Annex 7

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

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

These WHO Recommendations are intended to provide national regulatory authorities (NRAs) and vaccine manufacturers with guidance on evaluating the quality, safety and efficacy of live-attenuated Japanese encephalitis (JE) vaccines for use in humans to facilitate their international licensure and use.

The Recommendations replace the WHO Guidelines for the production and control of Japanese encephalitis vaccine (live) for human use (1), and should be read in conjunction with the other WHO Recommendations and Guidelines referred to in each part.

The scope of these Recommendations is limited to live-attenuated JE vaccines for human use. Other types of JE vaccines are outside the scope of these Recommendations. Revised WHO Recommendations for inactivated JE vaccines (2) are available separately.

Given the advances made in the immunization of humans with live-attenuated vaccines to prevent JE, there is increased interest in defining quality standards for the vaccines’ manufacture, developing guidelines for nonclinical studies, and in conducting clinical studies that will assess the vaccines’ efficacy and safety in humans as well as the risks the vaccine poses to public health and the environment. To this end, a group of international experts met in February 2012 to review the latest advances in this field, and to propose a revision of the 2002 Guidelines (1). The main changes introduced in this revision include:

- updating information on monitoring the use of animals from which primary hamster kidney cells are prepared;
- updating information on methods for testing and specifications for primary hamster kidney cell cultures used in vaccine production, according to the revised recommendations on cell substrates;
- updating information on testing for the attenuation of SA14-14-2 vaccine;
- the addition of recommendations on evaluating lot-release potency specifications, and information on the need for monitoring the upper limit of potency in addition to the existing minimum potency specification for the immunizing dose;
- the addition of information on the relationship between passage levels of vaccine seeds and production in the current production scheme for live JE vaccines;
- the addition of new specifications for the manufacture and quality control of a live JE vaccine based on an attenuated strain of yellow fever virus used as a viral vector and grown in Vero cell cultures;
the addition of guidelines for the nonclinical and clinical evaluation of new, live JE vaccines;
- the addition of guidelines for assessing the environmental risk of live JE vaccines derived using recombinant DNA technology;
- the addition of a model protocol for lot release, and a model certificate for vaccine release by NRAs.

These Recommendations are based on experience gained with live-attenuated JE vaccines that have been licensed through the procedures described below. The Recommendations will need to be updated as new data become available.

Part A sets out recommendations for manufacturing and quality control. Parts B, C and D provide guidelines specific to the nonclinical evaluation, clinical evaluation and environmental risk assessment of the vaccines, respectively. Part E provides recommendations for NRAs.

In the following section on general considerations, brief overviews of JE disease and vaccine development provide the scientific basis for formulating detailed technical recommendations (Parts A and E) and guidelines (Parts B, C and D).

**General considerations**

JE is caused by mosquito-borne Japanese encephalitis virus (JEV) infection, and is the most important viral encephalitis in Asia, accounting for at least 50,000 clinical cases, with 25–30% case-fatality rates annually (3). A study in 2011 estimated that approximately 67,900 JE cases typically occur each year in the 24 JE-endemic countries, giving an incidence of 1.8 cases per 100,000 overall population. Approximately 51,000 (75%) of these cases occur in children aged 0–14 years, which gives an estimated overall annual incidence of 5.4 per 100,000 in this age group (4). The high fatality rate and frequent residual neuropsychiatric sequelae in survivors make JE a considerable health problem. For example, a study from China demonstrated that significant neurological and overall functional disability were evident in a high proportion of JE survivors many years after infection, with 22% of JE patients having objective neurological deficits, and 28% having subnormal intelligence quotients (5).

Since the 1980s, JEV transmission has intensified in certain countries, and the disease has extended its geographical range to areas of Asia where it had not been previously recognized, as well as to northern Australia. Two epidemiological patterns of JEV infection are recognized. In northern temperate areas, JE occurs in summer epidemics, whereas in tropical areas, JE may occur all year round. In temperate zones and in the northern part of the tropical zone,
outbreaks have a marked seasonal incidence, occurring during the rainy season. In tropical areas, there is an endemic pattern of infection, with the occurrence of sporadic cases throughout the year. The incidence is highest in rural agricultural areas, and within that population it is highest in males because of their increased exposure to areas of rice cultivation.

JEV is a member of the genus Flavivirus, family Flaviviridae. JE disease was first reported in Japan in 1924, and subsequently reported in other Asian countries; it was first reported in Australia in 1955. JEV is the prototype of the JE antigenic complex (which includes the West Nile, Usutu, Murray Valley encephalitis and St Louis encephalitis viruses), and cross-reactions in neutralization antibody tests, and cross-protection in animals, have been demonstrated with other flaviviruses that are members of the complex. In the mouse model, considerable variation has been demonstrated in neurovirulence and peripheral pathogenicity. JEV has a genome comprising a positive-sense, single-stranded RNA molecule of approximately 11 kb that is capped at the 5’-end and is not polyadenylated at the 3’-end. It carries a single open-reading frame encoding a polyprotein that is processed into three structural proteins – core (C), membrane (M) and envelope (E) – and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), flanked by 5’- and 3’-nontranslated regions. Based on nucleotide sequencing of the C/PrM and E genes, five genotypes have been identified.

- Genotype I includes isolates from Australia, Cambodia, China and the Province of Taiwan, India, Japan, the Lao People's Democratic Republic, Malaysia, the Republic of Korea, Thailand and Viet Nam (1967–present).
- Genotype II includes isolates from Australia, Indonesia, Malaysia, Papua New Guinea, the Republic of Korea and Thailand (1951–1999).
- Genotype III includes isolates from mostly temperate regions in Asia – i.e. China and the Province of Taiwan, India, Indonesia, Japan, Malaysia, Myanmar, Nepal, the Philippines, the Republic of Korea, the Russian Federation, Sri Lanka, Thailand and Viet Nam (1935–present).
- Genotype IV includes isolates only from Indonesia (1980–1981).
- Genotype V virus was originally isolated from a human infected with JE in Malaysia and subsequently from mosquitoes in China and the Republic of Korea.

Until the later part of the 20th century, genotype III was the predominant JEV genotype involved in human infection. The genotypes of JEV have diverged in the order IV, III, II and I. Since the 1980s there has been a genotype replacement,
whereby genotype I is replacing genotype III as the dominant genotype. Although current JE vaccines are based on genotype III strains, all evidence indicates that these vaccines induce protective immunity to all genotypes of JEV.

JEV is maintained in an enzootic cycle, which typically involves culicine mosquitoes (primarily Culex tritaeniorhynchus) that breed in rice paddies, and domesticated swine or ardeidae (principally egrets and herons), which serve as virus-amplifying vertebrate hosts. Humans and other nonavian vertebrates are dead-end hosts because they fail to produce viraemias of sufficient titres to infect mosquitoes. However, infection of certain nonavian vertebrates, such as horses, can lead to clinical disease and encephalitis.

Humans of all ages are susceptible unless immunized by natural infection or vaccination. Evidence shows that effective vaccines will protect both animals and humans against clinical signs and disease. Although the control of mosquitoes and the vaccination of pigs are effective in certain circumstances, these measures are not practical means of preventing human illness. It is also important to recognize that humans are incidental hosts and for vaccination to be effective, coverage should be maintained indefinitely in all persons who may be exposed to the virus.

