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Annex 1
Guidelines for the production and quality control of candidate tetravalent dengue virus vaccines (live)

This document provides guidance to national regulatory authorities (NRAs) and vaccine manufacturers on the production and quality control of candidate live attenuated dengue virus vaccines by outlining the international regulatory expectations for product characterization. It should be read in conjunction with the WHO guidelines on nonclinical evaluation of vaccines (1), and the WHO guidelines on clinical evaluation of vaccines: regulatory expectations (2), to gain an understanding of the whole process of vaccine evaluation. Clinical evaluation of vaccines against dengue presents special challenges and WHO has developed specific guidance on clinical testing programmes (3), which should also be consulted. As candidate live attenuated dengue virus vaccines are still under development, the following text is presented in the form of guidelines rather than recommendations. Guidelines allow greater flexibility than recommendations with respect to expected future developments in the field. The document is thus provided for guidance to health administrators.

Introduction

In response to interest from many countries in the development of candidate live attenuated dengue virus vaccines, preliminary draft guidelines on production and quality control specifications for tetravalent dengue vaccine (live) were developed by a small drafting group established by WHO. These were presented to the WHO Expert Committee on Biological Standardization at its forty-eighth meeting in 1997 (*4*). The Committee advised that the field of dengue virus vaccine development needed to progress further before it would be appropriate for WHO to develop guidance on technical specifications for these vaccines. Since that time WHO had established the Task Force on Clinical Trials of Dengue Vaccines, and at the second meeting of this group, at Denver, Colorado, in November 2002, it was considered timely by the experts present for WHO to recommence the development of the production and quality control guidelines. The WHO Secretariat agreed and convened a small drafting group to review the previous draft and to advise on what changes should be made. The drafting group met in Geneva from 20–21 March 2003, reviewed the original document and, based on the conclusions from that meeting, subsequently developed a new draft. This draft was discussed in detail at a WHO consultation held in Philadelphia, USA, from 2–3 December 2003, and the current version of the document was prepared by the WHO Secretariat, taking into account the views expressed at that meeting (*5*) and the views of the fifty-fifth meeting of the Expert Committee on Biological Standardization.

The scope of this document covers candidate live attenuated tetravalent dengue virus vaccines. The aim of vaccination against dengue virus infection is to induce immunity against all four serotypes in one series of inoculations. The information available to the WHO Task Force on Clinical Trials for Dengue Vaccines in 2004 was that two tetravalent vaccine candidates had been generated by taking original patient isolates of each serotype and passaging these isolates in dog primary kidney cells to attenuate the viruses. Extensive testing has been done to define the attenuation phenotype for each of the vaccine candidates. Vaccine formulations are being developed based on the optimal degree of attenuation and immunogenicity. A third vaccine candidate had been generated from a molecular clone of dengue virus type 4. This virus contains a 30-nucleotide deletion in the 3′ noncoding region that attenuates the virus. To generate a tetravalent vaccine, chimeric vaccine candidates that contain the structural region of the other three dengue serotypes in a dengue virus type 4 backbone containing the 30-nucleotide deletion are being prepared

for clinical development. In addition the 30-nucleotide deletion has been introduced into the homologous region of dengue types 1, 2 and 3 to generate additional attenuated vaccine candidates. The fourth vaccine candidate was a chimeric vaccine made by expressing the dengue virus structural proteins, prM and E in a molecular clone of the yellow fever virus vaccine 17D backbone. A combination tetravalent vaccine was in development, which will have all four dengue serotypes, represented as chimeric dengue–yellow fever vaccines.

Clinical trial data (*6*, *7*) showed that vaccine candidates have induced human immune responses. Protection was also being measured in a human challenge study that was under way for at least one vaccine candidate. Additional data are required to define an acceptable level of immune response that correlates with protective immunity to candidate dengue vaccines. The information available to WHO suggested that the reactogenicity of the vaccine candidates being tested varied. To obtain maximum public health benefits, this vaccine was envisaged for use both in children and adults, thus it was important to establish and understand the safety profile and reactogenicity in all target age groups.

General considerations

An important consideration for the safety of any vaccine is the full passage history of the seed materials used for vaccine development. The purpose is to identify all substrates through which the seed materials had been passed to aid the development of appropriate programmes for testing for adventitious agents. The early passage history for the candidate dengue vaccines varies, but may include monkey kidney cells, or mosquito intermediates, or mouse brain or dog primary kidney cells, or a combination of these substrates. It will be essential to show that the virus seeds are free of adventitious agents relevant to the animal species used and from the substrates used in the derivation of the seeds.

A risk assessment for transmissible spongiform encephalopathies (TSE) would need to be included for the seed materials. The revised WHO Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (*8*) provide guidance on risk assessments for master and working seeds and should be consulted.

All of the vaccine candidates are claimed to be attenuated. However, for some vaccine candidates, this claim may be based on limited

clinical experience in humans as laboratory markers of attenuation are not well-defined. This is an area where further work is urgently needed to help assess consistency of production, especially when new viral seeds are produced. Potential laboratory markers include the sequence information on the seed viruses; viraemia levels in monkeys; and ability to replicate in or be transmitted by vector mosquitoes. In any laboratory test, the aim would be to show that a new seed material was similar to the previous seed and that each could be distinguished from the parent virus. Multiple passage of virus seed materials under defined conditions may be helpful to generate validation data on the chosen method. Studies on consistency of production would need to take into account the variability inherent in RNA virus replication and assess the presence of minority populations, as revealed for example by the occurrence of mixed plaque phenotypes or mixed base signals in sequencing studies. If minority populations are detected it will be necessary to assess their biological importance, for example, by carefully comparing the level of heterogeneity (e.g. plaque size) between the master or working seed and higher passage levels (e.g. clinical trial material).

