tropical Medicine and Hygiene*, 1989, 40:418–427.
- 75. Unni SK et al. Japanese encephalitis virus: from genome to infectome. *Microbes and Infection*, 2011, 13:312–321.
- 76. Guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 2.
- 77. Guidelines for independent lot release of vaccines by regulatory authorities. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 2.

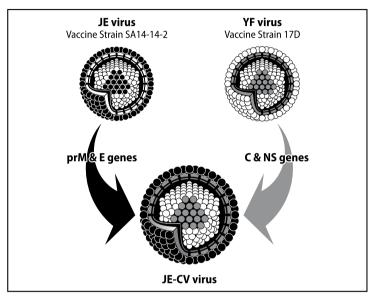
Passage history of Japanese encephalitis SA14-14-2 virus

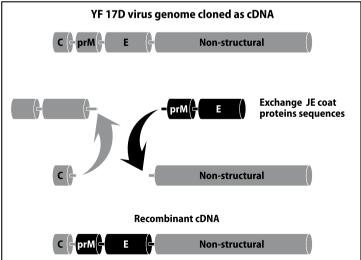


 $\label{eq:PCE} PCE = primary \ chick \ embryo; \\ PHK = primary \ hamster \ kidney.$

^a The notation SA14 clone 14-2 is abbreviated to SA14-14-2.

Derivation of Japanese encephalitis-yellow fever chimeric virus (CV) vaccine¹





Source: Chambers et al. Yellow fever/Japanese encephalitis chimeric viruses: construction and biological properties. *Journal of Virology*, 1999, 73:3095–3101.

Production and passage level of live-attenuated Japanese encephalitis vaccine

SA14-14-2 vaccine

Premaster seed (P 6)

Master seed (P 7)

Working seed (P 8)

Single harvest (P 9)

Virus pool

Final bulk

Final lot

Japanese encephalitis chimeric virus vaccine

Premaster seed (P 10)

Master seed (P 11)

Working seed (P 12)

Single harvest (P 13)

Bulk material

Final bulk

Final lot

Model protocol for the manufacturing and control of Japanese encephalitis vaccines (live, attenuated) for human use

The following protocol is intended for guidance. It indicates the information that should be provided as a minimum by a manufacturer to the NRA. Information and tests may be added or omitted as necessary, with the authorization of the NRA.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO Recommendations for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that will accompany the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot-release certificate from the NRA or from the NCL in the country where the vaccine was produced or released stating that the product meets national requirements as well as the recommendations in Part A of this annex.

1. Summary information on finished product (final lot)

International name:
Trade name/commercial name:
Product licence (marketing authorization) number:
Country:
Name and address of manufacturer:
Name and address of product licence holder, if different:
Virus strains:
Origin and short history:
Batch number(s):
Finished product (final lot):
Final bulk:
Type of container:
Number of filled containers in this final lot:
Number of doses per container:

Composition (antigen concentration)/volume of
single human dose:
Target group:
Expiry date:
Storage conditions:
2. Summary information on manufacture
Batch number of each monovalent bulk:
Site of manufacture of each monovalent bulk:
Date of manufacture of each monovalent bulk:
Batch number of final bulk:
Site of manufacture of final bulk:
Date of manufacture of final bulk:
Date of manufacture (filling or lyophilizing) of finished
product (final vaccine lot):
Date on which last determination of virus concentration
was started:
Shelf-life approved (months):
Storage conditions:
Volume of single dose:
Prescribed virus concentration per human dose:
-

A genealogy of the lot numbers of all vaccine components used in the formulation of the final product should be provided.

The following sections are intended for reporting the results of the tests performed during production of the vaccine.

3. Control of source materials

3.1 Cell cultures

Where primary hamster kidney cells are used

Animals

Information on how animal colonies were established and maintained should be provided at the time of licensing.

Information on the test programme to ensure freedom from specified pathogens and detectable antibodies to those pathogens described in Part A, section A.3.1.3.1, and test results should be provided at the time of licensing or on the establishment of new breeding colonies.

A summary table and details for hamster antibody production, mouse antibody production and rat antibody production tests and results should be provided at the time of licensing or on the establishment of new breeding colonies. A regular health-monitoring programme should be provided, and test results should be updated.