The virus replicates in a variety of cultured cells of vertebrate and nonvertebrate origin. Since the 1960s, both live and inactivated vaccines have been developed that provide active immunity against JEV. The development of these vaccines represented a major advance in the ability to control JEV infection and reduce the burden of disease. Viruses isolated from human patients in Japan in 1935 and in China in 1949 provided the prototype Nakayama and Beijing (Beijing-1) and P3 (Beijing-3) strains, respectively; these are the principal strains used in the production of inactivated JE vaccine. The SA14-14-2 strain, which was derived from a mosquito isolate, is widely used in the production of a live-attenuated JE vaccine, and is used as donor strain in a replicating recombinant vaccine. Systematic vaccination programmes — such as those in China (Province of Taiwan), Japan and the Republic of Korea — using an inactivated JE vaccine that meets international requirements, have controlled the disease to the point of elimination. However, in other countries the expense and complexity of producing the vaccine, and the need for repeated doses, have limited the use of this vaccine. In addition to the problems posed by multiple doses, use of the vaccine has been associated with hypersensitivity. A number of vaccine manufacturers have developed second-generation inactivated vaccines using African green monkey kidney-derived Vero cells.

As an alternative to inactivated vaccines, there are two different live-attenuated JE vaccines, namely the SA14-14-2-strain vaccine produced in primary hamster kidney (PHK) cells and the JE chimeric virus (JE-CV) vaccine produced in Vero cells. The SA14-14-2 vaccine was developed in China by empirical passage of a naturally occurring mosquito isolate (i.e. SA14) in mice,
hamster, chicken embryo and PHK cells; it is manufactured in PHK cells. Since its licensure in China in 1988, more than 300,000,000 doses of the live SA14-14-2 vaccine have been produced for administration to children during annual vaccination programmes. The vaccine is of considerable interest to countries where JEV is endemic and, as of 2012, had been licensed in Cambodia, the Democratic People's Republic of Korea, India, the Lao People's Democratic Republic, Myanmar, Nepal, the Republic of Korea, Sri Lanka and Thailand. The JE-CV vaccine is based on yellow fever (YF) vaccine strain 17D (YF-17D) that has been genetically modified to contain premembrane (prM) and E structural genes from JE vaccine strain SA14-14-2. The JE-CV vaccine is manufactured in Vero cells, and has been licensed in Australia and Thailand since 2010. JE-CV vaccine was previously known as ChimeriVax-JE and is now available under the tradenames IMOJEV and THAIJEV.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name should be Japanese encephalitis vaccine (live, attenuated) for human use. The proper name should be the equivalent of the international name in the language of the country of origin.

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

A.1.2 Descriptive definition

A live-attenuated JE vaccine defined in section A.1.1 should contain live-attenuated JEVs or replication-competent vector viruses that encode genes to express the major structural antigen of JEVs. The vaccine may be presented as a sterile, aqueous suspension or as freeze-dried material. The preparation should satisfy all the specifications given below.

A.1.3 International reference materials

No international reference materials commonly applicable for already licensed live, attenuated JE vaccines are available either for potency testing or for neurovirulence testing.

A.1.4 Expression of dose related to vaccine potency

The potency of a live-virus vaccine is typically expressed in terms of the number of infectious units of virus contained in a human dose, using a specified tissue culture substrate and based on the results of phase I and phase II clinical trials.
In the case of live-attenuated JE vaccines, potency will have to be assessed in terms of virus infectivity titres. When product-specific international reference standards for different types of vaccines become available, the dose related to vaccine potency should be calculated against a product-specific standard, and should be expressed in product-specific International Units (IUs) if this results in a reduction in variation among laboratories. Until then, alternatives are to use plaque-forming units (PFUs) or the median cell culture infectious dose (CCID₅₀) to express the potency and dose of the vaccine. The dose should also serve as the basis for establishing parameters for stability and for the expiry date.

A.1.5 Terminology

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

**Adventitious agents**: contaminating microorganisms of the cell culture or source materials, including bacteria, fungi, mollicutes (mycoplasmas or spiroplasmas), mycobacteria, rickettsia, protozoa, parasites, agents causing transmissible spongiform encephalopathies (TSEs), and viruses that have been unintentionally introduced into the manufacturing process of a biological product.

**Bulk material**: one or more single harvests after clarification or purification, or both, from which the final bulk is prepared.

**Cell bank**: a collection of appropriate containers whose contents are of uniform composition and that are stored under defined conditions. Each container represents an aliquot of a single pool of cells.

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

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

**Cell substrates**: cells used for the production of a vaccine.

**Final bulk**: the finished vaccine prepared from virus harvest pools or bulk, or both, held in a single vessel from which the final containers are filled.

**Final lot**: a collection of sealed final containers of finished vaccine that is homogeneous with respect to the risk of contamination during filling and, if applicable, freeze-drying. Therefore, all of the final containers should have been filled from one vessel of final bulk and, if applicable, freeze-dried under standardized conditions in a common chamber during one working session.

**Genetically modified organism**: an organism in which the genetic material has been altered using recombinant DNA techniques (or genetic
engineering techniques) in a way that does not occur naturally by mating or natural recombination and selection.

**Master cell bank:** a quantity of well characterized cells of animal or other origin that have been derived from a cell seed at a specific population doubling level or passage level, dispensed into multiple containers, cryopreserved and stored frozen under defined conditions, such as the vapour or liquid phase of liquid nitrogen, in aliquots of uniform composition. The master cell bank is prepared from a single homogeneously mixed pool of cells. It is considered best practice for the master cell bank to be used to derive working cell banks.

**Neuroinvasiveness:** the ability of a virus to replicate in peripheral tissues, induce viraemia and invade the central nervous system (CNS) (6).

**Neurovirulence:** the ability of a virus to initiate cytopathic infection in the CNS and to cause encephalitis (6). In animal experimental settings, clinical, virological and histopathological evaluations are often carried out after intracerebral inoculation of a virus.

**Plaque-forming unit (PFU):** the amount of a virus sufficient to cause a single visible focus of infection due to cytopathic effect in a cell culture monolayer after cells have been properly stained.

**Primary culture:** a culture started from cells, tissues or organs taken directly from one or more organisms. A primary culture may be regarded as such until it is successfully subcultured for the first time. It then becomes a cell line if it can continue to be subcultured at least several times.

**Production cell culture:** a collection of cell cultures used for biological production that have been prepared together from one or more containers from the working cell bank, or in the case of primary cell cultures, from the tissues of one or more animals.

**Single harvest:** a quantity of virus suspension harvested from production cell cultures and inoculated with the same virus working seed, and processed together in a single production run.

**Virus master seed:** A suspension of vaccine virus that has been aliquoted into identical vials and stored at a temperature and under conditions deemed to stabilize the virus in each container. The virus master seed is used as a source of infectious virus for the generation of each virus working seed lot.

**Virus pool:** a suspension of two or more single harvests of the virus collected into a single vessel.

**Virus working seed:** A quantity of virus of uniform composition, well characterized and derived from a virus master seed lot (see above) in a production cell. The working seed lot is used for the production of a single harvest.

**Working cell bank:** a quantity of well characterized cells of animal or other origin that have been derived from the master cell bank at a specific population doubling level or passage level, dispensed into multiple containers,
cryopreserved and stored frozen under defined conditions, such as in the vapour or liquid phase of liquid nitrogen, in aliquots of uniform composition. The working cell bank is prepared from a single homogeneously mixed pool of cells. One or more of the working cell bank containers is used for each production culture.

A.2 General manufacturing recommendations

The general manufacturing recommendations contained in the WHO Good manufacturing practices for biological products (7) should be applied with the addition of the following recommendations.

- All staff directly involved in the production and testing of live JE vaccine should be shown to be immune to JEV by appropriate neutralizing antibody tests.
- Written descriptions of the standard operating procedures used for the preparation and testing of live JE vaccine, together with evidence of appropriate validation for each production step, should be submitted for approval to the NRA as part of the licensing application. Proposals for any modifications to the manufacturing or control methods should be submitted for approval to the NRA before they are implemented.
- Production steps and quality control operations involving manipulations of live virus should be conducted under the appropriate biosafety level agreed with the NRA, and in accordance with national biosafety laws.

A.3 Control of source materials

A.3.1 Cell cultures for virus propagation

A.3.1.1 Conformity with WHO recommendations on cell substrates

Live JE viruses for vaccine production should be propagated in cell substrates that meet the WHO Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (8), and should be approved by the NRA. All information on the source and method of preparation of the cell culture system used should be made available to the NRA.