The need for a neurovirulence test should be based on evidence (*2*) either that the natural infection is neurotropic or that selection for neurotropism could have occurred during the passage history of the vaccine candidates. For example, this may occur if the attenuation process involved passage through central nervous system (CNS) tissue. Furthermore, if a neurovirulence test is indicated, any test specified should be able to reliably distinguish between acceptable and unacceptable preparations.

Because dengue viruses are not regarded as encephalitic, a neurovirulence test for each batch is not justified, and there is no need to test each working seed. However, it would be prudent to test at least the master seed to show that the derivation process for the seed did not lead to a change in the inherently attenuated neurovirulence phenotype of dengue viruses. For chimeric dengue candidate vaccines where one component of the chimera is derived from a virus with neurovirulence potential, such as dengue–yellow fever constructs, then more extensive neurovirulence testing may be required. Experience to date with dengue neurovirulence testing is based on using either the methodology described for live attenuated poliovirus (*9*) or for yellow fever vaccine (*10*). It is proposed that, because both yellow fever and dengue viruses are *Flaviviruses*, the specifications for the yellow fever test be applied to dengue virus vaccines in the future. Thus a neurovirulence test at the level of the master seed is included.

Two candidate vaccines are being developed in Vero cells. These are among the first examples of live-attenuated injectable vaccines for human use being developed in Vero cells. The existing WHO guidance on residual levels of cellular DNA (*11*, *12*) is incorporated into this draft. It is recognized that this specification will require that either a virus purification step and/or a DNA removal step be included in the production process.

For vaccines being developed in fetal rhesus lung diploid cells, existing WHO recommendations are applicable (*11*).

Although continuous cell lines or diploid cells are generally the preferred cell substrates for vaccine production, at least one candidate vaccine was produced in dog primary kidney cells. These cells are passaged a limited number of times and banked prior to use. Thus it is possible to conduct extensive characterization of adventitious agents in the cells. The principles of extensive testing of source animals plus extensive testing of each batch of cells, using as an example the guidance established for primary hamster cells for production of live attenuated Japanese encephalitis vaccine (*13*), have been applied in this document. Also the conclusions of a WHO Task Force meeting on cell substrates, which considered the issue of dog primary kidney cells (*14*) have been taken into account. Thus, for example, testing for canine retroviruses is included.

The infectivity of each serotype in a tetravalent mixture should be established and the plaque or focus-forming assay is specified for determinations of infectivity. Candidate titration standards do not exist at present and WHO should consider developing such reagents and their subsequent characterization by international collaborative study.

The thermal stability of the final tetravalent freeze-dried product should be determined in an appropriate stability study. This study should determine the thermal stability of each serotype in the tetravalent mixture. In addition to the stability of the freeze-dried product, the stability of the liquid vaccine after reconstitution should also be studied. Stability testing of intermediates, such as monovalent virus harvests prior to formulation as final tetravalent vaccine, is required in some countries. WHO is developing further guidance on this issue.

Based on the results of the stability testing programme, an accelerated degradation test should be conducted on each new batch of vaccine. This is to show the consistency of manufacture of the final stabilized formulation. For consistency with the testing done on other

vaccines the accelerated degradation test should be done at 37 °C for 1 week. A specification for the maximum allowable loss of titre during this period should be confirmed on the basis of experience yet to be accumulated.

Considerations for vectored vaccines for human use have been reviewed by WHO (*15*) and the general principles identified should be applied to the vaccine candidates derived by molecular methods.

Nonclinical testing of candidate dengue vaccines should follow conventional procedures (*1*), but in addition should address the issue of enhancement of antibody-mediated disease. There are no models that can be recommended at present, so each testing programme will need to be developed on a case-by-case basis. In addition, appropriate follow-up of vaccinees participating in human clinical trials for 3–5 years is recommended (3).

Theoretical concerns have been raised about adverse ecological events that may arise from recombination between a live attenuated dengue virus vaccine and a wild-type flavivirus (*16*). Scientific considerations show however that the likelihood of recombination between a wild-type flavivirus and a vaccine flavivirus is much less than that of recombination between two wild-type flavivirus. There is no evidence for generation of problematic recombinant flaviviruses (*17*). Dual infection laboratory studies between vaccine and wild-type strains are not recommended because the predictive value of such studies would be low (*18*).

Part A. Control of production

A.1 Definitions

A.1.1 *International name and proper name*

Although dengue vaccines are not yet licensed, the provision of a suggested international name at this early stage of development will aid harmonization of nomenclature if licensure is obtained. The international name should be "Live attenuated tetravalent dengue virus vaccine" or "Live attenuated tetravalent dengue–yellow fever virus chimeric vaccine" or "Live attenuated tetravalent dengue–dengue 4 virus chimeric vaccine". The proper name should be the equivalent to 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 specifications formulated below.

A.1.2 *Descriptive definition*

A candidate live attenuated dengue virus vaccine should be a sterile, aqueous suspension of the four serotypes of dengue vaccine strains, or viral vectors that express the four dengue serotypes, and which have been grown individually in mammalian cells. At least three types of dengue vaccine are in development. These are as follows:

- Live attenuated tetravalent dengue virus vaccine is a preparation of combined live attenuated dengue-1, dengue-2, dengue-3 and dengue-4 viruses grown in a suitable cell culture.
- Live attenuated tetravalent dengue–yellow fever virus chimeric vaccine is a preparation of combined live attenuated chimeric viruses, based on the live attenuated yellow fever virus vaccine and each expressing dengue-1, dengue-2, dengue-3 or dengue-4 virus envelopes.
- Live attenuated tetravalent dengue–dengue 4 virus chimeric vaccine is a preparation of combined live attenuated chimeric viruses, based on the live attenuated dengue-4 vector and each expressing dengue-1, dengue-2, dengue-3 or dengue-4 virus envelopes.

