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Meeting Report

WHO Working Group on Technical Specifications for Manufacture and Evaluation of Dengue Vaccines

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Summary

In May 2009, a group of international experts on dengue, vaccine quality and clinical evaluation met together i) to review disease, vaccine pipeline, quality issues in manufacturing, issues of environmental risk assessment, nonclinical and clinical evaluation of live recombinant dengue vaccines and ii) to initiate revising WHO guidelines for the production and quality control of candidate tetravalent dengue vaccines (live). This report summarizes an exchange of views on scientific and technical issues related to the quality, safety and efficacy of candidate dengue vaccines. Recognizing live dengue vaccines are the major vaccines in the clinical pipeline, the Working Group agreed i) to focus on live dengue vaccines in the revision of the WHO guidelines and ii) to add new guidelines on nonclinical and clinical evaluation, and environmental risk assessment for live dengue vaccines in the revision.

Keywords: Dengue vaccines; World Health Organization; Standards; Evaluation

1. Background and Objectives

Dengue is a common mosquito-borne viral disease of humans that has become a major international public health concern.. The geographical spread of both the mosquito vectors and the viruses has led to the global resurgence of epidemic dengue fever and emergence of dengue hemorrhagic fever (dengue/DHF) in the last 50 years with the development of hyperendemicity in many urban centers of the tropics. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-east Asia and the Western Pacific. South-east Asia and the Western Pacific are the most seriously affected. There is no specific treatment for dengue, but appropriate medical care frequently saves the lives of patients with the more serious dengue haemorrhagic fever. The only way to prevent dengue virus transmission is to combat the disease-carrying mosquitoes. Vaccination holds the potential for an effective intervention against dengue. Hence, there is an urgent need for developing dengue vaccines, especially to protect people from disease in endemic countries.

Efforts to develop a vaccine against disease caused by dengue virus (DENV) have mainly focused on live attenuated vaccine type. Success in the development and clinical use of live attenuated flavivirus vaccines such as 17D yellow fever (YF) vaccine and SA14-14-2 Japanese encephalitis (JE) vaccine has provided significant guidance on developing live attenuated DENV vaccines. Other vaccine candidates including inactivated vaccines, subunit vaccines that comprise premembrane/envelope (prM/E) proteins, and DNA vaccines that induce expression of DENV prM/E proteins in injected sites are in preclinical or early clinical stages of development.

To prevent the potential of antibody-dependent enhancement (ADE) of DENV infection by heterologous DENV antibodies resulting in severe dengue, including dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS), there is consensus that DENV vaccines should stimulate protective neutralizing antibodies to each of the four serotypes, preferably simultaneously.

Several strategies have been employed to develop live attenuated vaccines. DENV vaccine candidates passaged serially in primary dog kidney (PDK) cells at Mahidol University were shown to be insufficiently attenuated and subsequently were withdrawn from further consideration. However, the DENV serotype 2 (DENV-2) vaccine in this series, designated DENV-2 PDK53, is attenuated and provides the genetic background for development of a tetravalent DENV vaccine in which DENV-1, -3 and -4 component vaccines express the respective prM/E genes in the context of the DENV-2 PDK53 genome [1, 2].

The Walter Reed Army Institute of Research (WRAIR) independently attenuated four serotypes of DENV by serial passage in PDK cells and produced vaccine candidates in fetal rhesus lung (FRhL) cells. Tetravalent formulation of these vaccine candidates comprising attenuated four serotypes have been evaluated in Phase I clinical trials conducted by WRAIR and GlaxoSmithKline (GSK) and are currently in Phase II clinical trials [1-4].

An attenuation strategy used at the Laboratory of Infectious Disease of the National Institute of Allergy and Infectious Diseases (NIAID) has focused on introducing mutations into the 3'-

untranslated region (UTR) of DENV-4 and DENV-1 cDNA clones for attenuation [1-4]. The DENV-1 and DENV-4 vaccine candidates containing a 30-nucleotide (nt) deletion (nt 172-143), designated Δ 30, have a balance between attenuation and immunogenicity in nonhuman primate models. DENV-2 and DENV-3 vaccine candidates have been developed by inserting the prM and E proteins of DENV-2 and DENV-3 into the same region of the DEN4 Δ 30 candidate vaccine. A second DENV-3 candidate vaccine was developed by replacing the 3' UTR of a DENV-3 wild-type virus with that of the DEN4 Δ 30 UTR. Phase I trials have been conducted with Δ 30 and chimeric candidates as monovalent vaccines [1, 2, 5], and Phase I trials with the tetravalent formulation are scheduled in 2010.

The ChimeriVax™ platform is being used by Acambis-Sanofi Pasteur to create chimeric DENV vaccine candidates by substituting the prM and E genes from each of the four DENV serotypes into the YF17D vaccine strain of yellow fever virus. Tetravalent ChimeriVax™ DENV vaccines have been evaluated in Phase I and Phase II clinical trials and have demonstrated safety and immunogenicity. Phase IIb trials in children have begun in late 2009 to build a database for Phase III studies in Asia and the Americas [1, 2].

In May 2009, a group of international experts on dengue, vaccine quality and clinical evaluation met to review disease, vaccine pipelines, quality issues in manufacturing, issues of environmental-risk assessment, nonclinical and clinical evaluation of live recombinant dengue vaccines, and to initiate revising the World Health Organization (WHO) guidelines for the production and quality control of candidate tetravalent DENV vaccines (live). The meeting was held at WHO, Geneva.

In their opening remarks, Drs. D. Wood and J. Hombach (WHO) welcomed the participants on behalf of the Organization and explained the WHO role in providing guidance to public health authorities, national regulatory authorities, and the scientific community as well as to vaccine developers and manufacturers through standards and technical advice. For this reason, WHO prepares and continues to update reference documents relying on worldwide experts. Technical specifications for quality, efficacy and safety of new candidate recombinant genetically-engineered dengue vaccines need to be developed and the existing guidelines modified to include

recombinant live attenuated dengue vaccines, which are anticipated to be the first vaccines licensed for human use.

Dr. J. Shin (WHO) presented an overview of the meeting and role of the WHO global written standards for vaccines. He indicated that written standards for the quality, nonclinical and clinical evaluation of vaccines should help in the development of safe and efficacious vaccines. The standards are written as recommendations or guidelines intended to provide scientific and regulatory advice to vaccine developers. These guidelines provide guidance for regulatory authorities and manufacturers on international regulatory specifications for production and quality control of vaccines as well as the nonclinical and clinical evaluation of vaccines. In addition, they serve as reference for vaccine prequalification. They are living documents that undergo revisions in response to scientific advances. The process for production and endorsement of global written standards was reviewed and the structure of the written standards presented. The development of dengue vaccines has made significant progress since the adoption of the dengue written standards in 2004 [6] and they now need to be updated. Special considerations should be given to the quality-control aspects of live recombinant vaccines (e.g. genetic stability), environmental-safety aspects of live recombinant vaccines, and the use of Vero cells for the production of live parenteral vaccines.

Dr. P. Minor (National Institute for Biological Standards and Control, UK) agreed to act as Chair and Dr. D. Trent (individual consultant, USA) as Rapporteur.

2. Overview of Dengue and the Dengue Vaccine Pipeline

2.1 Disease and Epidemiology of Dengue

Dr. R. Edelman (University of Maryland, USA) reviewed dengue disease and epidemiology. Prior to the 1970s, only up to five countries located in Asia including Philippines and Thailand reported annual cases of dengue fever (DF) and DHF. However, DHF now occurs in more than 60 countries, including most of South-East Asia, South and Central America, the Caribbean and

South Pacific. DF is present in Africa as well, but disease outbreaks are rarely reported, possibly due to poor surveillance. Co-circulation of multiple serotypes is common within countries most affected by DENV.