A.3.1.2 Types of cell cultures

Live JE vaccines have been produced in either PHK cells or a continuous cell line – i.e. Vero cells. Sections A.3.1.3 and A.3.1.4 apply, respectively, to each type of cell.
A.3.1.3 PHK cells
A.3.1.3.1 Animals

Syrian hamsters aged between 10 days and 14 days may be used as the source of kidneys for cell culture. Only hamster stock derived from a healthy colony that is free from specific pathogens should be used as the source of tissue. The colony forms a group of animals that shares a common environment and has its own caretakers, who have no contact with other animal colonies. The animals should be tested according to a defined programme to ensure freedom from specified pathogens and from the antibodies to those pathogens. At the time the colony is established, all animals should be tested and show no detectable antibodies to Hantaan virus, Kilham rat virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse hepatitis virus, mouse poliovirus, pneumonia virus of mice, respiratory enteric orphan virus type 3, Sendai virus (murine parainfluenza virus type 1), simian virus type 5 and Toolans H-a virus. In addition, lysates of primary kidney cells should be tested for the presence of specific pathogens by inoculating hamsters, mice and rats; this should be followed by tests to detect antibodies in the animals' serum samples. No antibodies should be detected. Detailed lists of pathogens to be screened for in hamster, mouse and rat antibody production tests are summarized elsewhere (9). A test for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse transcriptase (RTase) assay should also be included. The results of such assays may need to be interpreted with caution because RTase activity is not unique to retroviruses, and may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (10). Nucleic acid amplification tests for retroviruses may also be used. A PCR test for hamster polyomavirus should be used on a selected number of hamster tissues – especially kidney – to qualify the colony, and should be repeated at intervals thereafter. When new animals are introduced into the colony, they should be maintained in quarantine in vermin-proof quarters for a minimum of two months, and should be shown to be free from these specified pathogens.

The parents of animals to be used as a source of tissue should be maintained in vermin-proof quarters. Neither the parent hamsters nor their progeny should previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored at regular intervals for zoonotic viruses and markers of contamination by following a defined programme.

Once the colony has been established, it should be monitored by testing for antibodies to the relevant pathogens in a representative group of animals – consisting of at least 5% of the animals – that are bled at intervals acceptable to the NRA.

For example, birds used in the production of chick embryo fibroblast cells for measles vaccine (11) are bled at monthly intervals.
In addition, the colony should be screened for pathogenic bacteria (including mycobacteria), fungi and mycoplasmas, as agreed with the NRA. The screening tests should be carried out on a regular basis over a defined period. The sample size, tests, method and testing intervals should be agreed with the NRA.

Any animal that dies should be investigated to determine the cause of death. If the presence of an infectious agent is demonstrated in the colony, the NRA should be informed, and the manufacture of live JE vaccine may be discontinued. Manufacturing should not be resumed until a thorough investigation has been completed and precautions have been taken to prevent the presence of the infectious agent in both the colony and the product; manufacturing should be resumed only with the approval of the NRA.

At the time of harvesting the kidneys, the animals should be visually examined for any gross abnormalities. If any kidney abnormalities or other evidence of pathology (e.g. abnormal size, protein ascites) is found, animals with the abnormalities should not be used for vaccine production.

Each group of control cultures derived from a single group of animals used to produce a single virus harvest should remain identifiable as such until all testing, especially for adventitious agents, has been completed.

A.3.1.3.2 Primary cell cultures

Kidneys derived from animals that fulfil the requirements in section A.3.1.3.1 should be dissected and homogenized under conditions approved by the NRA. A primary cell suspension is obtained after digestion with trypsin, and this is distributed, together with growth medium, into culture vessels. Penicillin and other beta-lactam antibiotics should not be used during any stage of manufacturing.

Minimal concentrations of suitable antibiotics, such as kanamycin, may be used if approved by the NRA.

A.3.1.4 Vero cells

The use of Vero cells for the manufacture of live JE vaccines should be based on the cell bank system. The cell seed should be approved by the NRA. The maximum number of passages or population doublings allowable between the cell seed, the master bank, the working cell bank and the production passage levels should be established by the manufacturer, and approved by the NRA.

Additional tests may include but are not limited to propagation of the master cell bank or working cell bank to or beyond the maximum in vitro age for production, and examination for the presence of retroviruses and tumorigenicity in an animal test system (8).
WHO has established a bank of Vero cells, designated as WHO Vero reference cell bank 10-87, which has been characterized as suitable in accordance with the Requirements for continuous cell lines used for biologicals production (12). The cell bank is available to manufacturers as a well characterized starting material for preparation of their own master cell bank and working cell bank.¹

In normal practice, a master cell bank is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer and approved by the NRA, at which point the cells are combined to give a single pool that is distributed into ampoules and preserved cryogenically to form the working cell bank.

The manufacturer’s working cell bank is used for the preparation of production cell culture, and thus for the production of vaccine batches.

The cell seed (if applicable), the master cell bank and working cell bank, and the end of production cells or extended cell bank, should be characterized according to the WHO Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (8).

A.3.1.5 Cell-culture medium

When serum is used for propagating cells for JE vaccine production, it should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas according to the requirements given in Part A, section 5.2 of the General requirements for the sterility of biological substances no. 6 (1973), and section 5.3 of the General requirements for the sterility of biological substances no. 6 (amended 1995) (13, 14), and freedom from infectious viruses should also be shown.

Detailed guidelines for detecting bovine viruses in serum used to establish master cell banks and working cell banks are given in Appendix 1 of the WHO Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (8). The principles outlined in the cell-substrate Recommendations should be applied as appropriate, and guidelines for detecting bovine viruses in serum used to establish the cell banks may be applicable to production cell cultures as well. In particular, validated molecular tests for bovine viruses may replace the cell culture tests on bovine serum if the NRA agrees. As an additional indicator of quality, serum samples may be examined to ensure they are free from

¹ Contact the Coordinator, Technologies Standards and Norms, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).
phages and endotoxins. Gamma radiation may be used to inactivate potentially contaminating viruses, while recognizing that some viruses are relatively resistant to gamma radiation.

The sources of animal components used in culture medium should be approved by the NRA. These components should comply with guidelines relating to animal-transmissible spongiform encephalopathies (15).

Human serum should not be used. If human albumin is used, it should meet the revised Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (16), as well as guidelines relating to human-transmissible spongiform encephalopathies (15).

The use of human albumin as a component of a cell culture medium requires careful consideration due to potential difficulties with the validity period of albumin (which is based on the length of time during which it is suitable for use in clinical practice) in relation to the potential long-term storage of vaccine intermediates. In addition, if human albumin is used, it should be tested according to the WHO Recommendations for cell substrates (8).

Penicillin and other beta-lactam antibiotics should not be used at any stage of manufacturing. Other antibiotics may be used during any stage, provided that the quantity present in the final product is acceptable to the NRA.

Any other substances added should be approved by the NRA.

Nontoxic pH indicators may be added (e.g. phenol red at a concentration of 0.002%).

If porcine or bovine trypsin is used for preparing cell cultures, it should be prepared, tested and found free from cultivable bacteria, fungi, mycoplasmas and infectious viruses as described in WHO Recommendations for cell substrates (8). The methods used to ensure this should be approved by the NRA.

If used, the source or sources of trypsin of bovine origin should be approved by the NRA, and should comply with guidelines relating to animal-transmissible spongiform encephalopathies (15).