The preparation should satisfy all of the specifications given below.

Live tetravalent dengue vaccine is blended with an appropriate stabilizer and may be freeze-dried.

A.1.3 *International reference materials*

No international reference materials are available at present, although candidate antiserum preparations to calibrate the neutralizing antibody response in vaccinees are under evaluation in a WHO collaborative study[a].

A.1.4 *Terminology*

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

Candidate vaccine
A vaccine under development which is used in human clinical trials to assess its safety and efficacy.

Cell seed
A quantity of well-characterized cells of human or animal origin stored frozen in liquid nitrogen in aliquots of uniform composition

[a] The 1st WHO Reference Regent for anti-dengue antibodies was established in 2005 by the 56th meeting of the Expert Committee on Biological Standardization. This material was assigned a unitage of 100 units per serotyse.

derived from a single tissue or cell, one or more of which would be used for the production of a master cell bank.

Cell substrates
A number of cell cultures derived from the same pool of cells, processed and prepared together.

Filling lot
A collection of sealed final containers of finished candidate vaccine that are homogeneous with respect to the risk of contamination during filling and freeze-drying. All the final containers must, therefore, have been filled from one vessel of final bulk in one working session and freeze-dried under standardized conditions in a common chamber.

Final bulk
The homogeneous finished tetravalent virus suspension prepared from one or more clarified virus pools in the vessel from which the final containers are filled.

Focus-forming unit (FFU)
The smallest quantity of virus suspension that can be defeated using dengue-specific antisera in monolayer cell cultures.

Master cell bank
A quantity of fully characterized cells of human, animal or other origin stored frozen at –70 °C or below in aliquots of uniform composition, one or more of which would be used for the production of a manufacturer's working cell bank.

Master seed lot
A quantity of virus derived from an original isolate, processed at the same time to assure a uniform composition and having been characterized to the extent necessary to support developing the working seed lot. The characterized master seed lot is used for the preparation of working seed lots.

Plaque-forming unit (PFU)
The smallest quantity of virus suspension that will produce a plaque in monolayer cell cultures.

Single harvests
A quantity of virus suspension derived from the batch of cell substrate that was inoculated with the same working seed lot and processed together in a single production run.

Working cell bank (WCB)
A quantity of cells derived from one or more ampoules of the master cell bank and of uniform composition, stored frozen at –70 °C or below in aliquots, one or more of which would be used for production purposes.

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 national authority. The cells are combined into one pool distributed into ampoules and preserved cryogenically to form the WCB.

Working seed lot
A quantity of virus of uniform composition, fully characterized, derived from a master seed lot. The working seed lot is used for the production of candidate vaccine lots.

Virus pool
A homogenous pool of single harvests collected into a single vessel before clarification.

A.2 Viruses for use in candidate vaccine production

A.2.1 *Virus strains*

The strains of dengue-1, dengue-2, dengue-3 and dengue-4 viruses used in the production of candidate tetravalent dengue vaccine should be identified by historical records, which will include information on the origin of each strain; method of attenuation; whether the strains have been biologically cloned prior to generation of the master seed; genetic sequence information; and the passage level at which attenuation for humans was demonstrated by clinical trials. Clinical signs, viraemia, and the immune response after human immunization with each dengue virus serotype must be determined to facilitate development of acceptable criteria for attenuation and immunogenicity of the vaccine viruses.

A.2.2 *Approval*

The four vaccine strains of dengue virus used in the production of candidate vaccine should have been shown to be safe by appropriate laboratory tests (see section A.4 of these guidelines) as well as by tests in susceptible humans. Only strains approved by the national regulatory authority should be used.

A.2.3 *Establishment of immunizing dose*

The immunizing dose, initially in PFU or FFU, of each serotype in the tetravalent vaccine that induces seroconversion when susceptible individuals are immunized with the tetravalent vaccine, should be established in a dose–response study. Any potential interference or potentiation between the serotypes in the plaque- or focus-forming assay should be evaluated prior to establishing this value. When international reference standards become available, the immunizing dose should be expressed relative to the standard. Expression of doses as a relative potency is encouraged because experience shows that this reduces variation between laboratories. The immunizing dose should also serve as a basis for establishing parameters for stability and expiry date.

A.3 General manufacturing requirements

The principles of good manufacturing practices for pharmaceutical and biological products, as appropriate to the different stages of vaccine development, should be applied by establishments manufacturing candidate tetravalent dengue vaccine (*22*), with the addition of the following:

Separate manufacturing areas for each of the four serotypes as well as tetravalent vaccine formulation are required. Alternatively, manufacturing areas may be used on a campaign basis with adequate cleaning between campaigns to ensure that cross-contamination does not occur.

Production steps and quality control operations involving manipulations of live virus should be conducted under biosafety level BSL 2.

A.4 Production control

A.4.1 *Control of source materials*

A.4.1.1 *Cell cultures for virus production*

A.4.1.1.1 Conformity with WHO requirements

Dengue viruses used in the production of tetravalent dengue vaccine should be propagated in cell substrate in conformity with the WHO requirements for use of animal cells as in vitro substrate for the production of biologicals (*11*, *12*) and approved by the national regulatory authority. All information on the source and method of preparation of the cell culture system used should be made available to the national regulatory authority (*11*).