DENV is transmitted to humans by the bite of infected *Aedes (A.) aegypti* mosquitoes, which are highly domesticated and the primary mosquito vector; however, *A. albopictus* can also sustain human-to-human transmission. The drastic increase in the incidence of DENV infection in the Americas during the past 30 years is primarily due to the geographical spread of *A. aegypti* following decline in vector-control efforts. The DENV that infects and causes disease in humans is maintained in a human-to-mosquito-to-human cycle and does not require a sylvatic cycle in non-human primates. Certain strains of DENV are known to be transmitted to non-human primates in western Africa and Malaysia; however, transmission to humans via mosquitoes from non-human primates is infrequent.

Most DENV infections are either asymptomatic or only mildly symptomatic. Most symptomatic infections present as classical DF, with an incubation period range of 3 to 14 days, but generally 4 to 7 days. DF presents as a sudden onset of fever accompanied by headache, pain behind the eyes, generalized myalgia and arthralgia, flushing of the face, anorexia, abdominal pain and nausea. It is believed that, following infection by the bite of an infected mosquito, the virus replicates in local dendritic cells, with subsequent infection of macrophages and lymphocytes followed by entry into the blood stream. Rash is common in DF and can present as a macular, maculopapular, morbilliform, scarlatiniform or petechial in character most often seen on the trunk, insides of the arms and thighs, and plantar and palmar surfaces. Severe dengue is clinically severe dengue syndromes, including DHF, DSS, and all episodes of organ dysfunction after infection (“complicated dengue”).

Containment of dengue through vector control has proven to be very difficult and costly. While vector control efforts should be sustained, vaccination holds substantive potential in the control of the diseases. Hence, there is an urgent need to develop dengue vaccines, especially to protect people from disease in endemic countries, such as those in South-East Asia and Latin America.

2.2 A View of the Current Dengue Vaccine Pipeline

Dr. J. Roehrig (US Centers for Disease Control and Prevention) introduced a dengue vaccine pipeline that includes attenuated DENV vaccine candidates at the most advanced stage of development. DENV is a member of the *Flavivirus* genus of the virus family *Flaviviridae*. The virus is a spherical particle of approximately 50 nm in diameter with a relatively smooth surface. The virion contains a single-stranded, positive-sense RNA genome of approximately 11 kb in length. The genome is positive in sense and has a single open reading frame that is translated into a large polyprotein which is processed by virus-encoded and host-cell proteases into three structural and at least seven non-structural (NS) proteins. The surface of the particle consists of a compact arrangement of 180 copies of the E and prM proteins. Immunity to the virus is mediated by neutralizing antibodies to the E protein, which contains epitopes that range from DENV serotype-specific to flavivirus group-reactive.

The primary antigen of the dengue virion is the E protein, although antibody responses are also directed towards other proteins, including the virion prM and nonstructural proteins. The E protein can be divided into three structural/functional domains designated I, II and III. Serotype-specific, neutralizing antibodies are elicited to epitopes on the surface of domain III, which has been implicated in cell-receptor binding. Although antibodies to domain I are generally non-neutralizing, antibodies that recognize epitopes in domain II, which contains the fusion peptide region, neutralize the virus by preventing fusion of the virion E protein with the cellular membrane. Antibodies to domain III have been shown to also block fusion for other flaviviruses.

Clinical trials of several candidate dengue vaccines have proceeded without a comprehensive understanding of the pathogenesis of severe dengue disease or an adequate animal model. Protective antibodies against DENV neutralize virus infectivity. The most efficacious virus-neutralizing antibodies are serotype-specific, however, virus-neutralizing antibodies cross-reactive between serotypes likely serve a role in protection. Serotype cross-reactive non-neutralizing antibodies, and sub-neutralizing levels of serotype-specific antibodies mediate enhanced viral entry into Fc-receptor bearing susceptible cells and subsequent replication in vitro. Therefore, dengue vaccines should contain each of the four serotypes to avoid the

theoretical risk of enhanced dengue disease severity due to cross-reactive non-protective antibody. Table 1 outlines dengue vaccine candidates that are at the most advanced stage of development.

During discussion, it was agreed that i) manufacture and quality control criteria for inactivated or subunit vaccines are different from those that apply to live vaccines and that ii) principles governing their manufacture and clinical implementation already exist (e.g. as applied to the existing hepatitis B vaccine [7, 8] and human papillomavirus vaccine [9]). There is a separate guidance document for DNA vaccines available for manufacture, quality control and nonclinical evaluation [10]. Dr. I. Knezevic commented that, to bring a candidate vaccine into the scope of a written standard, the status of entry into clinical phase III is important. It was agreed that vaccine-related issues that must be addressed for manufacturing, preclinical testing and clinical trials with the different DENV vaccines include:

- Safety, immunogenicity and efficacy of the final vaccine product
- Genetic stability of live attenuated vaccines
- Environmental safety of genetically modified viruses
- Consensus among national regulatory authorities and WHO on the regulatory issues that define the manufacture, clinical testing and licensure of live DENV vaccines

Table 1. Most advanced dengue vaccine candidates

Producers	Developer	Approach	Current Status
Sanofi Pasteur	Acambis (now Sanofi Pasteur Biologics)	YF17D chimera Tetravalent ChimeriVax™	Completed Phase I/II. Phase IIB trial in children 2009
GlaxoSmithKline	Walter Reed Army Institute of Research	Primary dog kidney passage - attenuation Tetravalent	Phase II studies in adults and children

Biological E, Butantan, Panacea	NIH Laboratory of Infectious Diseases	DEN4Δ30/DEN chimeras & non- coding 3'Δ30 deletion mutants Tetravalent	Phase I clinical trials with tetravalent formulations in 2010
Inviragen	Centers for Disease Control and Prevention	DEN2PDK53/DEN chimeras Tetravalent	Phase I trials in 2009 and 2010
Hawaii Biotech	Hawaii Biotech	DENV 80% E and NS1 recombinant proteins Monovalent and Tetravalent antigen	Process development Phase I by 2009 – 2010

3. Cell Culture Substrates Used for Dengue Virus Vaccine Production

3.1 Introduction

Dr. K. Eckels (WRAIR, USA) reviewed cell substrates for dengue vaccine production. Cell culture substrates currently used for production of live attenuated DENV vaccine candidates include Vero cells, a continuous line derived from the African green monkey (*Cercopithecus aethiops*) and DBS-FR_hL-2 (FR_hL), a normal, diploid cell derived from fetal lung tissue of the rhesus monkey (*Macca mulatta*) (Table 2).

Table 2. Cell cultures used for production of candidate dengue virus vaccines (attenuated)

Cell Substrate	Candidate Vaccine
Vero Cells	<ul style="list-style-type: none"> • Sanofi-Pasteur ChimeriVax™-DEN • NIH DEN 4Δ30DEN (chimera), DENΔ30' • CDC/Inviragen DENVax (chimera)
FRhL Cells	<ul style="list-style-type: none"> • WRAIR/GSK DENV

Vaccines produced in either Vero or FRhL cells have been licensed by national regulatory authorities (NRA) including the US Food and Drug Administration (FDA). As for all vaccines, licensure requires establishing and characterizing a master cell bank (MCB) and a working cell bank (WCB). A WHO Vero cell line (10-87) can be obtained from the American Type Culture Collection (ATCC) or the European Collection of Cell Cultures (ECACC) [11-13]. The WHO Vero 10-87 cell line is at passage 134, and FRhL cells deposited at the ATCC by the US FDA are at passage 10 [13]. In terms of in vitro culture passage, Vero cells are recommended to be used by manufacturers at no more than 150 passages due to their potential to become tumorigenic and FRhL cells at no more than 60 passages due to the development of senescence [14]. FRhL cells are normal and have a finite life span.