A.3.2 Virus seeds

A.3.2.1 Vaccine virus strain certification

Only a designated strain of virus for live JE vaccine that has been approved by the NRA should be used in the production of vaccine. The strain should be identified by historical records, which should include information on the strain's origin, its method of attenuation, whether the strain has been biologically or molecularly cloned prior to generation of the master seed, and the passage level (or levels) at which attenuation, immunogenicity, safety and efficacy were demonstrated by clinical studies. The entire genome sequence of both the master seed and working seed viruses should be determined.
A.3.2.2 Strains derived by recombinant DNA techniques

In some countries, if a vaccine seed derived by recombinant DNA technology is used, and because it is a live-attenuated vaccine, the candidate vaccine is considered to be a genetically modified organism (GMO) and should comply with the regulations of the producing and recipient countries regarding GMOs (see Part D).

The entire nucleotide sequence of any complementary DNA (cDNA) clone used to generate vaccine-virus stocks should be determined prior to any further nonclinical or clinical trial. The cell substrate used for transfection to generate the virus should be appropriate for human vaccine production, and should be approved by the NRA.

Entire genomes of preseed lot virus stocks derived from passaging of the primary virus stock should also be sequenced as part of a nonclinical evaluation.

A.3.2.3 Virus-seed lot system

The production of vaccine should be based on the virus-seed lot system, which includes a master seed and a working seed. The virus working seed lot used for the production of vaccine batches should be prepared from a qualified virus master seed lot by means of a method approved by the NRA.

Seed lots should be prepared in the same type of cells and using the same conditions for virus growth (other than scale) as those used for production of the final vaccine. The virus working seed should have a well defined relationship to the virus master seed, with respect to passage level and method of preparation, so that the virus working seed retains all of the in vitro and in vivo phenotypes and the genetic character of the virus master seed.

The maximum passage level of master seed and working seed lots should be approved by the NRA. The inoculum for infecting cells used in the production of vaccine should be from a virus working seed lot without intervening passage in order to ensure that no vaccine is manufactured that is more than one passage level from the working seed.

The virus master seed and working seed lots used to produce live-attenuated JE vaccines should be demonstrated to be safe and immunogenic using appropriate laboratory tests. All virus seed lots should be stored in a suitable manner to ensure their stability over prolonged periods. The tests specified in sections A.3.2.4 and A.3.2.5 should be performed on both the master seed and working seed. However, the master seed is a crucial feature of the production of a safe vaccine, and should ideally be made in large amounts to avoid the need to remake it. The use of additional tests to provide further assurance of quality should be considered; these are detailed in Part B.
A.3.2.4 Control cell cultures for virus seeds
In agreement with NRAs, tests on control cell cultures may be required and should be performed as described in section A.4.1.

A.3.2.5 Tests on virus master seed and working seed lots
A.3.2.5.1 Identity test
Each virus master seed and working seed lot should be identified as JE vaccine seed virus by serological methods approved by the NRA.

Appropriate serological methods include enzyme immunoassays that use a vaccine seed virus-specific monoclonal antibody, immunofluorescence or neutralization assays that use a reference serum or monoclonal antibody specific to JEV.

A test for genetic identity should also be performed.

A.3.2.5.2 Virus titration for infectivity
The infectivity of each virus master seed and working seed lot should be established using an assay that is acceptable to the NRA. Manufacturers should determine the appropriate titre necessary to produce vaccine reliably.

Depending on the type of vaccines and available data, plaque assays, CCID₃₀ assays or CCID₅₀ with a molecular read-out, such as quantitative PCR, may be used.

All assays should be validated.

For intra-assay validation, titration should be carried out in parallel with titration of a reference vaccine, as approved by the NRA.

A.3.2.5.3 Genetic and phenotypic characterization
In addition to the infectivity titration, it will be necessary to examine genetic and phenotypic stability relevant to the consistency of production. The applicable tests will be identified in the course of the nonclinical evaluation of the strains. Each seed should be characterized by full-length consensus nucleotide sequence determination and by other relevant laboratory and animal tests, which will provide information on the consistency of each virus seed.

Mutations introduced during the derivation of each vaccine strain should be maintained in the consensus sequence unless spontaneous mutations induced during tissue culture passage are shown to be innocuous in nonclinical and small-scale clinical trials. Some variations in the nucleotide sequence of the virus population on passage are to be expected, but the determination of what is acceptable should be based on experience in production and clinical use.
For any new virus master seed and working seed, it is recommended that the first three consecutive consistency bulk-vaccine lots should be analysed for consensus genome sequence changes from the virus master seed. The nucleotide sequence results should be used to demonstrate the consistency of the production process.

A.3.2.5.4 Tests for bacteria, fungi, mycoplasmas and mycobacteria

Each virus master seed and working seed lot should be shown to be free from bacterial, fungal and mycoplasmal contamination using the appropriate tests specified in Part A, section 5.2 of the General requirements for the sterility of biological substances no. 6 (1973), and section 5.3 of the General requirements for the sterility of biological substances no. 6 (amended 1995) (13, 14).

Nucleic acid amplification techniques (NATs) – used alone or in combination with cell culture and an appropriate detection method – may be used as alternatives to one or both of the compendial mycoplasma detection methods, if they have been validated and the NRA is in agreement (8).

Seed lots should be shown to be free from mycobacteria by a method approved by the NRA.

NATs may be used as an alternative to microbiological methods for culturing mycobacteria or to the in vivo guinea-pig test for the detection of mycobacteria after they have been validated and approved by the NRA (8).

A.3.2.5.5 Tests for adventitious agents

Each virus master seed and working seed lot should be tested in cell cultures and in animals for adventitious viruses relevant to the passage history of the seed virus.

Where antiserum is used to neutralize JEV or the recombinant JEV, the antigen used to generate the antiserum should be produced in a cell culture from a species different from that used for the production of the vaccine, and it should be free from extraneous agents.

Depending on the derivation of the seed lot, a volume of each virus master seed and working seed lot of at least 10 ml should be tested for adventitious agents as described below:

- For virus grown in hamster or its cultured cells, the neutralized virus should be tested for adventitious agents by inoculating it on to cultures of human cells, mouse cells, simian cells, mosquito cells (e.g. C6/36), baby hamster kidney (BHK)-21 cells and PHK cells.
The cell culture should not be from the same batch as that used in the preparation of the virus seed. Uninoculated control cell cultures should be included in the tests. All cell cultures (except mosquito cells) should be observed for at least 14 days. At least one subculture of one cell culture fluid should be made, and should be observed for 14 days in order to enhance the opportunity to detect adventitious agents.

* For virus grown in simian or human cells, the neutralized virus should be tested on separate cultures of simian and human cells. If other cell systems are used, cells of that species, but from a separate batch, are also inoculated. At the end of the observation period, the cells should be tested for haemadsorbing viruses. If the virus master seed was prepared in primary cell cultures, at least one subculture of one cell culture fluid should be made, and should be observed for 14 days in order to enhance the opportunity to detect adventitious agents.

The cells should be observed microscopically for cytopathic changes. At the end of the observation period, the cells or fluids should be tested for haemadsorbing viruses. For the test to be valid, 20% or fewer of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test. For the seed virus to be satisfactory, no cytopathic changes or adventitious agents should be detected. Tests for a number of agents should be conducted on the virus master seed lot to rule out the presence of adventitious agents associated with any primary cell cultures that were used in the adaptation of the JEV. For rodent primary cell substrates, such agents include mouse viruses identified using the mouse antibody production assay; hamster viruses identified using the hamster antibody production assay; human, porcine and bovine viruses identified by PCR; and endogenous retroviruses identified using a sensitive assay such as product-enhanced RTase (see section A.3.2.5.7 for details). Consideration should be given to using electron microscopy with the negative-stain technique in order to obtain additional information on extraneous agents that may not be detected by other methods.

Each virus master seed and working seed lot should also be tested in animals; these animals may include guinea-pigs and mice, as appropriate (8).