A.4.1.1.2 Types of cell culture

Dengue vaccine candidates have been produced in fetal rhesus lung diploid cells, in continuous cell lines and in dog primary kidney cells. For fetal rhesus lung diploid and continuous cells, sections A.4.1.1.3 and A.4.1.1.4 should apply; for dog primary kidney cells, section A.4.1.1.5 should apply to the source materials. Section A.4.1.1.6 applies to all types of cell culture.

A.4.1.1.3 Master cell bank and working cell bank

The use of a cell line such as fetal rhesus lung diploid cells or Vero cells for the manufacture of dengue vaccines should be based on the cell bank system. The cell seed should be approved by the national regulatory authority. The maximum number of passages (or population doublings) allowable between the cell seed and the WCB should be established by the national regulatory authority. Additional tests for Vero cells include:

— propagation of the MCB or WCB cells to or beyond the maximum in vitro age; and
— examination for the presence of retroviruses and tumorigenicity in an animal test system (*11*).

WHO has established a cell bank of Vero cells characterized in accordance with the requirements in the report of the WHO Expert Committee on Biological Standardization (*11*), which is available to manufacturers as a well-characterized starting material (*12*) for preparation of their own master and working cell seeds on application to the Coordinator, Quality Assurance and Safety of Biologicals, WHO, Geneva, Switzerland.

A.4.1.1.4 Identity test

The master cell bank should be characterized according to the *Requirements for animal cells lines used as substrates for the production* of *biologicals* (*11*), as appropriate to continuous cells or fetal rhesus diploid cells.

The WCB should be identified by means, inter alia, of biochemical (e.g. isoenzyme analysis), immunological, and cytogenetic marker tests, approved by the national regulatory authority.

A.4.1.1.5 Sources of dog kidney cells

If cultures of dog kidney cells are used for the propagation of dengue vaccine viruses, dogs less than 2 months old may be used as the source of kidneys for cell culture. Only dog stock approved by the national

regulatory authority should be used as the source of tissue and should be derived from a closed, healthy colony. A closed colony is a group of animals sharing a common environment and having their own caretakers who have no contact with other animal colonies. The animals are tested according to a defined programme to ensure freedom from specified pathogens and their antibodies. When new animals are introduced into the colony for breeding purposes, they should be maintained in quarantine in vermin-proof quarters for a minimum of 2 months and shown to be free from these specified pathogens. The parents of animals to be used as a source of tissue should also be maintained in vermin-proof quarters. Neither parent dogs nor their progeny should previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored for zoonotic viruses and markers of contamination at regular intervals.

At the time the colony is established, all animals should be tested to determine freedom from antibodies to the following pathogens: rabies, canine parvovirus, canine distempervirus, canine adenovirus 1, canine adenovirus 2, parainfluenzavirus 3, Sendai virus, SV-5 virus, reovirus types 1, 2, 3, *Mycobacterium tuberculosis*, infectious canine hepatitis and leptospirosis. Following this initial screening, a monitoring programme should be implemented to ensure that the colony remains free of the specified pathogens.

In some countries, the whole group of animals is bled on the establishment of the colony, and thereafter 5% of the animals should be bled each month. The screening programme should test all of the animals over a defined period of time, as agreed with the national regulatory authority. The serum samples should be screened to establish freedom from antibodies to the pathogens above.

Consideration should also be given to testing the colony for hepatitis E virus, Japanese encephalitis, canine circovirus, canine coronavirus, canine herpesviruses and bordetella bronchiseptica.

The colony should be tested for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse transcriptase (Rtase) assay. The results of such assays may need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (*19*). If a positive result is obtained in this screening, it is then important to determine whether replication-competent retroviruses are present. It should be noted that dogs have many classes of defective endogenous retroviruses but, as yet, no definitely characterized exogenous retrovirus.

Any animal that becomes ill or dies should be investigated to determine if the cause of illness or death may be of infectious origin. Similarly, ill health of animals within the colony should be investigated to determine if the cause is infectious in origin. If an infectious agent is detected in the colony, specific steps must be put in place to ensure that the agent is excluded from the candidate vaccine.

The dog kidney cell cultures currently in use are generated from harvested tissue that is passaged up to three times and then stored as a working cell bank. This enables these cells to be characterized in more detail than if they were used without intervening passage. Nevertheless, these cells are still considered primary cells. Given the inherent variability of primary cell cultures and to ensure consistency of manufacturing, it is recommended that the characterization of any new dog primary kidney cell bank (third passage cells) include tests comparing cells currently in use with newly harvested and passaged cells.

A.4.1.1.6 Cell culture medium

Serum used for the propagation of cells should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised *Requirements for biological substances*, No. 6 (*20*), and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the *Recommendations for production and control of poliomyelitis vaccine (oral)* (*9*).

Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera.

As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin.

Irradiation may be used to inactivate potential contaminant viruses.

The acceptability of the sources(s) of any components of bovine, sheep or goat origin used in culture media should be approved by the national regulatory authority. These components should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (*8*).

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* (Requirements for Biological Substances No. 27) (*21*), as well as current guidelines in relation to human transmissible encephalopathies (*8*).

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 for which it is suitable for use in clinical practice) in relation to the potential long-term storage of monovalent bulks of each dengue serotype.

Penicillin and other beta-lactams should not be used at any stage of the manufacture. Other antibiotics may be used at any stage in the manufacture provided that the quantity present in the final product is acceptable to the national regulatory authority. Nontoxic pH indicators may be added, e.g. phenol red at a concentration of 0.002%. Only substances that have been approved by the national regulatory authority may be added.