Primary hamster kidney cells
Date of preparation:
Methods of preparation:
Gross examination of kidneys
Specification:
Date:
Result:
Microscopic observation of prepared cells
Specification:
Date:
Result:
Where continuous cell cultures are used
General information on the cell banking system
Information and results in relation to characterization tests on the cell banking
system from cell seed – if applicable, the master cell bank, working cell bank, en-
of production cells, or the extended cell bank – should be provided according to
WHO Recommendations for the evaluation of animal-cell cultures as substrate
for the manufacture of biological medicinal products and for the characterization
of cell banks.
Name and identification of cell substrate:
Origin and short history (attach a flowchart
if necessary):
Lot number and date of preparation of each bank:
Date each bank was established:
Date of approval by the NRA:
Total number of ampoules stored for each bank:
Passage level or population-doubling level of each bank:
Maximum passage level or population-doubling level
approved for each bank:
Storage conditions:
Date of approval of protocols indicating compliance
with the requirements of the relevant monographs and
with the marketing authorization:

Characterization tests on cell seed (if applicable), the master cell bank, working cell bank, end of production cells, or extended cell banks

A summary table for characterization tests on each bank should be provided.

Characterization tests performed on each bank
Methods:
Specification:
Date of test:
Results:
Cell-culture medium
Serum used in cell-culture medium
Animal origin of serum:
Batch number:
Vendor:
Country of origin:
TSE-free certificate reference number:
Tests performed on serum
Methods:
Specification:
Date of test:
Results:
Teodito.
Trypsin used for preparation of cell cultures
Animal origin of trypsin:
Batch number:
Vendor:
Country of origin:
TSE-free certificate reference number:
Tests performed on trypsin
Methods:
Specification:
Date of test:
Results:
Antibiotics
Nature and concentration of antibiotics or selecting
agent(s) used in the production cell culture's
maintenance medium:
municentialed inediani,

Other source material
Identification and source of starting materials used in preparing
production cells, including excipients and
preservatives (particularly any materials of human or
animal origin – e.g. albumin, serum):
ummur origin e.g. uibummi, serum).
3.2 Virus seeds
Vaccine virus strain(s) and serotype(s):
Substrate used for preparing seed lots:
Origin and short history of virus seeds:
Authority that approved virus strain(s):
Date approved:
Information on conduct managed in
Information on seed lot preparation
Virus master seed
Source of virus master seed lot:
Virus master seed lot number:
Name and address of manufacturer:
Passage level:
Date of inoculation:
Date of harvest:
Number of containers:
Conditions of storage:
Date of establishment:
Maximum passage level approved for virus
master seed:
Date approved by the NRA:
V:
Virus working seed
Virus working seed lot number:
Name and address of manufacturer:
Passage level from virus master seed lot:
Date of inoculation:
Date of harvest:
Number of containers:
Conditions of storage:
Date of establishment:
Date approved by the NRA:

Tests on virus seeds
<i>Identity test</i>
Method:
Specification:
Lot number of reference reagents:
Dates of test (start, end):
Result:
Virus titration for infectivity
Method:Specification:
Dates of test (start, end):
Result:
Genetic and/or phenotypic characterizations
Method:
Reference reagents:
Specification:
Dates of test (start, end):
Result:
result.
Tests for bacteria and fungi
Method:
Specification:
Media:
Number of containers tested:
Volume of inoculum per container:
Volume of medium per container:
Temperatures of incubation:
Dates of test (start, end):
Result:
Test for mycoplasmas
Method:
Specification:
Media:
Volume tested:
Temperature of incubation:
Positive controls:
Dates of test (start, end):
Result:

Test for mycobacteria
Method:
Specification:
Media:
Volume tested:
Temperature of incubation:
Dates of test (start, end):
Result:
Tests for adventitious agents
Volume of virus seed samples for neutralization
and testing:
Batch number(s) of antisera or antiserum used for
neutralization of virus seeds:
Tests in tissue cultures for adventitious agents
Test in human cells
Type of human cells:
Quantity of neutralized sample inoculated:
Incubation conditions:
Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Test in simian cells
Type of simian cells:
Quantity of neutralized sample inoculated:
Incubation conditions:
Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Other cell types (if appropriate)
Type of cells:
Quantity of neutralized sample inoculated:
In substian conditions:

Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Test in animals for adventitious agents
Method:
Specification:
Dates of test (start, end):
Result:
Tests by molecular methods for adventitious agents
Method:
Specification:
Dates of test (start, end):
Result:
Tests for retroviruses
Method:
Specification:
Dates of test (start, end):
Result:
Tests for attenuation
The items tested and a detailed protocol should be provided.
Tests in mice for neurovirulence, reversion and
neuroinvasiveness, when appropriate
Method:
Specification:
Dates of test (start, end):
Result:
Tests in nonhuman primates for neurovirulence
Method:
Specification:
Dates of test (start, end):
Result:

4. Control of vaccine production

4.1 Control of production cell cultures

Information on preparation				
Lot number of master cell bank:				
Lot number of working cell bank:				
Date of thawing of ampoule of working cell bank:				
Passage number of production cells:				
Date of preparation of control cell cultures:				
Result of microscopic examination:				
Total of interest pre examination.				
Tests on control cell cultures				
Number of control cultures, or ratio of control				
cultures to production cell cultures:				
Incubation conditions:				
Period of observation of cultures:				
Dates started and ended:				
Proportion of cultures discarded, and reason:				
Results of observation:				
Date supernatant fluid collected:				
•				
Test for haemadsorbing viruses				
Quantity of cells tested:				
Method:				
Specification:				
Dates of test (start, end):				
Result:				
Test for adventitious agents on supernatant culture fluids				
Test in simian cells				
Type of simian cells:				
Quantity of pooled sample inoculated:				
Incubation conditions:				
Method:				
Specification:				
Dates of test (start, end)				
Proportion of cultures viable at end of test:				
Result:				
Test in human cells				
Type of human cells:				
Quantity of pooled sample inoculated:				

Incubation conditions:
Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Other cell types (if appropriate)
Type of cells:
Quantity of pooled sample inoculated:
Incubation conditions:
Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Test for identity of cells, if appropriate
Method:
Specification:
Dates of test (start, end):
Result:
Cells used for vaccine production
Observation of cells used for production (if appropriate)
Specification:
Date:
Result:
4.2 Single harvests
Information on manufacture
Batch number(s):
Date of inoculation:
Date of harvesting:
Lot number of virus master seed lot:
Lot number of virus working seed lot:
Passage level from virus working seed lot:
Methods and date of purification, if relevant:
Volume(s), storage temperature, storage time and duration
of approved storage period:

7	t
-	٠,
Š	-
c	١
000	Š
Q	C
C	
(
2	>
Corror	Ĺ
. 2	
S	Ļ
7	
(
2	1
2	L
٥	-
7	
hni	Ξ
2	
٠,	J
5	
c	
4	
2	2

Tests on single harvests
Test for identity
Method:
Specification:
Lot number of reference reagents:
Specification:
Date of test:
Result:
Result.
Virus titration for infectivity, if appropriate
Method:
Specification:
Dates of test (start, end):
Result:
Test for bacteria and fungi
Method:
Specification:
Media:
Number of containers tested:
Volume of inoculum per container:
Volume of medium per container:
Temperatures of incubation:
Dates of test (start, end):
Result:
T. (f 1
Test for mycoplasmas Moth od
Method:
Specification:
Media: Volume tested:
Temperature of incubation:
Positive controls:
Dates of test (start, end):
Result:
Result.
Test for mycobacteria
Method:
Specification:
Media:
Volume tested:

Temperature of incubation:
Dates of test (start, end):
Result:
Test for adventitious agents
Test in simian cells
Type of simian cells:
Quantity of neutralized sample inoculated:
Incubation conditions:
Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Test in human cells
Type of human cells:
Quantity of neutralized sample inoculated:
Incubation conditions:
Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Other call tupes (if appropriate)
Other cell types (if appropriate) Type of cells:
Type of cells:
Incubation conditions:
Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
TOO GET
4.3 Virus pool or bulk material
Information on manufacture
Batch number(s):
Date of preparation:
Methods and date of purification, if relevant: Volume(s) storage temperature storage time and duration
Volume(s), storage temperature, storage time and duration
of approved storage period:

Tests on virus pool or bulk material
Test for identity, if appropriate
Method:
Specification:
Lot number of reference reagents:
Specification:
Dates of test (start, end):
Result:
Virus titration for infectivity
Method:
Specification:
Dates of test (start, end):
Result:
Test for bacteria and fungi
Method:
Specification:
Media:
Number of containers tested:
Volume of inoculum per container:
Volume of medium per container:
Temperatures of incubation:
Dates of test (start, end):
Result:
Test for adventitious agents, if not performed on the single harvest
Test in simian cells
Type of simian cells:
Quantity of neutralized sample inoculated:
Incubation conditions:
Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Test in human cells
Type of human cells:
Quantity of neutralized sample inoculated:
In substice and discuss

Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Other cell types (if appropriate)
Type of cells:
Quantity of neutralized sample inoculated:
Incubation conditions:
Method:
Specification:
Dates of test (start, end):
Proportion of cultures viable at end of test:
Result:
Test for host-cell proteins, if applicable
Please provide these results as part of the characterization studies submitted for
the licensing application.
Method:
Specification:
Date of test:
Result:
Test for residual cellular DNA
Method:
Specification:
Dates of test (start, end):
Result:
Test for retroviruses, if applicable
Method:
Specification:
Dates of test (start, end):
Result:
Test for consistency of virus characteristics
Method:
Specification:
Dates of test (start, end):
Desult.

4.4 Final bulk
Information on manufacture
Batch number(s):
Date of formulation:
Total volume of final bulk formulated:
Monovalent virus pools used for formulation (serotype,
lot number, volume added, virus concentration):
Name and concentration of added substances, if relevant
(e.g. diluent, stabilizer):
Volume(s), storage temperature, storage time and duration
of approved storage period:
Tests on final-bulk lot
Virus titration for infectivity, if applicable
Method:
Specification:
Dates of test (start, end):
Result:
Test for bacteria and fungi
Method:
Specification:
Media:
Number of containers tested:
Volume of inoculum per container:
Volume of medium per container: Temperatures of incubation:
Dates of test (start, end):
Result:
Result.
Test for neurovirulence in mice
Method:
Specification:
Dates of test (start, end):
Result:
5. Filling and containers
Lot number:
Date of filling:
Type of container:

Volume of final bulk filled:				
Filling volume per container:				
Number of containers filled (gross):				
Date of lyophilization:				
Number of containers rejected during inspection:				
Number of containers sampled:				
Total number of containers (net):				
Maximum approved period of storage:				
Storage temperature and period:				
6. Control tests on final lot				
6.1 Tests on final vaccine lot				
Inspection of final containers				
Appearance:				
Specification:				
Date of test:				
Results:				
Before reconstitution:				
After reconstitution:				
Diluent used:				
Lot number of diluent used:				
Test for identity				
Method:				
Specification:				
Dates of test (start, end):				
Result:				
Test for potency				
Method:				
Batch number of reference vaccine and assigned potency:				
Specification:				
Dates of test (start, end):				
Result:				
Thermal stability				
Method:				
Specification:				
Dates of test (start, end):				
Result for each serotype:				

٩	4	
,	_	
ì		
1	7	2
(1	V
(5
ć	X	١
ì	ŝ	1
١	J.	١
	_	
	C	
٠	-	,
4	\geq	
	U	,
	ŏ	ļ
	ġ,	4
	0	j
t	2	
٠	٠	d
	ì	
	2	١
	a	۱
	ŝ	J
C	1	
•	_	
	7	5
	`	í
	>	
	0	
	ì	
	C	j
	Q	j
ŀ	_	
٠,		
(١
2	=	4
į	э.	
•	<	
4	\leq	
-	_	

Test for bacteria and fungi
Method:
Specification:
Media:
Volume tested:
Temperatures of incubation:
Dates of test (start, end):
Result:
General safety (unless omission of test authorized)
Tests in mice
Date of inoculation:
Number of animals tested:
Volume and route of injection:
Dates of observation period:
Specification:
Results (give details of deaths):
Tests in guinea-pigs
Date of inoculation:
Number of animals tested:
Volume and route of injection:
Dates of observation period:
Specification:
Results (give details of deaths):
Test for pH
Method:
Specification:
Date of test:
Result:
Residual moisture, if applicable
Method:
Specification:
Dates of test (start, end):
Result:
Residual antibiotics, if applicable
Method:
Specification:

Dates of test (start, end):
Result:
Endotoxins
Method:
Specification:
Dates of test (start, end):
Result:
6.2 Diluent
Name and composition of diluent:
Lot number:
Date of filling:
Type of diluent container:
Appearance:
Filling volume per container:
Maximum approved period of storage:
Storage temperature and period:
Other specifications:
7. Certification by the manufacturer
Name of the manufacturer
Name of head of production (typed)
Certification by the person from the control laboratory of the manufacturing company taking responsibility for the production and control of the vaccine
I certify that lot no of Japanese encephalitis vaccine (live, attenuated) for human use, whose number appears on the label of the final container, meets all national requirements and/or satisfies Part A^1 of the WHO Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use (2014) .
Signature
Name (typed)
Date

¹ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

² WHO Technical Report Series, No. 980, Annex 7.

8. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 5), a label from a final container and an instruction leaflet for users.

Model certificate for the release of Japanese encephalitis vaccines (live, attenuated) for human use by NRAs

Lot-release certificate
Certificate no
The following lot(s) of Japanese encephalitis vaccine (live, attenuated) for human use produced by
The release decision is based on
The coutif acts may include the following information.

The certificate may include the following information:

- name and address of manufacturer
- site(s) of manufacturing
- trade name and common name of product
- marketing authorization number
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)

¹ Name of manufacturer.

² Country of origin.

³ If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.

With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

⁵ WHO Technical Report Series, No. 980, Annex 7.

⁶ WHO Technical Report Series, No. 961, Annex 3.

⁷ WHO Technical Report Series, No. 822, Annex 1.

⁸ WHO Technical Report Series, No. 978, Annex 2.

⁹ Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.

WHO Technical Report Series No. 980, 2014

- type of container used
- number of doses per container
- number of containers or lot size
- date of start of period of validity (e.g. manufacturing date) and/or expiry date
- storage conditions
- signature and function of the person authorized to issue the certificate
- date of issue of certificate
- certificate number.

The Director of the NRA (or other appropriate authority)

Name (typed)	
Signature	
Date	

Annex 8

Biological substances: WHO International Standards and Reference Reagents

A list of WHO International Standards and Reference Reagents for biological substances was issued in Annex 4 of WHO Technical Report Series, No. 897 (2000) and an updated version is available at: http://www.who.int/biologicals. Copies of the list may be obtained from appointed sales agents for WHO publications or from: WHO Press, World Health Organization, 1211 Geneva 27, Switzerland (tel.: +41 22 791 3264; fax: +41 22 791 4857; e-mail: bookorders@who.int; web site: http://www.who.int/bookorders).

At its meeting in October 2012, the WHO Expert Committee on Biological Standardization made the changes shown below to the previous list.

Vaccines and related substances; blood products and related substances; cytokines, growth factors and biotherapeutics other than blood products; and in vitro diagnostic device reagents are held and distributed by the National Institute for Biological Standards and Control, Potters Bar, Herts, EN6 3QG, England. Antibiotic reference preparations are held by the European Directorate for the Quality of Medicines & HealthCare, Council of Europe, 7 allée Kastner, Cs 30026 F-67081, Strasbourg, France.

Additions

Preparation	Activity	Status	
Antibiotics			
Neomycin	19 050 IU per vial	Third WHO International Standard	
Neomycin B	17 640 IU per vial	Second WHO International Standard	
Biotherapeutics other than blood products			
Erythropoietin, recombinant, for bioassay	1650 IU per ampoule	Third WHO International Standard	
Follicle stimulating hormone (FSH), urinary, human and luteinizing hormone (LH), urinary, human	183 IU FSH and 177 IU LH per ampoule	Fifth WHO International Standard	