Additional testing for adventitious viruses may be performed using a validated NAT-based assay. New molecular methods with broad capabilities are being developed to detect adventitious agents. These methods include: (i) degenerate NAT for whole virus families that analyses the amplicons by hybridization, sequencing or mass spectrometry; (ii) NAT with random primers that is followed by analysis
of the amplicons on large oligonucleotide microarrays of conserved viral sequencing or digital subtraction of expressed sequences; and (iii) high-throughput sequencing. These methods may be used in the future to supplement existing methods, or as alternatives to both in vivo and in vitro tests after appropriate NRA validation and agreement.

A.3.2.5.6 Tests for attenuation

The tests applied to the SA14-14-2 and JE-CV live vaccines differ slightly because of the nature of the viruses concerned. Reference preparations to be included in each test should be identified as a high priority, and could take the form of working seeds shown to produce satisfactory vaccines.

Section A.3.2.5.6.1 applies to SA14-14-2 and section A.3.2.5.6.2 applies to JE-CV.

A.3.2.5.6.1 Tests for neurovirulence of SA14-14-2 seeds

Test for neurovirulence in weanling mice

Both master seed and working seed should be tested for neurovirulence in weanling mice.

Ten animals of the Kunming strain of Swiss mice aged 17–19 days should be inoculated by the intracerebral route with 0.03 ml of the master seed or working seed at a titre of not less than 5.7 log_{10} PFU per ml. Mice are observed daily for 14 days after inoculation. Mice that die within three days of inoculation are considered to have died from brain trauma, and are not included in the evaluation of the test. If more than 20% of mice die within three days, the test is considered invalid. If any mice die or show clinical signs of JE infection, the preparation is unacceptable.

Test for reversion in suckling mice

Both master seed and working seed should be tested for reversion to virulence in suckling mice.

Each of 10 animals of the Kunming strain of Swiss mice aged 3–5 days should be inoculated by the intracerebral route with 0.02 ml of the master seed or working seed at a titre of not less than 5.7 log_{10} PFU per ml. Mice showing clinical signs or dying within three days of inoculation are not included in the evaluation. All mice are expected to develop clinical signs of encephalitis over a period of 6–8 days. The first three animals showing clinical signs of encephalitis should be euthanized, their brains aseptically removed, and a pooled 10% brain homogenate prepared. Dilutions of 1:10, 1:100, 1:1000 and 1:10000 of the homogenate should be made, and 0.03 ml of each dilution should be inoculated intracerebrally into each of 4 Kunming strain Swiss mice.
aged 17–19 days. The mice are observed daily for 14 days. Mice that die within three days of injection are considered to have died from injection trauma, and are not included in the evaluation. If the end-point titre of the 10% brain homogenate is greater than 3 log_{10} LD_{50}, the virus is considered to have undergone unacceptable reversion on passage in the young mice, and the preparation is unacceptable for use.

**Test for neuroinvasiveness in weanling mice**

Master seed should be tested for neuroinvasiveness in weanling mice.

Ten mice of the Kunming strain of Swiss mice aged 17–19 days should be conditioned by injecting a sterile needle intracerebrally to locally destroy the blood–brain barrier; the mice are then inoculated subcutaneously (between the leg and the abdomen) with 0.1 ml of the master-virus seed. The mice are then observed for 14 days. If any mice show clinical signs of encephalitis with JEV (such as convulsions) during the observation period, the preparation is considered unsuitable. Omission of this test on a new virus master seed lot may be considered with the approval of the NRA.

**Test for neurovirulence in monkeys**

New virus master seed lots of SA14-14-2 should be tested for neurovirulence in monkeys. To avoid the unnecessary use of monkeys, virus master seed lots should be prepared in large quantities. A reference preparation should be included in each test.

An alternative test may be used, with the agreement of the NRA, if equal or greater sensitivity has been demonstrated. Testing of the working seed of SA14-14-2 in monkeys is not required.

A.3.2.5.6.2 Tests for neurovirulence of JE-CV

**Test in mice**

Both master seed and working seed should be tested for neurovirulence in mice.

Groups consisting of eight HSD:ICR (CD-1) outbred infant mice (eight days of age) should be inoculated by the intracerebral route with 0.02 ml of either the test article (2.0 × 10^2 PFU, 2.0 × 10^4 PFU and 2.0 × 10^4 PFU), a negative control, or YF-17D vaccine as a control. The animals are observed for 21 days for clinical signs, and euthanized if necessary. The numbers of dead or severely diseased animals and their survival times are recorded. The test material is acceptable if it is statistically less virulent than the YF-17D control. The assay is considered valid if 80% of the mice in the negative control group survive, and no more than two mice per litter die within the first 48 hours.
Test in monkeys

Because JE-CV vaccine is based on YF vaccine, both master seed and working seed should be tested in monkeys by following the WHO Recommendations for YF vaccine (17).

A.3.2.5.7 Test for retroviruses

Seeds should be examined for retroviruses using an assay for RTase that is acceptable to the NRA.

Highly sensitive PCR-based assays for RTase may be considered as tests for retroviruses, but the results need to be interpreted with caution because RTase activity is not unique to retroviruses and it may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (10). NAT assays for retroviruses may also be used.

A.4 Control of vaccine production

A.4.1 Control of production cell cultures

A fraction equivalent to at least 5% of the total volume of the cell suspension (or 500 ml, or 100,000,000 cells) should be used to prepare control cultures.

The control cells should be maintained under conditions similar to those of the infected cells in terms of time, temperature and media. The control cultures should be observed microscopically at regular intervals for cytopathic and morphological changes attributable to the presence of adventitious agents at a temperature of 35–37°C for at least 14 days after the day of inoculation of the production cultures, or until the time of virus harvest, whichever is later. At the end of the observation period, at least one fourth of the cell-culture flasks should be checked for haemadsorbing viruses as described in section A.4.1.1.

Samples that are not tested immediately should be stored at −60°C or below. If any tests in control cultures show evidence of any adventitious agents, the harvest of virus should not be used for vaccine production. For the test to be valid, 20% or fewer of the control culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test.

A.4.1.1 Test for haemadsorbing viruses

At the end of the observation period, 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea-pig red blood cells. If the red cells have been stored, the duration of storage should not have exceeded seven days, and the temperature of storage should have been in the range of 2–8°C.
In some countries, the NRA requires that the control cell cultures described above be tested for the presence of haemadsorbing viruses at the end of the production culture incubation period instead of 28 days of observations following subpassage. If this is the case, the test for haemadsorbing viruses described here may be omitted. In some countries, the NRA requires that other types of red cells, including cells from humans (blood group O), monkeys and chickens (or other avian species) be used in addition to guinea-pig cells. In all tests, readings should be taken after cells that have been incubated for 30 minutes at 2–8 °C, and after a further incubation for 30 minutes at 20–25 °C. For tests using monkey red blood cells, readings should also be taken after a final incubation for 30 minutes at 34–37 °C. For the tests to be valid, 20% or fewer of the control culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test.

A.4.1.2 Tests for adventitious agents in control cell-culture fluids

At the time of harvest, a sample of 10 ml of the pooled fluid from each group of control cultures should be tested in the same type of cell culture, but not the same batch, as that used for virus production. The test should also be performed in both human and cercopithecid cell cultures.

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

The cultures should be incubated at 35–37 °C, and should be observed for at least 14 days. During this observation period, the cultures should be examined at intervals for cytopathic changes.

The tests are satisfactory if no cytopathic changes attributable to adventitious agents are detected in the test sample. For the tests to be valid, 20% or fewer of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test.

A.4.1.3 Identity of cells

Depending on the type of cells used at the production level, the cells – especially those propagated from the working cell bank – should be identified by means of tests approved by the NRA.

Suitable methods include but are not limited to biochemical tests (e.g. isoenzyme analyses), immunological tests (e.g. major histocompatibility complex assays), cytogenetic tests (e.g. for chromosomal markers) or tests for genetic markers (e.g. DNA fingerprinting).
A.4.2 Production and control of a single virus harvest

A.4.2.1 Cells used for virus inoculation

On the day of inoculation with the working seed virus, each production cell culture flask (or bottle) or control cell culture flask should be examined for cytopathic effects potentially caused by infectious agents. If the examination shows evidence of an adventitious agent, all cell cultures should be discarded.