If trypsin is used for preparing cell cultures it should be tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially bovine or porcine parvoviruses, as appropriate. The methods used to ensure this should be approved by the national regulatory authority.

The source(s) of trypsin of bovine origin, if used, should be approved by the national regulatory authority. Bovine trypsin, if used, should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (*8*).

A.4.1.2 *Virus seeds*

A.4.1.2.1 **Virus strains**

Virus strains of dengue viruses used for master and working seeds to produce vaccine candidates should comply with the specifications of section A.2. Strains derived by molecular methods may be used, provided that guidance on vectored vaccines is taken into account (*15*). Viruses may be passed in continuous, diploid, and/or primary cell lines. The candidate vaccine strains should be approved by the national regulatory authority.

If molecularly derived strains are used, and because this is a live attenuated vaccine, the candidate vaccine should be considered a genetically modified organism (GMO) and should comply with the regulations of the producing and recipient countries regarding GMOs. An environmental risk assessment should be undertaken.

A.4.1.2.2 **Molecularly derived strains**

The genomes of the viruses in these candidate vaccines may be genetically altered and may consist of intentionally introduced mutations or

deletions, genetic elements from one or more of the dengue virus strains, or genetic elements of related flaviviruses. The primary virus seed is made from the transfection of in vitro-generated viral RNA transcripts that are synthesized from a well-characterized, full-length cDNA clone template into an appropriate cell substrate.

The sequence of any cDNA clone used to generate vaccine virus stocks must be determined prior to transfection of viral RNA into the defined host cell substrate. Virus stocks to be used as working virus seeds, derived from passaging of the primary virus stock, should also be sequenced.

Viral vaccine seeds rederived by cDNA cloning to reduce the risk of TSE contamination are considered as new vaccine candidates and appropriate bridging studies, including clinical studies, should be performed to demonstrate similarity to the starting virus seed.

A.4.1.2.3 Virus seed lot system

The production of vaccine should be based on the master and working seed lot system. Seed lots should be prepared in the same type of cells as those used for production of final vaccine.

Virus seed lots should be stored in a dedicated temperature-monitored refrigerator at a temperature that ensures stability. It is recommended that a large working virus seed lot be set aside as the basic material for use by the manufacturer for the preparation of each batch of vaccine.

A.4.1.2.3.1 Tests on virus master seeds

1. Identity

Each master seed lot should be identified as dengue virus type-1, 2, 3 or 4 by immunological assay or by sequencing.

2. Genotype/phenotype characterization

Each seed should be characterized by full-length sequence and by other relevant laboratory and animal tests. Genotype and phenotype stability of the seeds upon passage should be measured using relevant assays to ensure uniformity of vaccine lots. It should be noted that full-length sequencing may not identify minority populations of variants that may be present in candidate vaccines.

3. Freedom from bacteria, fungi and mycoplasmas

Each master seed lot should be shown to be free from bacterial, mycotic and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the revised *Requirements*

for biological substances, No. 6 (General Requirements for the Sterility of Biological Substances, 1995) (*20*).

4. Tests for adventitious viruses

Each master seed lot should be tested in cell cultures for adventitious viruses relevant to the passage history of the seed virus. Neutralization of dengue virus is necessary for many tests because the virus is cytopathogenic. Antisera used for this purpose should be shown to be free from antibodies that may neutralize the specific adventitious virus being tested for. The cells inoculated should be observed microscopically for cytopathic changes. At the end of the observation period, the cells should be tested for haemadsorbing viruses.

Each master or working seed lot should also be tested in animals that include guinea-pigs, mice and embryonated chicken eggs, as appropriate.

Additional testing for adventitious viruses may be performed using PCR amplification techniques.

5. Testing in non-human primates

Neurotropism test

To provide some level of assurance that a candidate vaccine will not be unusually neurovirulent, each master seed lot of each serotype should be tested for neurovirulence in monkeys by inoculation of *Macaca mulatta* (rhesus), Cynomolgus or other susceptible species of monkey. Tests should follow the WHO Requirements for yellow fever vaccine (*10*). Groups of at least 10 monkeys, demonstrated to be non-immune to dengue viruses and yellow fever virus immediately prior to inoculation of the seed virus, should be inoculated intracerebrally into the frontal lobe. A control group of 10 monkeys, also demonstrated to be non-immune to dengue viruses and yellow fever virus immediately prior to inoculation of the seed virus should receive yellow fever vaccine strain 17D as the control group.

The neutralizing antibody test should be used to assess immune status to dengue virus and yellow fever virus.

All monkeys should be observed for a period of 30 days for signs of encephalitis. Clinical scores, and the severity of histological lesions of the central nervous system, of the test group should not exceed scores of the control (yellow fever vaccine) group.

Viscerotropism test

For some vaccine candidates, evaluation of the master seed virus of each serotype for viscerotropism by assay of viraemia may be con-

sidered as an additional characterizing parameter. The method and specifications for yellow fever virus vaccine (*10*) should be followed.

6. Virus titration for infectivity

Each master seed lot should be assayed for infectivity in a sensitive assay in cell cultures.

- A plaque assay may be used in Vero or other sensitive cells. Titre should be determined by counting the number of visible plaques developed, and results recorded as PFU/ml.
- An immunofocus assay may also be used to measure virus titre. The assay is based on the visualization of infected areas of a cell monolayer by probing with dengue serotype-specific monoclonal antibodies. Results should be recorded as FFU/ml.
- A tissue culture infectious dose assay may also be used to determine virus titre. Results should be recorded as cell-culture infectious dose $(CCID)_{50}$/ml.