Each of these cell types has advantages and disadvantages for production of live attenuated DENV vaccines. Vero cells can be grown on microcarriers in large volume bioreactors in serum-free medium. DENV grows to high titer in Vero cells but the vaccine virus requires purification to remove cellular proteins and cellular DNAs. Attenuated dengue vaccine viruses replicate to high titer in FRhL cells grown in stationary cell culture systems. Harvested virus does not have to be highly purified as FRhL cells are a normal diploid cells.

In the discussion, Dr. K. Eckels indicated PDK cells are no longer being considered as a manufacturing substrate for dengue vaccines, while MRC-5 cells, a normal, human diploid cell, could be considered as a manufacturing substrate. Potential concerns about live vaccines derived from a continuous cell line such as Vero cells led to a discussion of the purity of candidate dengue vaccines. In the case of oral polio vaccine derived from Vero cell cultures, the vaccine is highly purified. Dr. L. Mallet informed the group that purification of the polio virus was very

efficient, resulting in reduction of the quantity and size of host cell-derived DNAs to meet regulatory standards. Dr. L. Markoff indicated cellular proteins are present in attenuated vaccines and the Center for Biologics Evaluation and Research (CBER) is contemplating establishing criteria to limit the amount of cellular protein in vaccines. Dr. K. Peden commented it would be difficult to quantify cellular proteins and DNA in vaccines if they are present in very small amounts.

3.2 Risk of DNA from Neoplastic Cell Substrates

Dr. K. Peden (US FDA) elaborated on the potential risks associated with the presence of residual cellular DNA in vaccines. If the vaccines are produced in tumorigenic cells, a risk assessment should be carried out and the risk factors defined, as far as possible in a quantitative manner. However, risks may be difficult to define qualitatively or quantitatively because the endpoints are either theoretical or unmeasurable.

The potential risk associated with use of tumorigenic cells in manufacturing vaccines should be evaluated against the benefits of intended clinical use of the product. Residual cellular DNA is perceived to be one of the risks to vaccine recipients; the other is the presence of adventitious agents. The major issues associated with residual DNA from continuous cell lines are its oncogenicity and infectivity. The oncogenic activity would arise from DNA that contains activated dominant oncogenes, while infectivity would arise from DNA that encodes an infectious virus genome (e.g. from a retrovirus), which could generate an infectious virus following inoculation. The approach taken to determine the risk of residual cell-substrate DNA in vaccines was to develop quantitative assays to measure the infectivity activity and the oncogenic activity of DNA. From the results of these assays, estimates of risk were made based on the minimum amount of DNA active in the most sensitive assay; such estimates, therefore, are conservative. In addition, the assays were used to determine how much reduction in DNA activity can be achieved with such treatments as nuclease digestion and chemical inactivation. The combination of reducing the amount of DNA and reducing its biological activity can be

factored into risk estimates. For a more detailed discussion of DNA issues, see Sheng-Fowler et al (2009) [15].

The Vero cell line is the only continuous cell line used for licensed vaccines in the US at the time of this meeting. Vero cells are used for producing live attenuated and inactivated poliovirus vaccines, live attenuated smallpox vaccine, and a live attenuated reassortant rotavirus vaccine. The live poliovirus vaccine and the rotavirus vaccine are administered orally; the only licensed live vaccine manufactured in Vero cells that is administered parenterally, is the smallpox vaccine. However, Vero cells are currently being used for producing experimental vaccines, under investigational new drug (IND) applications, such as influenza, rabies, and hepatitis A vaccines and live vaccinia-vectored vaccines.

In conclusion, WHO has established DNA limits for parenterally administered vaccines: i) vaccines produced in primary and diploid cells have no limit of residual DNA; and ii) vaccines produced in continuous cell lines have a limit of ≤ 10 ng of DNA per single parenteral dose. Therefore, dengue vaccines produced in Vero cells should contain no more than 10 ng of DNA per dose. The situation for FRhL-2 cells might be different, as these are diploid cells, and the NRA should be consulted for guidance if these cells are used.

Current US FDA guidance for clearance of DNA from vaccines that are manufactured in certain continuous cell lines (e.g., tumorigenic cells or cells derived from human tumors) and that are administered parenterally is, like WHO recommendations, to reduce the amount of DNA to ≤ 10 ng per dose but with the additional recommendation to reduce the median size of the DNA to 200 base pairs or smaller.

3.3 WHO Recommendations for Evaluation of Cell Substrates for Production of Biologicals

Dr. I. Knezevic (WHO) introduced the main issues in the revision of WHO recommendations for evaluation of cell substrates for the production of biologicals. The selection of an appropriate cell

substrate for use in the production of biological products has been a recurring focus for the past 50 years. The central question has always been “is the product manufactured in a given cell substrate going to be safe to use in humans?” The safety issue has expanded to include consideration of elements within the cell other than microbial agents that could be transmitted to humans. Concern has been expressed regarding the transmission of activated oncogenes present in the cell-substrate DNA (see above). Another example of a transmissible cellular element would be prions known to be the causative agents for certain encephalopathies.

The need to revise requirements for cell substrates was identified by the WHO Expert Committee on Biological Standardization (ECBS). In response, WHO established a Study Group in 2006 that prepared updated recommendations for evaluating cell substrates for producing biologicals. A review of scientific evidence during 2006 and 2007 to support revision of the recommendations focused on evaluating new cell lines, tumorigenicity of continuous cell lines, oncogenicity and infectivity of cell DNA, and tests for quantifying residual cell DNA. Details of the changes proposed by the Study Group in 2007 were published [16]. In 2008, discussions with manufacturers and regulators led to the conclusion that guidance on good cell-culture practice should be included in the updated recommendations. In 2009, a draft revision was circulated to regulators, manufacturers and other experts for review and comments.

The Study Group held its third meeting in April 2009 to review progress in the revision and to propose further updates. This meeting provided clarifications of the rationale for in vivo testing as well as the potential for applying new in vitro testing methods to detect microbial agents. In addition, the need for reference preparations, reference cell banks and standardized test methodologies was discussed. The draft revision provides comprehensive information on the advantages and disadvantages of various animal models for testing tumorigenicity in vivo. Selection of the appropriate animal model for a particular cell substrate should be made by manufacturers and agreed by NRAs.

New cell lines for producing live attenuated vaccines need to be carefully reviewed and considered. Cells from dogs and insects are considered as potential hosts for vaccine production. The examples of new cell lines include Madin-Darby canine kidney, 293, PER.C6 and *Trichoplusia ni* Hi5 insect cells. New testing methodologies for characterization and their

specifications to determine quality and safety of novel substrates need to be developed and validated.

WHO has been requested to take the lead in a global consultation process to set standards for the safety assessment of new vaccine cell substrates. These standards should allow introduction of new cell substrates for production of new vaccines (e.g. vaccines against human immunodeficiency virus/acquired immunodeficiency syndrome) that cannot be produced in currently accepted substrates or for improving production yields, such as dengue, rabies and influenza vaccines. After consultations with a broad audience of regulators, manufacturers and other experts in this field, the Study Group plans to submit a draft revision of recommendations on cell substrates to the ECBS in 2010.