If animal serum is used in the growth medium, the medium should be removed from the cell culture either before or after inoculation with the virus working seed. The cell cultures should be rinsed, and the growth medium replaced with serum-free maintenance medium.

Penicillin and other beta-lactam antibiotics should not be used during any stage of manufacturing.

Minimal concentrations of other suitable antibiotics may be used if approved by the NRA.

A.4.2.2 Virus inoculation and incubation

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

The multiplicity of infection, temperature range and duration of incubation depends on the vaccine strain and the production method, and specifications should be defined by each manufacturer.

A.4.2.3 Harvest of vaccine virus

Vaccine virus fluid should be harvested when the cytopathic effect becomes obvious. Vaccine virus is harvested within a defined period after inoculation or for a defined level of cytopathic effect (CPE).

Samples of single virus harvests should be taken for testing. If they are not processed immediately, they should be stored at −60°C or below. The manufacturer should submit data to support the conditions chosen for these procedures.

Harvests derived from cultures of continuous cell lines should be subjected either to further purification to minimize the amount of cellular DNA or treatment with DNase to reduce the size of the DNA, or both.

Alternatively, this step may be performed on a virus pool.

If the harvests are not processed immediately, they should be stored at a temperature agreed with the NRA.
A4.2.4 Tests on a single virus harvest
A4.2.4.1 Tests for bacteria, fungi, mycoplasmas and mycobacteria

A sample of each single-harvest or virus-culture supernatant should be tested for bacterial, fungal and mycoplasmal sterility as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances no. 6 (1973), and section 5.3 of the General requirements for the sterility of biological substances no. 6 (amended 1995) (13, 14). If contamination is detected, the harvest should be discarded.

NAT-based assays used alone or in combination with cell culture and an appropriate detection method, may be used as alternatives to one or both of the compendial mycoplasma detection methods if they have been validated and the NRA agrees.

The harvest should be shown to be free from mycobacteria by a method approved by the NRA.

NAT-based assays may be used as an alternative to microbiological methods for culturing mycobacteria or to the in vivo guinea-pig test for detection if they have been validated and the NRA agrees (8).

A4.2.4.2 Virus titration for infectivity

In the case of pooling of viral harvests, the virus content of each single harvest should be tested with an infectivity assay that is acceptable to the NRA to determine the acceptability of the material for further processing, and to confirm the consistency of production. For information on selecting and validating a test method see section A3.2.5.2.

A4.2.4.3 Test for identity

A test for identity should be performed if this has not been done on the virus pool or on the bulk material.

A4.2.4.4 Test for adventitious agents

If the single harvests are not pooled on the same day, a test for adventitious agents should be performed on each single harvest.

A4.3 Preparation and control of virus pool or bulk material

A4.3.1 Preparation of virus pool or bulk material

Only virus harvests meeting the recommendations for sterility and virus content should be pooled.

The vaccine virus pool should be clarified or filtered by a method that maximizes the removal of cells and cell debris. Samples of the clarified bulk
suspension should be taken immediately after clarification in order to ensure that no microscopically observable cells or cell particles remain. Samples should also be taken to confirm the identity and determine the content of infectious virus in the pool. If not tested immediately for virus content, the samples should be stored below −60 °C until testing is done.

A.4.3.2 Tests on virus pool or bulk material
A.4.3.2.1 Virus titration for infectivity
The virus content of the pool should be assayed by titrating in cell culture against a reference preparation of live JE vaccine as described in section A.3.2.5.2 of these Recommendations, and should be approved by the NRA.

A.4.3.2.2 Test for identity
A test for identity should be performed if it was not carried out on the single harvest. However, it is not necessary to perform the genetic identity test on the virus pool.

A.4.3.2.3 Tests for bacteria and fungi
After clarification, the virus pool should be tested for bacterial and fungal sterility in accordance with Annex 4, Part A, section 5.2 of the WHO General requirements for the sterility of biological substances (13), and associated amendments (14), or by a method approved by the NRA.

A.4.3.2.4 Test for adventitious agents
A test for adventitious agents should be performed if it was not carried out on the single virus harvests (see section A.3.2.5.5).

A.4.3.2.5 Test for residual materials
Each manufacturer should demonstrate, by testing each virus pool or by validating the manufacturing process, that any residual materials used in manufacturing – such as animal serum, antibiotics, residual cellular DNA and DNase – are consistently reduced to a level acceptable to the NRA.

The host-cell protein profile should be examined as part of characterization studies (8).

For viruses grown in continuous cell-line cells, purified bulk material should be tested for the amount of residual cellular DNA, and the total amount of cell DNA per dose of vaccine should be not more than the upper limit agreed by the NRA. If this is technically feasible, the size distribution of the DNA should be examined as a characterization test, taking into account the amount of DNA detectable using state of the art methods approved by the NRA.
A.4.3.2.6 Test for retroviruses for vaccine prepared on primary cells

Samples from the filtered virus pool should be examined for the presence of retroviruses using an RTase assay that is acceptable to the NRA. Confirmation that the assays used will detect retroviruses potentially present in PHK cells should be presented.

Highly sensitive PCR-based assays for RTase may be considered as tests for retroviruses, but the results need to be interpreted with caution because RTase activity is not unique to retroviruses and it may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (10). NAT assays for retroviruses may also be used.

A.4.3.2.7 Test for consistency of virus characteristics

The virus pool or bulk material should be tested to compare it with virus working seed or a suitable comparator to ensure that the vaccine virus has not undergone critical changes during multiplication in the production culture system.

Relevant assays should be identified in nonclinical studies and may include, for example, virus yield in tissue culture, plaque phenotype or temperature sensitivity.

Other identifying characteristics may also be applicable, such as consensus nucleotide sequencing, to ensure the integrity of attenuating mutations.

The test may be omitted as a routine test once the consistency of the production process has been demonstrated on a significant number of batches, and if the NRA agrees. When there is a significant change in the manufacturing process, the test should be reintroduced.

A.4.4 Preparation and control of final bulk
A.4.4.1 Preparation of final bulk
A.4.4.1.1 Pooling of virus pool or bulk material

More than one virus pool or bulk material satisfying the control tests of these Recommendations may be pooled and diluted to form the final bulk.

A.4.4.1.2 Added substances

In the preparation of the final bulk, only substances approved by the NRA may be added, such as diluents or stabilizers. The concentration of the substances should be approved by the NRA. The substances should have been shown by appropriate tests not to impair the safety or effectiveness of the vaccine.
A.4.4.2 Tests on final bulk
A.4.4.2.1 Virus titration for infectivity
If the live-virus content of the final bulk was pooled, it should be assayed by
titration in cell culture of live JE vaccine as described in section A.3.2.5.2 of these
Recommendations, and should be approved by the NRA.

A.4.4.2.2 Tests for bacteria and fungi
Each final bulk should be tested for bacterial and fungal sterility in accordance
with Annex 4, Part A, section 5.2 of the WHO General requirements for the
sterility of biological substances (13), and associated amendments (14), or by a
method approved by the NRA.

A.4.4.2.3 Test for neurovirulence in mice
Each final bulk should be tested for neurovirulence in mice. This test should be
validated if it has not been tested previously with final bulk.

The test may be performed on the final product or the bulk material,
whichever is acceptable to the NRA, and as described in sections
A.3.2.5.6.1 and A.3.2.5.6.2.

The test for neuroinvasiveness is not required for single harvests, the pooled
harvest or the final product.

A.4.4.3 Storage
Prior to filling, the final bulk suspension should be stored under conditions
shown by the manufacturer to allow the final bulk to retain the desired viral
potency.

A.5 Filling and containers
The requirements concerning good manufacturing practices for biological
products appropriate to a vaccine apply (7).