A.4.1.2.3.2 Tests on virus working seeds

The virus working seed lot is used for the production of vaccine batches and is prepared from a qualified virus master seed lot approved by the national regulatory authority. The working seed lot should be limited to a specified number of passages in cell culture beyond the master seed lot.

1. Identity

Each working seed lot should be identified as dengue virus type-1, 2, 3 or 4 by immunological assay or by sequencing.

2. Genotype/phenotype characterization

Each working seed should be characterized by full sequence and by other relevant laboratory and animal tests. Genotype and phenotype stability of the seeds upon passage should be measured using relevant assays to ensure uniformity of vaccine lots. Samples from vaccine lots that have been used for human clinical trials should be available in sufficient amounts to serve as future reference materials.

3. Freedom from bacteria, fungi and mycoplasmas

Each working seed lot should be shown to be free from bacterial, mycotic and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the revised *Requirements for biological substances* No. 6 (General Requirements for the Sterility of Biological Substances 1995) (*20*).

The absence of interference by the test articles in the sterility tests should be demonstrated.

4. Tests for adventitious viruses

Each working seed lot should be tested in cell cultures for adventitious viruses appropriate to the passage history of the seed virus. Neutralization of dengue virus is necessary for many tests because the virus is cytopathogenic. Antisera used for this purpose should be free from antibodies that may neutralize the adventitious virus being tested for. The cells inoculated should be observed microscopically for cytopathic changes. At the end of the observation period, the cells should be tested for haemadsorbing viruses.

Additional testing for adventitious viruses may be performed using PCR amplification techniques.

5. Virus titration for infectivity

Each working seed lot should be assayed for infectivity in a sensitive assay in cell cultures.

- A plaque assay may be used in Vero or other sensitive cells. Titre should be determined by counting the number of visible plaques developed, and results recorded as PFU/ml.
- An immunofocus assay may also be used to measure virus titre. The assay is based on the visualization of infected areas of a cell monolayer by probing with dengue serotype-specific monoclonal antibodies. Results should be recorded as FFU/ml.
- A tissue culture infectious dose assay may also be used to determine virus titre. Results should be recorded as $CCID_{50}$/ml.

If international reference standards become available, immunizing doses should be expressed as relative potencies rather than $CCID_{50}$ because experience shows that this reduces variability between assays.

A.4.2 *Control of vaccine production*

A.4.2.1 *Control cell cultures*

From the cell suspension used to prepare cell cultures for growing attenuated dengue viruses, an amount of processed cell suspension equivalent to at least 5% or 500 ml of cell suspension, whichever is greater, should be used to prepare control cultures of uninfected cells. These control cultures should be observed microscopically for changes attributable to the presence of adventitious agents for at least 14 days after the day of inoculation of the production cultures, or until the time of final virus harvest, if this is later. At the end of the observation period, fluids collected from the control culture should be tested for the presence of adventitious agents as described below (A.4.2.1.2). Samples that are not tested immediately should be stored at –60 °C or lower.

In some countries, samples of fluid from each control vessel are collected at the time of harvest. If several virus harvests are made from the same cell culture lot, the control fluid taken at each harvest is frozen and stored at or below –60 °C until the last virus harvest from that tissue culture lot has been taken. The control fluids are then pooled and submitted for testing.

If any test shows evidence of the presence of any adventitious agent in control cultures, the harvest of virus from these cultures should not be used for vaccine production.

For the test to be valid, not more than 20% of the control culture vessels should have been discarded for nonspecific accidental reasons by the end of the test period.

A.4.2.1.1 Test for haemadsorbing viruses

At the end of the observation period, cells comprising no less than 25% of the control cells should be tested for the presence of haemadsorbing viruses, using guinea-pig red blood cells. If the red blood cells have been stored, the duration of storage should not have exceeded 7 days, and the storage temperature should have been in the range of 2–8 °C.

In some countries, the national regulatory authority requires that additional tests for haemadsorbing viruses be performed using red blood cells from other species including those from humans (blood group O), monkeys and chickens (or other avian species). For all tests, readings should be taken after incubation for 30 minutes at 0–4 °C, and again after a further incubation for 30 minutes at 20–25 °C. The test with monkey red cells should be read once more after an additional incubation for 30 minutes at 34–37 °C.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific accidental reasons by the end of the test period.

A.4.2.1.2 Tests for cytopathic, adventitious agents in control cell fluids

Control cell fluids collected at the time of harvest should be used for testing. A 10-ml sample of the pool should be tested in the same substrate, but not the same batch as that used for virus growth, and an additional 10-ml sample of each pool should be tested in both human and monkey cells.

Each sample should be inoculated into cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not

exceed 1 in 4. The area of the cells should be at least 3 cm^2 per ml of pooled fluid. At least one bottle of the cell cultures should remain uninoculated as a control.

The inoculated cultures should be incubated at a temperature of 35–37 °C and should be examined at intervals for cytopathic effects over a period of at least 14 days.

Some national regulatory authorities require that, at the end of this observation period, a subculture is made in the same culture system and observed for at least 7 days. Furthermore, some national control authorities require that these cells should be tested for the presence of haemadsorbing viruses.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific accidental reasons by the end of the test period.

A.4.2.1.3 Additional tests if dog cell cultures are used

If dog cell cultures are used, a sample of fluids pooled from the control cultures should be tested for retroviruses, by a method approved by the national regulatory authority.

A test for retroviruses using a sensitive PCR-based reverse transcriptase (Rtase) assay may be used. The results of such assays need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (*19*). Nucleic acid amplification tests for retrovirus may also be used.