4. Quality Control Aspects of Manufacturing Live Recombinant Dengue Vaccines

Dr. D. Trent (individual consultant, USA) started his talk by introducing the legal basis of vaccine regulation in the USA and the definitions of vaccines, safety, purity, potency, and standards in the US statutes [17].

The genetic stability of recombinant dengue viruses can be determined by nucleotide sequence analysis. The best approach is to confirm the presence of the mutations or deletions that have been introduced for attenuation of a vaccine virus and to ensure these mutations are present in the vaccine master and working seeds. Dideoxy nucleotide sequence methods confirm sequence analysis of $\pm 90\%$ of the virus population. More sensitive TaqMan mismatch amplification mutation assay (TaqMAMA) can be applied to determine the sequence of $\pm 0.01\%$ of the population for a specific nucleotide change.

Potency of the vaccine can be measured by determining virus infectivity in tissue culture, in terms of either plaque-forming units or focus-forming units or 50% cell culture infective dose per

unit of volume, once the immunizing dose for humans has been established via clinical trial results. The quality of the vaccine measures production consistency, and it can be determined by, the number of genome equivalents per infectious unit for each serotype component of a tetravalent vaccine. This type of analysis would provide insight into consistent efficacy of the vaccine and genetic changes which may affect attenuation.

All vaccines must be developed by processes that include preparation of a pre-master seed, master seed, working seed, single harvest, monovalent vaccine bulk substance, pooled vaccine bulks to form the tetravalent bulk vaccine substance, and vaccine product. All of the foregoing steps require the prior establishment and complete characterization of MCB and WCB. Quality control testing of the virus vaccine at each stage of development is well documented and outlined in detailed guidance documents applicable to development, manufacture, licensure and use of vaccines [17-19]. Standards for quality control testing of recombinant DENV vaccines to ensure genetic stability of mutations introduced into the vaccine (in turn, to ensure attenuation and absence of reversion to virulence) must consider testing both genetic sequence and the linked phenotype of the vaccine. These tests supplement the standard quality control testing currently recommended by US FDA, EMA (the European Medicines Agency) and WHO for live attenuated vaccines. This process is as follows: i) seed lot system specifying a limited number of passages from master seed virus to vaccine; and ii) confirmation that genetically engineered attenuation markers and specific phenotypic markers present in the master and working seeds are present in the vaccine product.

In discussion, the main subject of attention was about ways to deal with phenotypic and genotypic markers for attenuation of recombinant dengue vaccines in quality control (QC) terms. Table 3 shows a summary of proposed QC tests, for dengue vaccines at different stage of vaccine production, that were agreed in principle. Issue of product-specific tests were raised but not discussed in detail.

There were many questions that may be important for future reference:

- Does plaque size variation correspond to genetic change?

- Would suckling mouse neurovirulence test be useful in testing safety of plaque size variants?
- Are there significant genetic and biological differences in a dengue vaccine with YF backbone with those who have an attenuated DENV backbone in the mouse model versus and vaccinated human?
- How to judge the possible significance of the detection of spontaneous mutations in vaccine virus genomes that may occur during production?
- What is the role for monkey neurovirulence testing in evaluating vaccine virus seeds or in lot release?
- Would the mouse models for immunogenicity and pathogenesis of the DENV vaccine be relevant to research only or also for product registration?
- Would batch-to-batch testing for genetic stability be useful?
- Would it be possible to develop mutant analysis by PCR - polymerase chain reaction - and restriction enzyme cleavage (MAPREC) or similar kinds of assay to monitor genetic stability of live vaccines.

In wrapping up the discussion, Dr. Minor suggested that, as quality is the marker of manufacturing consistency, phenotypic stability - despite lack of association with virulence - can be used as a quality check. He suggested that the use of a reference preparation would facilitate assay standardization.

Table 3. Proposed quality control testing of dengue virus recombinant vaccines (monovalent seed and/or vaccine bulk)

Quality control test	Virus vaccine preparation			
	Pre-master	Master seed	Working seed	Bulk substance
Potency	X	X	X	X
Plaque size	X	X	X	X
Mouse	X	X	X	

neurovirulence*1				
Mosquito replication, dissemination, transmissibility	X	X		
Total genome sequence	X	X	X	
Specific mutation sequence	X	X	X	
Quantitative TaqMAMA*2 specific mutations	X	X	X	

*1 Monkey neurovirulence for master seed lot is needed for a certain vaccine

*2 TaqMAMA: TaqMan mismatch amplification mutation assay

5. Environmental Risk and Population Safety of Live Recombinant Vaccines

5.1 Scientific Considerations

Dr. D. Bleijs (National Institute for Public Health and the Environment, The Netherlands) introduced environmental risk assessment of genetically modified organism (GMO). The risk assessment of GMO is based on the likelihood of its unintended transfer or transmission to humans other than the intended person, to animals or to the environment at large, as well as the extent of its impact on the environment.

Environmental risk assessment (ERA) requires identification and evaluation of potential adverse effects of the GMO on human health and the environment on a case by case process. The specific purposes of the investigation are to identify needs for risk management and to propose methods that can be employed.

The methodology of risk assessment involves identification of the hazard, estimation of the likelihood, risk estimation, risk management and estimation of the overall risk. These processes should identify the potential adverse effects by comparing the GMO with non-modified organisms under the same conditions and by evaluating the potential consequences of each potential adverse event. For example, does the GMO pose a threat of disease to humans including allergenic or toxic effects; disease to animals; or have effects on other organisms in the environment and/or altered susceptibility to pathogens that would compromise prophylactic or therapeutic treatment?

DENVs that have been attenuated by passage in primary dog kidney cells have acquired a combination of mutations that attenuate the virus for humans; however these viruses are not regarded as GMO. All of the DENV vaccines in which the genetic material has been genetically modified by recombinant DNA technology are considered to be GMOs. The chimeric DENV vaccines have been constructed by cloning of the prM and E genes of DENV into the backbone of another attenuated flavivirus. The attenuated virus vectors that are currently being used to create attenuated chimeras are for example YF-17D, DEN4Δ30 and DEN2-PDK53. Important aspects of an environmental risk assessment for these recombinant vaccines include: 1) parental organism, 2) recipient organism, 3) vector characteristics, 4) characteristics of the donor sequence, 5) genetic modification, 6) intended release and 7) the receiving environment.

Risk factors of significance include: 1) pathogenicity of the of vaccine virus for man, mosquitoes and other potential hosts, 2) virus host range, 3) tropism of the vaccine virus, 4) survival of the virus in the environment, 5) transmissibility of the virus by mosquito vectors from vaccinated humans and 6) potential for recombination of the vaccine virus with other viruses in the environment including humans, animals and mosquitoes.

An environmental risk assessment is required for the recombinant GMO DENV vaccines. The factors that must be considered for the assessment include i) genetic stability of the viruses (reversion to virulence), ii) potential for transmission from vaccinated person, iii) the potential for recombination between the vaccine virus and other flaviviruses, which may be present in mosquitoes, and iv) the immune status of population to be vaccinated because the presence of pre-existing immunity due to earlier exposure to DENVs is likely to reduce the extent and duration of vaccine virus biodistribution and shedding.