Care should be taken to ensure that the materials from which the container and, if applicable, the closure are made do not adversely affect
the infectivity (potency) of the vaccine under the recommended conditions
of storage.

A final filtration stage may be included during the filling operations to
assure sterility.

The manufacturer should provide the NRA with adequate data to prove
that the product is stable under appropriate conditions of storage and shipping.
A.6  Control of final lot

A.6.1  Inspection of final containers

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

The appearance of the freeze-dried or liquid vaccine should be described with respect to form and colour. In the case of freeze-dried vaccines, a visual inspection should be performed of the vaccine, the diluent and a sample of the reconstituted vaccine.

A.6.2  Tests on the final lot

A.6.2.1  Identity test

An identity test, as described in section A.3.2.5.1, should be performed on at least one final, labelled container from each filling lot after the vaccine has been reconstituted according to the manufacturer's instructions for preparing the vaccine for human administration. However, it is not necessary to perform the genetic identity test on the final lot.

A.6.2.2  Potency test

The potency (virus content) in each of at least three containers randomly selected from the final lot should be determined individually. The virus content should be determined after the freeze-dried product has been reconstituted with the approved diluent. Limits for determining the accuracy and precision of virus titration should be agreed with the NRA. Since no international reference materials have been established for live JE vaccine, no recommendations for potency based on such materials can be formulated. The NRA should provide or approve a reference preparation of live JE vaccine for use in tests to determine virus concentration. The NRA should specify the minimum amount of vaccine virus that one human dose should contain. Consideration should be given to establishing the upper limit of the lot-release specification (see section C.2.3).

A.6.2.3  Thermal stability test

The purpose of the thermal stability test is to demonstrate the consistency of production. Additional guidance on the evaluation of vaccine stability is provided in the Guidelines on stability evaluation of vaccines (18). At least three containers of the final vaccine lot should be incubated at the appropriate elevated temperature for the appropriate time (e.g. 37 °C for seven days). The geometric mean titre (GMT) of infectious virus in the containers should not have decreased during the period of exposure by more than a specified amount (e.g. 1 log10) that has been justified by the production data and approved by the NRA. Titration of non-exposed and exposed containers should be carried out in parallel. A reagent for intra-assay validity control should be included in each assay.
A.6.2.4 Sterility test for bacteria and fungi
Each final lot should be tested for bacterial and fungal sterility as specified in Part A, section 5.2, of the General requirements for the sterility of biological substances no. 6 (1973) (13).

A.6.2.5 General safety tests
Each final lot should be tested for unexpected toxicity (i.e. abnormal toxicity) using a general safety test approved by the NRA.

This test may be omitted for routine lot release once the consistency of production has been established to the satisfaction of the NRA and when good manufacturing practices are in place.

Each lot, if tested, should pass a general safety test.

A.6.2.6 Test for pH values
The pH of the final lot should be tested in a pool of final containers. A pH value approved by the NRA should be maintained when freeze-dried vaccine is dissolved using the approved diluent, and this value should be within the range of values found in vaccine lots shown to be clinically safe and effective.

A.6.2.7 Test for residual moisture
The residual moisture in a representative sample of each freeze-dried lot should be determined by a method approved by the NRA. The upper limit for moisture content should be approved by the NRA using results from stability testing.

Moisture levels of 3% or lower are generally considered acceptable.

A.6.2.8 Test for residual animal serum protein
If applicable – i.e. when animal serum has been used during production – a sample of the final lot should be tested to verify that the level of serum albumin in the final reconstituted vaccine is less than 50 ng per human dose.

Alternatively, this test may be performed on the clarified virus pool or on the final bulk.

A.6.2.9 Test for residual antibiotics
If any antibiotics were added during production, the content of the residual antibiotics should be determined, and this should be within the limits approved by the NRA.

Alternatively, this test may be performed on the clarified virus pool or on the final bulk.
A.6.2.10  Test for endotoxins

In some countries, determination of endotoxin content may be required, and specifications will be approved by the NRA.

A.6.3  Control of diluents

The recommendations given in WHO good manufacturing practices for pharmaceutical products: main principles (19) should apply to the manufacturing and control of the diluents used to reconstitute live-attenuated JE vaccines. An expiry date should be established for the diluent using the stability data. For lot release of the diluent, tests should be carried out to assess the appearance, identity (if applicable), volume, sterility and content of key components.

A.7  Records

The recommendations in Good manufacturing practices for biological products (7) apply, as appropriate to the level of development of the candidate vaccine.

A.8  Retained samples

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

A.9  Labelling

The recommendations provided in section 7 of Good manufacturing practices for biological products (7) that are appropriate to a candidate vaccine apply.

In addition, the label on the carton enclosing one or more final containers, or the leaflet accompanying the container, should include:

- a statement that the candidate vaccine complies with Part A of these WHO Recommendations;
- a statement on the nature of the preparation, specifying the designation of the strain of JE or recombinant virus contained in the live-attenuated JE vaccine;
- the minimum number of infective units per human dose, the nature of any cellular systems used for the production of the vaccine, and whether the vaccine strains were derived by molecular methods;
- a statement of the nature and quantity, or upper limit, of any antibiotics present in the vaccine;
- a statement that contact with disinfectants should be avoided;
• a statement concerning the photosensitivity of the vaccine, based on photostability data;
• a statement indicating the volume and nature of diluent to be added to reconstitute the vaccine, and specifying that the diluent to be used is that supplied by the manufacturer;
• a statement advising that after the vaccine has been reconstituted, it should be used without delay or if not used immediately, it should be stored at 2–8 °C and protected from light for the maximum period defined by the stability studies.

A.10 Distribution and transport

The recommendations given in Good manufacturing practices for biological products (7) that are appropriate to a candidate vaccine apply. Shipments should be maintained within specified temperature ranges, and packages should contain cold-chain monitors (20).

A.11 Stability, storage and expiry date

The recommendations given in Good manufacturing practices for biological products (7) and the Guidelines on stability evaluation of vaccines (18) that are appropriate to a candidate vaccine apply. The statements concerning storage temperature and expiry date that appear on the primary or secondary packaging should be based on experimental evidence, and should be submitted to the NRA for approval.

A.11.1 Stability testing

Stability testing should be performed at different stages of production, namely on stored intermediates (including single harvests, purified bulk and final bulk) and the final lot. Stability-indicating parameters should be defined or selected according to the stage of production. It is advisable to assign a shelf-life to all in-process materials during vaccine production, particularly to stored intermediates such as single harvests, purified bulk and final bulk.

The stability of the vaccine in its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRA on at least three lots of final product. Accelerated thermal-stability studies may be undertaken on each final lot to provide additional information on the overall stability of a vaccine (see section A.6.2.3).

The vaccine should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with the NRA.
A.11.2 Storage conditions
Before being distributed by the manufacturer or before being issued from a storage site, the vaccine should be stored at a temperature shown by the manufacturer to be compatible with a minimal loss of titre. The maximum duration of storage should be fixed and approved by the NRA, and should ensure that all quality specifications for the final product, including the minimum titre specified on the label of the container (or package), will be maintained until the end of the shelf-life.

A.11.3 Expiry date
The expiry date should be approved by the NRA and should be based on the shelf-life, as also approved by the NRA. If the vaccine is stored at a temperature lower than that used for stability studies and is intended to be released without re-assay, the expiry date should be calculated from the date of removal from cold storage. The expiry dates for the vaccine and the diluent may be different unless they are in the same package.

A.11.4 Expiry of reconstituted vaccine
For single-dose containers, the reconstituted vaccine should be used immediately. For multidose containers, the container should be kept in the dark at 2–8 °C unless photostability studies have shown that this not required; the expiry time for the use of an opened container should be defined by stability studies and approved by the NRA but it should not exceed 6 hours.