A.4.3 *Production and harvest of monovalent vaccine virus*

A.4.3.1 *Cells used for vaccine production*

On the day of inoculation with the working seed virus, each cell culture and/or cell culture control should be examined for degeneration caused by infectious agents. If such examination shows evidence of the presence in a cell culture of any adventitious agent, the whole group of cultures concerned should not be used for vaccine production.

If animal serum is used in the growth medium for the cell cultures, the serum should be removed from the cell culture either before or after inoculation of working seed virus. Prior to beginning virus harvests, 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 at any stage of manufacture.

Minimal concentrations of other suitable antibiotics may be used if approved by the national regulatory authority.

A.4.3.2 *Virus inoculation*

Cell cultures with a complete monolayer of cells are inoculated with dengue working seed virus at an optimal multiplicity of infection. After viral adsorption, cell cultures are fed with maintenance medium and incubated at a temperature within a defined range and for a defined period of time.

The optimal multiplicity of infection, temperature range and duration of incubation will depend on the vaccine strain and production method, but specifications should be given by each manufacturer. For multiplicity of infection, the specified range should not be greater than 10-fold and for temperature, the specified range should not be greater than +/– 1.5 °C.

A.4.3.3 *Monovalent virus harvest pools*

Vaccine virus is harvested within a defined time-period post-inoculation. A monovalent harvest may be the result of one or more single harvests. If several single harvests are taken, each single harvest should be stabilized and stored at 2–8 °C until pooling. No antibiotics should be added at the time of harvesting or at any later stage of manufacture. Samples of monovalent virus harvest pools should be taken for testing and stored at a temperature of –60 °C or below.

The monovalent virus harvest pool may be clarified or filtered to remove cell debris and stored at a temperature that ensures stability before being used to prepare final bulk for freeze-drying.

The national regulatory authority may require the further purification of harvests derived from continuous cell lines to remove cellular DNA, and/or the use of DNAase treatment to reduce the size of DNA fragments. If the harvests are derived from human diploid or primary cell cultures, further purification is not required.

A.4.3.4 *Tests on monovalent virus harvest pools*

1. Identity

Each monovalent virus harvest pool should be identified as the appropriate dengue virus serotype by immunological assay or by sequencing (see section A.6.1).

2. Freedom from bacteria, fungi and mycoplasmas

Each monovalent virus harvest pool should be shown to be free from bacterial, mycotic and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the revised *Requirements for biological substances* No. 6 (General Requirements for the Sterility of Biological Substances, 1995) (*20*).

3. Tests for adventitious viruses

Each monovalent virus harvest pool should be tested in cell cultures for adventitious viruses appropriate to the passage history of the seed virus. Neutralization of dengue virus is necessary for many tests because the virus is cytopathogenic. Antisera used for this purpose should be free from antibodies that may neutralize the adventitious virus being tested for. The cells inoculated should be observed microscopically for cytopathic changes. At the end of the observation period, the cells should be tested for haemadsorbing viruses.

Additional testing for adventitious viruses may be performed using PCR amplification techniques.

4. Virus titration for infectivity

Each monovalent virus harvest pool should be assayed for infectivity in a sensitive assay in cell cultures.

- A plaque assay may be conducted using Vero or other sensitive cells. Titre should be determined by counting the number of visible plaques developed, and results recorded as PFU/ml.
- An immunofocus assay may also be used to measure virus titre. The assay is based on the visualization of infected areas of a cell monolayer by probing with dengue serotype-specific monoclonal antibodies. Results should be recorded as FFU/mL.
- A tissue culture infectious dose assay may also be used to determine virus titre. Results should be recorded as $CCID_{50}$/ml.

5. Tests for cellular DNA

For viruses grown in continuous cells the monovalent harvest pool should be tested for residual cellular DNA. The removal process, at production scale, should be shown to reduce consistently the level of cellular DNA to less than 10 ng per human dose. This test may be omitted, with the agreement of the national regulatory authority, if the manufacturing process is validated to achieve this specification.

6. Test for consistency of virus characteristics

The dengue virus in the monovalent harvest pool should be tested to compare it with the working seed virus, or suitable comparator, to

ensure that the vaccine virus has not undergone critical changes during its multiplication in the production culture system. The results of these tests for successive batches of vaccine should enable an assessment to be made of the consistency of vaccine production.

Attenuation assays for dengue viruses include reduced titre in tissue culture, small plaque phenotype, temperature sensitivity, and decrease in pathogenesis in an animal model. Other assays may be used if validated for this purpose.

Assays for the attenuation of dengue–yellow fever virus chimeric vaccines include tests in suckling and adult mice. Intracerebral inoculation of suckling mice with 10-fold dilutions of vaccine and yellow fever 17D is followed by the determination of the mortality ratio and survival time. Intracerebral inoculation of adult mice with undiluted virus seed or vaccine as compared with yellow fever 17D strain is also performed.

7. Storage

Monovalent virus harvest pools should be stored at a temperature that ensures stability until tetravalent formulation.

A.4.3.5 *Final tetravalent bulk lot*

The final tetravalent vaccine lot should be prepared from bulk lots of the four dengue virus subtypes using a defined virus concentration of each component.

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

In preparing the final bulk, any substance, such as diluent or stabilizer, that is added to the product should have been shown to the satisfaction of the national regulatory authority not to impair the safety and efficacy of the vaccine in the concentration used.

A.4.3.5.1 **Tests on the final tetravalent bulk lot**

Residual animal serum protein. A sample of the final bulk should be tested to verify that the level of serum is less than 50 ng per human dose. Alternatively the test may be performed on the clarified monovalent bulk.

Sterility. Each final bulk suspension should be tested for bacterial and mycotic sterility according to Part A, sections 5.2 and 5.3 of the Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (*20*), or by a method approved by the national regulatory authority.