Issues outlined in the WHO Technical Report Series, No. 932 (2006) need to be considered in communication with the country in which the vaccine trials are to be conducted. Each national jurisdiction may have specific regulations regarding vaccines that are considered to be a GMO.

In the discussion, Dr. Bleijs indicated the principle of ERA methodology would be similar regardless of how it is approached by different jurisdictions. The manufacturing, use, and trans-boundary shipping of such recombinant vaccines should comply with the environmental regulations of the producing and recipient countries regarding GMO. Further clarification for the role of ERA in the EU was raised. To obtain marketing authorization in the EU, a single assessment for submission to the EMA would be sufficient, but outside the EU, the respective NRA should be consulted on ERA issues related to clinical trial approval. A question to ask is whether it is appropriate to impose ERA only because it is a GMO despite low pathogenicity, while highly pathogenic viruses are present in nature, e.g. avian influenza. Dr. Bleijs indicated, that in many countries, to comply with environmental regulations, an ERA should be undertaken if a live recombinant vaccine is being tested in a clinical trial and the vaccine will be administered to the general public. The objective of an ERA is to identify and evaluate, on a case-by-case basis, potential adverse effects of the application of a GMO on public health and the environment. Ideally, the result of an ERA is based on quantitative data, and expressed in quantitative terms. However, the critical information in an ERA may be qualitative for the reason that quantification is often hard to accomplish. The ERA does not have to be based on the scenario that is expected to occur; but may be based on a worst-case scenario. A worst case scenario is usually applied in cases where there is a high degree of scientific uncertainty. It is anticipated that the worst-case scenario will be more often applicable to replication-competent

live recombinant viral vaccines as compared with replication-defective viral vaccines. The process of using models for evaluating safety is applied, because precise data on the environmental fate of the live vaccine will generally be lacking. It was agreed that to help resolve safety issues it can be helpful to conduct an ERA if the vaccine has been genetically manipulated and safety issues are of concerns.

5.2 Development of Regulatory Documents in Europe

Dr. J. Robertson (NIBSC, UK) outlined European guidelines on ERA. Despite the existence of European legislation on ERA, there has been a tendency for individual member states to interpret the legislation in their own way, resulting in different interpretations of regulatory processes and policies. Product class specific guidelines for ERA have been developed at the EMA by three different working parties, while the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) Gene Therapy Expert Group is drafting considerations for general principles of how to address viral/vector shedding.

The EMA Biologics Working Party (BWP) outlines the legislative frame work for the ERA in the marketing application for medicines containing a GMO including:

- i) Administrative and procedural details that define the applications
- ii) A description of the ERA methodology and scientific issues that must be addressed
- iii) Recommendations for a pre-licensure submission meeting of applicant with the EMEA
- iv) ERA review based on factual data but may involve theoretical assumptions
- v) If quantitative data are insufficient and no animal model is available, the EMEA review will resort to a worst-case scenario
- vi) If unacceptable risks are defined, then the risk-reducing measures must be defined, and a conclusion of the environmental acceptability overall has to be made

The EMA Gene Therapy Working Party (GTWP) outlines steps in the ERA review of the GMO as follows:

- i) Characteristics of the GMO that cause adverse effects
- ii) Evaluation of potential consequences of each hazard
- iii) Evaluation of the likelihood of occurrence of each hazard
- iv) Estimation of the risk
- v) Application of risk management strategies
- vi) Determination of the overall risk

Draft ICH considerations of general principles to address viral/vector shedding include: i) a clear definition of the concept of shedding, biodistribution, and transmission; ii) factors to consider in designing non-clinical and clinical shedding studies, iii) analysis and impact of resulting data in the assessment for the risk of transmission and possible consequences; iv) differing opinions on the availability, quality, and quantity of virus or vector shedding data and the potential value of these studies relative to the GMO risk.

The EMA Vaccine Working Party (VWP) has also developing guidance on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines, and the finalized guidance (EMA/CHMP/VWP/141697/2009) is available on its website .

In discussion, it was noted that the requirement or outcome of an ERA would be different depending on endemic and non-endemic areas. A function of WHO written standards for vaccines is to serve as the basis of WHO technical specifications for prequalification of vaccines purchased by UN agencies and guidance on ERA needs to be considered in the revision of dengue written standards: feedback on this issue would be helpful.

5.3 FDA Policy on “Medicinal GMOs” and its Application to Vaccines

Dr. L. Markoff (US FDA) presented information regarding the FDA perspectives on GMO issues. Flavivirus recombinant vaccines have genomes that are chimeric and/or the genome has been genetically modified by site-directed mutagenesis. Questions related to the use of live recombinant vaccines include tissue tropism and virulence of the wild-type virus and the

recombinant. FDA guidance related to the phenotype of recombinant viruses (21 CFR312.23 p8) describes testing to be considered when evaluating the safety of GMOs. The FDA has rescinded elements of this CFR that require neurovirulence testing of all recombinant vaccines.

Neurovirulence of the vaccine is considered on a case-by-case basis. The FDA is developing an algorithm by which decisions regarding neurovirulence testing will be determined, but this document has not been finalized. A draft document of this algorithm for neurovirulence testing indicates that decisions regarding neurovirulence testing could be based on whether nervous systems, either central or peripheral or both, are the primary targets for the wild-type virus. If any nervous systems are identified as viral targets, then the NRA may consider whether the method of attenuation eliminates all risks of neurovirulence. An example of a “GMO” DENV vaccine that was tested for neurovirulence is ChimeriVax™-DEN, because the genetic basis for this vaccine relies upon the attenuated phenotype of YF strain 17D, where the parent virus is neurovirulent in primates. Results of the WHO neurovirulence test in non-human primates indicated this vaccine was less neurovirulent than a YF vaccine YF-VAX® . The ChimeriVax™-DEN vaccine was also not viscerotropic in non-human primates, with limited replication in the liver, spleen and organs of the reticuloendothelium system. Acambis/Sanofi Pasteur has published data supporting use of the mouse as a surrogate for non-human primates for neurovirulence testing of the ChimeriVax™ class of vaccines [20].

The effect of pre-immunity of the vaccinees to the vector component of a chimeric DENV vaccine on safety and efficacy of the chimera has been investigated. Studies by Acambis/Sanofi Pasteur with the YF17D virus vector expressing the prM and E genes of JEV and DENVs have shown no interference in stimulation of antibodies to the JEV prM and E proteins in individuals who had been immunized with YF-VAX® . Prior immunity to the YF-17D structural genes did not inhibit but did enhance the immunological response to the chimeric JEV or DENV prM and/or E proteins [21].

Additional questions that need to be considered regarding the chimeric DENV vaccines include concerns that these viruses do not express the non-structural genes of the respective targeted pathogens. Will the lack of immunity to the nonstructural (NS) viral proteins adversely affect long-term immunity or cell-mediated immunity (CMI) responses that are critical to the protective

immune response? It is unknown whether the flavivirus capsid and NS proteins are critical elements in stimulation of long term protective immunity.

Studies showing the genetic stability of recombinant flavivirus vaccine viruses have been published for ChimeriVax™ vaccines vectored by 17D YFV, DENV PDK53 (DENVax) , rDEN4ΔDEN chimeras, and rDEN1-4Δ30 DEN 1-4 viruses. Attenuation of the DENV chimeras is dependent on retention of specific mutations introduced into DENV genes cloned into the chimera and/or chimerization with a virus that is attenuated. In all cases tested, vaccines produced by recombinant technology are genetically stable and retain the attenuated phenotype. To ensure the attenuated phenotype is retained, the vaccines need to be tested for both phenotypic and genotypic markers during the manufacturing process and in the final product.