Preparation	Activity	Status	
Interleukin-2 (IL-2), human, recombinant	210 IU/ampoule	Second WHO International Standard	
Interleukin-29 (IL-29), human, recombinant	5000 units per ampoule	First WHO Reference Reagent	
Blood products and related	substances		
Factor II and X concentrate	Factor II: 9.4 IU per ampoule Factor X: 8.1 IU per ampoule	Fourth WHO International Standard	
Factor VII concentrate	10.6 IU per ampoule for clotting methods 9.8 IU per ampoule for chromogenic methods	Second WHO International Standard	
Factor XIa	10 units per ampoule	First WHO Reference Reagent	
Fibrinogen concentrate	Clottable protein 10.9 mg/ampoule Total protein 15.0 mg/ampoule	Second WHO International Standard	
Heparin, low molecular weight	Anti-Xa 1068 IU per ampoule Anti-Ila 342 IU per ampoule	Third WHO International Standard	
Urokinase, high molecular weight	3200 IU per ampoule	Second WHO International Standard	
In vitro diagnostic device re	agents		
Human immunodeficiency virus (HIV) genotype panel	No assigned values	Second WHO Subtype Reference Panel	
Substances for the control of parenteral pharmaceutical products			
Endotoxin	10 000 IU/vial	Third WHO International Standard	
Vaccines and related substances			
Antibody to human papillomavirus type 18	8 IU per ampoule when reconstituted in 0.5 ml of water	First WHO International Standard	

Preparation	Activity	Status
Antibody to influenza A/ California/7/2009 (H1N1) virus	1200 IU per ampoule for use in HI assay	Second WHO International Standard
BCG vaccine of Moreau-RJ substrain	3.1 million CFU and 24.69 ng ATP per ampoule	First WHO Reference Reagent
Diphtheria antitoxin, human	2 IU per ampoule	First WHO International Standard
Tetanus vaccine	260 IU/ampoule when assayed in mice	Fourth WHO International Standard

SELECTED WHO PUBLICATIONS OF RELATED INTEREST

WHO Expert Committee on Biological Standardization

Sixty-second report.

WHO Technical Report Series, No. 979, 2013 (xiii + 366 pages)

WHO Expert Committee on Biological Standardization

Sixty-first report.

WHO Technical Report Series, No. 978, 2013 (xi + 384 pages)

WHO Expert Committee on Biological Standardization

Sixtieth report.

WHO Technical Report Series, No. 977, 2013 (viii + 231 pages)

WHO Expert Committee on Biological Standardization

Fifty-ninth report.

WHO Technical Report Series, No. 964, 2012 (viii + 228 pages)

WHO Expert Committee on Biological Standardization

Fifty-eighth report.

WHO Technical Report Series, No. 963, 2011 (viii + 244 pages)

WHO Expert Committee on Biological Standardization

Fifty-seventh report.

WHO Technical Report Series, No. 962, 2011 (viii + 206 pages)

Web site: http://www.who.int/biologicals

Further information on these and other WHO publications can be obtained from WHO Press, World Health Organization, 1211 Geneva 27, Switzerland (tel.: +41 22 791 3264; fax: +41 22 791 4857; e-mail: bookorders@who.int; order online: http://www.who.int/bookorders)

This report presents the recommendations of a WHO Expert Committee commissioned to coordinate activities leading to the adoption of international recommendations for the production and control of vaccines and other biologicals, and the establishment of international biological reference materials.

Following a brief introduction, the report summarizes a number of general issues brought to the attention of the Committee. The next part of the report, of particular relevance to manufacturers and national regulatory authorities, outlines the discussions held on the development of revised WHO Recommendations and Guidelines for a number of vaccines, blood products and related substances. Specific discussion areas included the development of WHO guidance on the quality, safety and efficacy of poliomyelitis vaccines; recombinant malaria vaccines; diphtheria vaccines; tetanus vaccines; combined vaccines based on diphtheria and tetanus vaccines; and Japanese encephalitis vaccines.

Subsequent sections of the report then provide information on the current status and proposed development of international reference materials in the areas of vaccines and related substances; blood products and related substances; in vitro diagnostic device reagents; biotherapeutics other than blood products; and antibiotics.

A series of annexes are then presented which include an updated list of WHO Recommendations, Guidelines and other documents on biological substances used in medicine (Annex 1), followed by a series of WHO Recommendations and Guidelines adopted on the advice of the Committee (Annexes 2–7). All additions made during the meeting to the list of International Standards and Reference Reagents for biological substances maintained by WHO are then summarized in Annex 8, and are also available at: http://www.who.int/biologicals.