A.4.3.5.2 Storage

Until it is distributed into containers and lyophilized, the final bulk suspension should be stored under conditions shown by the manufacturer to retain the desired immunogenic activity.

A.5 Filling and containers

The requirements concerning good manufacturing practices for biological products (*22*) appropriate to a developmental vaccine should apply.

Care should be taken to ensure that the materials of which the container and, if applicable, the closure is made do not adversely affect the virus content of the vaccine under the recommended conditions of storage.

A final filtration could be included during the filling operations.

The manufacturer should provide the national regulatory authority with adequate data to prove the stability of the product under appropriate conditions of storage and shipping.

A.6 Control tests on final product

The following tests should be carried out on the final product.

1. Identity

Each tetravalent vaccine virus lot should be identified as dengue virus type-1, 2, 3 or 4 by immunological assay or by sequencing.

2. Virus titration for infectivity

Each tetravalent vaccine virus lot should be assayed for infectivity in a sensitive assay in cell cultures in which interference or potentiation between serotypes does not occur. The titre of each individual serotype should be determined.

- A plaque assay may be used in Vero or other sensitive cells. Titre should be determined by counting the number of visible plaques developed, and results recorded as PFU/ml.
- An immunofocus assay may also be used to measure virus titre. The assay is based on the visualization of infected areas of a cell monolayer by probing with dengue serotype-specific monoclonal antibodies. Results should be recorded as FFU/ml.
- A tissue culture infectious dose assay may also be used to determine virus titre. Results should be recorded as $CCID_{50}$/ml.

3. Accelerated degradation tests

Three containers of the final freeze-dried tetravalent vaccine should be incubated at 37 °C for 7 days. The geometric mean infectious

virus titre of the containers that have been exposed should not have decreased by more than a specified amount during the period of exposure. Titration of non-exposed and exposed vials should be done in parallel and results expressed in terms of PFU or $CCID_{50}$ or FFU per human dose. A reference reagent of each of the four dengue serotype viruses should be included in each assay to validate the assay.

The maximum allowable loss of titre during the accelerated degradation test should be confirmed on the basis of experience yet to be accumulated.

4. Sterility test

Reconstituted vaccine should be tested for bacterial and mycotic sterility according to the requirements in Part A, section 5.2 of the Requirements for biological substances No. 6 (Requirements for the sterility of biological substances) (*20*), by acceptable methods approved by the national regulatory authority.

5. General safety tests

Each filling lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety (innocuity) test approved by the national regulatory authority.

This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the national regulatory authority and when good manufacturing practices are in place. Each lot, if tested, should pass a test for abnormal toxicity.

6. Residual moisture

The residual moisture in a representative sample of each freeze-dried lot should be determined by a method approved by the national regulatory authority and an appropriate limit should be set by them.

Generally, moisture levels of 2% and less are considered satisfactory although some candidate vaccines formulations may be satisfactory at levels of up to 4%.

7. Inspection of final containers

Each container in each final lot should be inspected visually and those showing abnormalities should be discarded.

A.7 Records

The requirements of Good manufacturing practices for biological products (*22*) pp. 27–28, should apply, as appropriate for the level of development of the candidate vaccine.

A.8 Samples

A sufficient number of samples should be retained for future studies and needs. Vaccine lots that are to be used for human clinical trials may serve as reference materials in the future and a sufficient number of vials should be reserved, and appropriately stored, for that purpose.

A.9 Labelling

The requirements of Good Manufacturing Practices for Biological Products (*20*) pp. 26–27, appropriate for a candidate vaccine should apply, with the addition of the following:

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 fulfils Part A of these Requirements;
— a statement of the nature of the preparation, specifying the designation of the strains of dengue viruses contained in the live attenuated tetravalent vaccine, the minimum number of infective units per human dose, the origin of the substrates used in the preparation 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 antibiotic present in the vaccine;
— an indication that contact with disinfectants is to be avoided;
— a statement concerning the photosensitivity of the vaccine, cautioning that both lyophilized and reconstituted vaccine should be protected from light;
— 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; and
— a statement that after the vaccine has been reconstituted, it should be used without delay, or if not used immediately, stored between 2 °C and 8 °C and in the dark for a maximum period defined by validation studies.

A.10 Distribution and shipping

The requirements of Good Manufacturing Practices for Biological Products (20) appropriate for a candidate vaccine should apply.

Shipments should be maintained at temperatures of 8 °C or below and packages should contain cold-chain monitors.

A.11 Storage and expiry date

The Requirements given in Good Manufacturing Practices for Biological Products (*22*) appropriate for a candidate vaccine should 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 for approval to the national regulatory authority.

A.11.1 *Storage conditions*

Before being distributed by the manufacturing establishment or 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. After distribution, live tetravalent dengue vaccine should be stored at all times at a temperature not more than 8 °C. The maximum duration of storage should be fixed with the approval of the national regulatory authority and should be such as to ensure that the minimum titre specified on the label of the container (or package) will be maintained after release by the manufacturing establishment until the end of the shelf-life, if the conditions under which the vaccine is stored are in accordance with those stated on the label. The maximum duration of storage at 2–8 °C or below –20 °C may be specified.

A.11.2 *Expiry date*

An expiry date should be fixed and should relate to the date of the last satisfactory determination, performed in accordance with Part A, section 6.2, of virus concentration, i.e., the date on which the cell cultures were inoculated.

Part B. National control requirements

The national regulatory authority may give directions to manufacturers concerning the dengue virus strains to be used in candidate vaccine production and concerning the proposed human dose(s) to be tested.

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