Questions have been raised regarding the possibility that genetically engineered vaccines may recombine with virulent flaviviruses in nature. Thus far, there is no convincing evidence to favor the hypothesis that such recombination events occur. Furthermore, all live flavivirus vaccines under development display phenotypes (e.g. very low peak titers in both vaccinees and mosquito vectors) that dramatically reduce any risk of intragenic recombination to a level below the very low risk for such an event occurring between wild-type virus genomes [22-24].

During the discussion, a question was raised without an immediate answer: whether US FDA considers ERA for live recombinant dengue vaccines or whether other institutions (such as the National Institutes for Health or Environmental Protection Agency or Institutional Review Board) will require ERA. For example both the Canadian and Australian regulatory authorities require an ERA for approval of a GMO as a commercial vaccine. It is recognized that different institutions or authorities in different countries do have different oversight roles. It was proposed that the WHO should consider a process for developing a product-specific and geographically-specific ERA. It is recognized that, from the WHO perspective, it is important that the Organization assists in characterizing and managing risks, especially in developing countries.

5.4 Prospective in Developing Countries

5.4.1 Overview for dengue vaccine trials in Brazil

Dr. S. Nishioka (WHO), who had served as a Brazilian regulator before joining WHO, and other Brazilian participants provided the following information. At the current time (2009), DENV-1, -2 and -3 are circulating in Brazil and continue to cause clinical disease. Introduction of the recombinant DENV vaccines is a strategic issue and GMO issues must be resolved by appropriate governmental authority. Files for approval for clinical trials with vaccines containing a GMO will be evaluated by the Brazilian NRA and the evaluation will include ERA. Generally, clinical trials with vaccines sponsored by American or European producers need to be conducted in the country of manufacture before studies are initiated in Brazil.

5.4.2 Overview of dengue vaccine trials in Thailand

Mrs. P. Thanaphollert (Thai FDA) emphasized that an ERA needs to be undertaken for the vaccine products being registered for clinical trials and/or marketing approval. The Thai NRA recognizes significant environmental concerns with introduction of recombinant DENVs into Thailand. The live attenuated chimeric DENV vaccine candidates now being studied in clinical trials in Thailand are recognized as being GMO vaccines. Therefore, the clinical trials and licensure of the vaccines in Thailand must be approved on the basis of their science, technology and safety. One dengue vaccine manufacturer has submitted a proposal to undertake a clinical trial in Thailand, and it is under review by the ethics committee. Currently, Thailand and other countries in tropical zones are receiving applications for conducting clinical studies with dengue and JEV vaccines that are not licensed in industrialized countries where dengue and JE do not occur. This fact requires special attention by the Thai NRA.

In the discussion, it was questioned whether an ERA is required for clinical trial approval or for marketing approval and there was no consensus on this issue. Some experts favored the former

view (requirement of an ERA as clinical trial approval), as there has been no separate mandate or guidance other than the EU; there was attention to this issue during development phase but not at formalized process; and, in the USA, ERA review is carried out as part of clinical review. Dr. Minor asked if participants object to developing ERA guidance as an appendix to the revision of WHO dengue written standards. Dr. A. Barrett added that it would be good to include an example with illustration. The main points of this discussion are captured in the conclusion section of this report.

6. Clinical Evaluation of Dengue Vaccines

6.1 WHO Guidelines for Clinical Evaluation of Dengue Vaccines in Endemic Areas

Dr. R. Edelman (University of Maryland, USA) reviewed the 2008 revision of *WHO Guidelines for Clinical Evaluation of Dengue Vaccines in Endemic Areas* [25, 26]. DENV is spreading rapidly through the world where *A. aegypti* is the principal vector. Basic and clinical research has received increased financial support from industry, WHO, government and the Pediatric Dengue Vaccine Initiative (PDVI). Under this support significant strides have been made in understanding the biology of dengue disease, vector competency and immunology of the protective immune response. Many candidate vaccines are in preclinical development and three attenuated vaccines are in Phase I/II clinical trials. The 2008 revision has provided detailed guidance on establishing clinical end points, immune correlates of protection, and the impact of previous flavivirus infection and vaccination against other flaviviruses on immunization for dengue. The guidelines outline Phase II and III bridging studies with considerations for Phase III and IV (post-licensure trials). Successful vaccination against dengue must provide protection against disease from all 4 serotypes simultaneously and evaluate the role of neutralizing antibody in protection. It is critical that long-term vaccine safety and protection efficacy of a vaccine be

evaluated in areas that are endemic for flavivirus infection and that clinical follow-up of vaccine volunteers is extended for 3-5 years post immunization.

A primary efficacy endpoint for Phase III efficacy studies mandates that all febrile patients are identified and blood is collected to test for DENV viremia in a patient with at least 2 days of fever and no later than day 5 after dengue onset. Detection of a surrogate DENV antigen such as NS1 in blood, virus isolation, and/or PCR-based assays should be employed to confirm dengue viremia. The only practical primary trial endpoint is detection of virus in the sera of patients with at least 2 days of fever irrespective of dengue severity. “Severe dengue” as an endpoint is not practical because it is likely that the number of such cases will be low, and severity might be reduced by careful management of patients in a trial. Thus, the size and therefore the cost of Phase III efficacy trials would increase to unacceptable levels.

Secondary efficacy endpoints that can assist in interpreting clinical, virological and immunological data are essential. A positive serological result without virus isolation may be confounded by serological cross-reactions between related flaviviruses. In such cases, a four-fold rise in DENV plaque reduction neutralization (PRNT) antibodies provides a presumptive diagnosis but not definite diagnosis. Other issues that should be considered include the tabulation of virologically-confirmed cases stratified by age group and gender, cases confirmed after two or more vaccinations, and the severity of virologically confirmed cases.

The long-term objective of Phase III trials for dengue is to demonstrate protective efficacy against each of the four DENV serotypes in absence of any long-term safety concerns. It is unlikely that all four serotypes will circulate at a single site during a single transmission season, hence multi-centric, multi-seasonal studies will be needed. Efficacy measures for licensure could be based on pooled efficacy estimates from different sites and serotypes. Bridging studies could be conducted if an immune correlate of protection is identified in the initial Phase III studies.

6.2 Points to Consider for Conduct of Nonclinical and Clinical Studies to Evaluation of Dengue Vaccines in Endemic Areas

Dr. M. Powell (Medicines and Healthcare Products Regulatory Agency, UK) introduced points to consider for the conduct of nonclinical and clinical studies to evaluate dengue vaccines in endemic areas. The WHO disease-specific written standards include an introduction, manufacturing recommendations (Part A), nonclinical evaluation (Part B), clinical evaluation (Part C) and recommendations to the national regulatory authorities (Part D). Each section is specific to the disease to be prevented and, as appropriate, to the types of vaccine that fall within the scope. Cross-reference is made to the relevant general guidance documents, e.g. WHO nonclinical and clinical evaluation of vaccines [27, 28].

Clinical guidance given as Part C of WHO written standards usually comprises: i) assessment of immunogenicity, ii) discussion of assays designed to measure immunogenicity of the vaccine, iii) selection of dose and regimen, iv) protective efficacy studies, v) co-administration/sequential administration issues, vi) special safety issues and vii) effectiveness and post-market surveillance (PMS) data. The appropriate application of information in these documents for dengue vaccines should take into account and cross-refer to the WHO Guidelines IVB/08.12, which provide very specific guidance on the development of dengue vaccines.

Dr. Powell led the discussion of the possible content of Parts B and C by presenting a number of questions. One of the main points of the discussion involved the choice of an animal model as addressed in Part B. There is no suitable animal model that mimics human dengue disease. The macaque monkey is the most useful for dengue virus/vaccine immunologic and virological studies. The monkey model is useful for assessing neurovirulence (if appropriate to test for neurological disease, e.g. ChimeriVax™-DEN), exploring immunogenicity, measuring viremia post vaccination and for challenge studies. Mouse models have been used for neurovirulence studies but many aspects of studies with mouse-adapted DEN viruses are of limited value. More recently "humanized" mouse and AG129 models have been developed that need to be reviewed carefully.

Issues in Part C of the revised WHO dengue written standards that need further resolution were also considered. They included the following items:

1. DENV neutralizing antibody can be assayed by DENV PRNT [29]). Tests for IgM, IgG and HI antibodies other than virus neutralization may be helpful but are not recognized as being essential to assess potential vaccine efficacy. There was no consensus on the need for specialized serological tests (e.g. E-protein DIII domain specific responses) to be performed in sera from subsets of vaccinees as part of the overall assessment of immunogenicity.
2. It is possible that a vaccine could elicit a “notably” lower antibody titer to one or more of the DENV serotypes, which could have implications for vaccine safety and efficacy. If this occurs during a Phase III tetravalent DENV vaccine trial, it was agreed that modification of the immunization schedule with further investigation of the poor immune response is essential.
3. The minimum PRNT titer that should be met for each vaccine type before proceeding to Phase III efficacy studies has not been definitively established. In the conduct of DENV clinical trials, serological endpoints are established by the seroconversion rate (SCR) and geometric mean titer (GMT) that can be applied to compare serological responses between individuals in the vaccination and placebo groups.
4. Data on cell-mediated immunity may not be critical for assessing immunogenicity of dengue vaccines but should be explored because it may have implications for understanding the long-term memory response and the vaccine safety profile (see WHO/IVB/08.12) [25]. The former is particularly true for ChimeriVax™ because it has YFV nucleocapsid that may carry CMI epitopes specific for a viral strain or common to the flavivirus family.
5. During conduct of Phase III efficacy clinical trials the case definition for protective efficacy must be based on data from studies where sensitive and validated tests are used

to establish viremia in the vaccinated patient who is ill with suspected dengue. The routine test for viremia should be virus isolation in cell culture; however, quantitative PCR tests may be applied if they are validated.

6. Secondary endpoints used in protective efficacy studies have been outlined and are described in WHO/VB/08.12. Secondary endpoints are usually study-specific and are tailored to the specific vaccine under consideration.
7. The need to evaluate serological responses to booster doses of vaccine (e.g. in subjects living in a non-endemic area) should take into account data on antibody persistence and information whether waning antibody titers still confer protection from infection over time.
8. The potential need to conduct more than one protective efficacy study in different geographic regions and the possible reliance on a single study, with or without study sites in different regions (with different circulating strains and disease incidence) was discussed. This is a very complex problem and undoubtedly will depend upon the circumstances and the countries involved. It is possible that a single pre-licensure placebo-controlled efficacy study might be supplemented by post-approval effectiveness studies in different regions where the vaccine was introduced. This matter will require further consideration.
9. In the discussions, it was proposed that, if a pre-licensure protective efficacy (Phase III) study was conducted under conditions that meet requirements for conduct of the study in another region/population, the immune correlate of protection may be extrapolated to demonstrate efficacy to other populations based primarily of serological studies designed to show non-inferiority (but see below).
10. There may be distinct clinical and immunological issues that need to be resolved with specific vaccines (i.e. live vs. inactivated, DNA vaccine, chimeric, etc.). It is expected that each vaccine will elicit an immunological response specific for the antigens

expressed by the attenuated vaccine that may involve both structural and nonstructural proteins. In principle, the titer of virus neutralizing antibody stimulated by one vaccine for a specific DENV serotype would be correlated with protection stimulated by a different vaccine for this DENV serotype. In theory this titer could be considered generally applicable to all the other DENV vaccine types. At present, there is no evidence to support such an assumption; however, it seems to be theoretical reasonable.

11. All clinical trials need expert oversight by an independent data safety monitoring boards (DSMBs) that are fully competent to evaluate adverse events (AEs) and serological and efficacy data.
12. Expert statistical advice should be taken when determining the safety database needed to assess the theoretical risk of ADE. Modeling may be helpful to predict the expected incidence of severe dengue or dengue hemorrhagic fever in those immunized. In all instances the risk of ADE should be further assessed in the post-approval period.

7. Conclusions

The group reviewed the main points of discussion and agreed on the following:

- The purpose of revising the WHO Technical Report Series No. 932 is to provide updated recommendations to NRAs and manufacturers to assure quality, safety and efficacy of dengue vaccines. These recommendations will also serve as the basis of WHO technical specifications for prequalification.
- Disease caused by DENV will be described in the introductory part of the revision.
- The revision will focus on tetravalent live recombinant vaccines. Inactivated virus vaccines, DNA vaccines and subunit vaccines will be briefly described in an introductory part and WHO guidance on hepatitis B or HPV vaccines will be referred to as background specifications.

- PDK cell substrate is no longer used so that it will not be considered in the revision. Other cells that have potential for producing live dengue vaccines need to be explored during revision process.
- The molecular genetics approach for development of live attenuated DENV vaccine will be the focus of the revised standards. The potency will be expressed as plaque forming units (PFU) or other measures of virus infectivity, but for the manufacturing consistency, the ratio of PFU/ genomic equivalents (GEQ) may be considered as a quality parameter.
- The revision will include new sections on environmental risk assessment, nonclinical and clinical evaluation of live dengue vaccines.

References

- [1] Vaughn DW, Whitehead S, Durbin A. Dengue. In: Barrett AD, Stanberry L, editors. Vaccines for biodefense and emerging and neglected diseases. New York: Elsevier Inc, 2009: 287-324.
- [2] Halstead SB, Vaughn DW. Dengue vaccines. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines. New York: Elsevier Inc., 2008: 115-1162.
- [3] Whitehead SS, Blaney JE, Durbin AP, Murphy BR. Prospects for a dengue virus vaccine. *Nat Rev Microbiol* 2007 Jul;5(7):518-28.
- [4] Hombach J. Vaccines against dengue: a review of current candidate vaccines at advanced development stages. *Rev Panam Salud Publica* 2007 Apr;21(4):254-60.
- [5] Blaney JE, Jr., Sathe NS, Goddard L, Hanson CT, Romero TA, Hanley KA, et al. Dengue virus type 3 vaccine candidates generated by introduction of deletions in the 3' untranslated region (3'-UTR) or by exchange of the DENV-3 3'-UTR with that of DENV-4. *Vaccine* 2008 Feb 6;26(6):817-28.
- [6] World Health Organization. Guidelines for the production and quality control of candidate tetravalent dengue virus vaccines (live). In: WHO Expert Committee on Biological Standardization. Fifty-fifth report. Geneva, World Health Organization, 2006, Annex 1 (WHO Technical Report Series No. 932)

- [\[http://www.who.int/biologicals/publications/trs/areas/vaccines/dengue/TRS932Annex%201_Dengue%20virus%20vacc%20live.pdf\]](http://www.who.int/biologicals/publications/trs/areas/vaccines/dengue/TRS932Annex%201_Dengue%20virus%20vacc%20live.pdf).
- [7] World Health Organization. Requirements for hepatitis B vaccines made by recombinant DNA techniques (requirements for biological substances no. 45). In: WHO Expert Committee on Biological Standardization. Thirty-ninth report. Geneva, World Health Organization, 1989, Annex 2 WHO Technical Report Series, No. [\[http://www.who.int/entity/biologicals/publications/trs/areas/vaccines/hepatitis/WHO_TRS_786_A2.pdf\]](http://www.who.int/entity/biologicals/publications/trs/areas/vaccines/hepatitis/WHO_TRS_786_A2.pdf).
- [8] World Health Organization. Requirements for hepatitis B vaccines made by recombinant DNA techniques (requirements for biological substances no. 45, amendment 1997). In: WHO Expert Committee on Biological Standardization. Forty-eighth report. Geneva, World Health Organization, 1999, Annex 4 (WHO Technical Report Series, No. 889) [\[http://www.who.int/biologicals/publications/trs/areas/vaccines/hepatitis/WHO_TRS_889_A4.pdf\]](http://www.who.int/biologicals/publications/trs/areas/vaccines/hepatitis/WHO_TRS_889_A4.pdf).
- [9] World Health Organization. Guidelines to assure the quality, safety and efficacy of recombinant human papillomavirus virus-like particle vaccines. In: WHO Expert Committee on Biological Standardization. Geneva, World Health Organization, (Adopted 2006), (in press) [\[http://www.who.int/biologicals/publications/trs/areas/vaccines/human_papillomavirus/HVg%20Final%20BS%202050%20.pdf\]](http://www.who.int/biologicals/publications/trs/areas/vaccines/human_papillomavirus/HVg%20Final%20BS%202050%20.pdf).
- [10] World Health Organization. Guidelines for assuring the quality and non-clinical safety evaluation of DNA vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-sixth report. Geneva, World Health Organization, 2007, Annex 1 (WHO Technical Report Series No. 941) [\[http://www.who.int/biologicals/publications/trs/areas/vaccines/dna/Annex%201_DNA%20vaccines.pdf\]](http://www.who.int/biologicals/publications/trs/areas/vaccines/dna/Annex%201_DNA%20vaccines.pdf).
- [11] World Health Organization. Requirements for the use of animal cells as in vitro substrates for the production of biologicals (requirements for biological substances no. 50). In: WHO Expert Committee on Biological Standardization. Forty-seventh report. Geneva, World Health Organization, 1998, Annex 1 (WHO Technical Report Series, No. 878)

- [\[http://www.who.int/biologicals/publications/trs/areas/vaccines/cells/WHO TRS 878_A1Animalcells.pdf\]](http://www.who.int/biologicals/publications/trs/areas/vaccines/cells/WHO_TRS_878_A1Animalcells.pdf).
- [12] World Health Organization. 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, Annex 4 (WHO Technical Report Series, No. 927)
[\[http://www.who.int/biologicals/publications/amendment_cell_substrates_ecbs_nov_2003.pdf\]](http://www.who.int/biologicals/publications/amendment_cell_substrates_ecbs_nov_2003.pdf).
- [13] U. S. Food and Drug Administration. Guidance for Industry: Characterization and qualification of cell substrates and other biological starting materials used for the production of viral vaccines for the prevention and treatment of infectious diseases. 2010.
[\[http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/UCM202439.pdf\]](http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/UCM202439.pdf).
- [14] Petricciani J, Sheets R. An overview of animal cell substrates for biological products. *Biologicals* 2008 Nov;36(6):359-62.
- [15] Sheng-Fowler L, Lewis AM, Jr., Peden K. Issues associated with residual cell-substrate DNA in viral vaccines. *Biologicals* 2009 Jun;37(3):190-5.
- [16] Knezevic I, Stacey G, Petricciani J. WHO Study Group on cell substrates for production of biologicals, Geneva, Switzerland, 11-12 June 2007. *Biologicals* 2008 May;36(3):203-11.
- [17] Baylor NW, Midthun K. Regulation and Testing of Vaccines. In: Plotkin SA, Orenstein WA, Offit PA, editors. *Vaccines*. New York: Elsevier Inc., 2008: 1611-27.
- [18] Krause PR. Regulatory issues related to vaccines against emerging infectious diseases. In: Barrett AD, Stanberry L, editors. *Vaccines for biodefense and emerging and neglected diseases*. New York: Elsevier Inc, 2009: 191-210.
- [19] Novak JM, Ruckman J, Trent DW. The pre-IND/IND regulatory process: Vaccines. In: Barrett AD, Stanberry L, editors. *Vaccines for biodefense and emerging and neglected diseases*. New York: Elsevier Inc, 2009: 172-89.
- [20] Monath TP, Myers GA, Beck RA, Knauber M, Scappaticci K, Pullano T, 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 Sep;33(3):131-44.

- [21] Guirakhoo F, Kitchener S, Morrison D, Forrat R, McCarthy K, Nichols R, et al. Live attenuated chimeric yellow fever dengue type 2 (ChimeriVax-DEN2) vaccine: Phase I clinical trial for safety and immunogenicity: effect of yellow fever pre-immunity in induction of cross neutralizing antibody responses to all 4 dengue serotypes. *Human Vaccine* 2006 Mar-Apr;2(2):60-7.
- [22] Ishikawa T, Widman DG, Bourne N, Konishi E, Mason PW. Construction and evaluation of a chimeric pseudoinfectious virus vaccine to prevent Japanese encephalitis. *Vaccine* 2008 May 23;26(22):2772-81.
- [23] Guy B, Guirakhoo F, Watson M, Higgs S, Monath TP. Safety of flavivirus chimeric vaccines: answer to Ishikawa et al. [*Vaccine* 26 (22) (2008) 2772-2781]. *Vaccine* 2008 Aug 5;26(33):4107-8.
- [24] Guy B, Barban V, Mantel N, Aguirre M, Gulia S, Pontvianne J, et al. Evaluation of interferences between dengue vaccine serotypes in a monkey model. *Am J Trop Med Hyg* 2009 Feb;80(2):302-11.
- [25] World Health Organization. Guidelines for the clinical evaluation of dengue vaccines in endemic areas. Geneva: World Health Organization; 2008.
- [26] Edelman R, Hombach J. "Guidelines for the clinical evaluation of dengue vaccines in endemic areas": summary of a World Health Organization Technical Consultation. *Vaccine* 2008 Aug 5;26(33):4113-9.
- [27] World Health Organization. WHO guidelines on clinical evaluation of vaccines: regulatory expectations. In: WHO Expert Committee on Biological Standardization. Fifty-second report. Geneva, World Health Organization, 2004, Annex 1 (WHO Technical Report Series, No. 924)
[\[http://www.who.int/biologicals/publications/trs/areas/vaccines/clinical_evaluation/035-101.pdf\]](http://www.who.int/biologicals/publications/trs/areas/vaccines/clinical_evaluation/035-101.pdf).
- [28] World Health Organization. WHO guidelines on nonclinical evaluation of vaccines. In: WHO Expert Committee on Biological Standardization. Fifty-fourth report. Geneva, World Health Organization, 2005, Annex 1 (WHO Technical Report Series, No. 927)
[\[http://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%20Nonclinical.P31-63.pdf\]](http://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%20Nonclinical.P31-63.pdf).

- [29] Roehrig JT, Hombach J, Barrett AD. Guidelines for plaque-reduction neutralization testing of human antibodies to dengue viruses. *Viral Immunol* 2008 Jun;21(2):123-32.

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