Part B. Nonclinical evaluation of Japanese encephalitis vaccines (live, attenuated) for human use

B.1 General principles
The nonclinical evaluation of new JE vaccines should be based on the WHO guidelines on nonclinical evaluation of vaccines (21). Testing should confirm the safety and efficacy of the candidate vaccine. Tests should include product characterization at each stage of manufacturing (including quantification of contaminants, such as cellular proteins and DNA), proof of concept immunogenicity studies (including dose-ranging studies in animals), toxicity (if required by the NRA), a test for vaccine potency to ensure efficacy throughout the anticipated shelf-life, and safety testing in animals. The following specific issues should be considered in the context of developing new live-attenuated JE vaccines. The nonclinical studies conducted during the development of the vaccine should be discussed with the NRA prior to and during the process of licensure.
8.2 Product characterization and process development

It is vital that vaccine-production processes are standardized and controlled to ensure consistency in manufacturing, and that the safety and potential efficacy of a vaccine to be used in humans are indicated by nonclinical data. Such standardization and control are prerequisites for entering the clinical-trial phase.

New, live JE vaccine formulations should be characterized to define, as far as is practical, the critical genetic and phenotypic markers of attenuation that indicate that the vaccine virus genome is stable following tissue culture passage. Each vaccine virus should be evaluated using in vitro and in vivo approaches to determine whether the genetic basis of attenuation is stable enough to demonstrate a lack of risk of reversion to virulence during either manufacturing or replication in a vaccinee. To this end, laboratory and animal studies should define genetic changes that have occurred in the virus genome during attenuation. Phenotypic markers may be useful for detecting reversion events and differentiating vaccine strains from wild-type virus strains during epidemiological surveillance following human immunization.

Qualification of a vaccine-seed strain should include obtaining the consensus nucleotide sequence of the entire genome of the candidate vaccine by using the consensus nucleotide sequence of the genome of the parent virus as a comparator. This is essential for documenting mutations in the phenotype of the vaccine virus genome that may correlate with the attenuated phenotype. It is also good practice to document any in vitro studies that might serve as indicators of the stability of the mutations that differentiate the vaccine virus from its virulent parent. Such markers include but are not limited to plaque size, replication efficiency in mosquito vectors, induction of viraemia in nonhuman primates, neurovirulence, neuroinvasion, virulence in any other animal model, and temperature sensitivity. Identifying changes in virus phenotype markers may facilitate the detection of minor or quasispecies genomes present in the master-virus seed that have emerged during vaccine production, and which are different and may have a wild-type virus phenotype. Developers should bear in mind that consensus genome sequencing is unsuitable for identifying minor or quasispecies genomes in a vaccine seed or batch (22).

The investigative use of next-generation sequencing or microarray technology to establish polymorphism is encouraged. These methods must be validated if they are to be used for regulatory purposes.

8.3 Nonclinical immunogenicity and protection

Assessing the innate and adaptive immune responses to the JE vaccine in animals provides evidence that the virus has replicated in the host, and has stimulated the production of antibodies and a virus-specific T cell immune response. Animals, especially mice and nonhuman primates, have been appropriate
hosts in which to assess the various elements of the immune response to live-attenuated JE vaccines. Specific correlates of protection have been interpreted from clinical studies conducted with inactivated JE vaccines (23–25). It is generally accepted that individuals with a serum neutralizing antibody titre of at least 1:10 are protected from JEV disease. Immunization of 21-day-old mice and nonhuman primates with live-attenuated JE vaccines stimulates neutralizing antibodies that protect against virulent virus challenge (26–33). In neutralization assays and passive protection studies, antibodies stimulated by JEV genotype III SA14-14-2 virus or JE-CV show protection against JEV of genotypes I, II, III and IV (31). Immunization of nonhuman primates with the live-attenuated JE vaccines stimulates high titre neutralizing antibodies that are protective against intracerebral virulent JEV challenge (32, 33). The ability of vaccine to stimulate virus-specific CD4+ and CD8+ T cells, as well as levels of proinflammatory cytokines, and an increase in immunomodulatory interleukin 4 and interleukin 5 cytokines that may enhance the survival of the animals, may be considered. Prior to the initiation of clinical studies with new, live-attenuated JE vaccines, immunogenicity should be determined at least in nonhuman primates and a second species, as should protection from JEV disease arising from active virus challenge (26, 27, 32, 33). It is recommended that antibodies stimulated by new vaccines in animal models should be tested for neutralization of JEV isolates representing all genotypes of the virus (34–38).

8.4 Nonclinical toxicity and safety

8.4.1 Toxicity and safety testing

General guidance on the nonclinical assessment of toxicity and the design of nonclinical studies that apply to vaccines is provided in the WHO guidelines on nonclinical evaluation of vaccines (21). The term toxicity is generally associated with the untoward consequences of the administration of a nonreplicating medicine or biological that is directly related to the dose-dependent effect in the test animal. Thus, toxicity studies entail the careful analysis of all major organs, as well as tissues proximal to and distal from the site of administration, in order to detect any unanticipated, direct toxic effects over a range of doses of a biological that is replicating; these studies should include doses that sufficiently exceed the intended clinically relevant dose or amount. It is generally expected that if a live, attenuated vaccine does not replicate in the test animal, then direct toxic effects are unlikely to be detected. For live-attenuated vaccines the emphasis is on the demonstration of nonclinical safety as a consequence of vaccine virus replication in an animal that is susceptible to infection with the vaccine virus.

Single-dose toxicity or repeat-dose toxicity, an assessment of viraemia and vaccine virus excretion, the tissue distribution of the vaccine virus, and
local tolerance may be considered on a case by case basis, according to WHO guidelines (21).

Genotoxicity and carcinogenicity studies may not be necessary. If the live JEV vaccine is intended to be used to immunize women of childbearing age, studies of developmental and reproductive toxicity should be performed, according to WHO guidelines (21).

Nonclinical safety studies of live vaccines are required for live-attenuated vaccines during the early stages of development and testing. These studies are designed for the primary purpose of demonstrating that the vaccines are less virulent in animal models than comparable wild-type viruses, and that the vaccine does not exhibit any unexpected harmful tissue tropism and damage, and does not have the capacity to elicit a harmful immune response. For instance, the viraemia and tissue-distribution profile may be used as a marker for tropism; viraemia and invasion of the central nervous system may be used as correlates for neurovirulence. Nonhuman primates and mice are good animal models for evaluating the neurovirulence of JEV and YF viruses, respectively (29–32). To support clinical trials, the design of nonclinical safety studies should reflect the proposed route and frequency of vaccine administration (21).

8.4.2 Neurovirulence in mice and monkeys

JEV infection has been studied in many different mouse models (39–41). When appropriate, a mouse model may be selected to evaluate the attenuation of a candidate vaccine virus relative to the parent wild-type strain. In mouse experiments, the titre of virus in the blood, brain and other tissue at various times after infection may be evaluated to determine the pathogenesis of the viral encephalitis.

If viral replication is not detected in the mouse, tests in monkeys should be considered.

The tests for attenuation described in Part A, section A.3.2.5.6, may be applicable. These include: (i) neurovirulence tests in mice and nonhuman primates; (ii) reversion to neurovirulence in susceptible mice with either the vaccine seed beyond the level of passages in production or the seed passaged in the brains of suckling mice or vaccine viruses recovered from viraemic patients in clinical studies; and (iii) tests for neuroinvasiveness in mice.

The use of additional tests should be considered. A reference preparation should be prepared and included as a positive control to validate each test. The selection of one or more reference preparations is a matter of high priority, and should be made in consultation with experts in neurovirulence testing who should advise also on the development and implementation of a collaborative study to validate the ability of the test system to reliably distinguish suitable vaccine preparations from those that are unsuitable.
Modifications to these neurovirulence tests in mice may be used to evaluate a new virus master seed lot if approved by the NRA. Female ICR mice aged 28–32 days provide a neurovirulence test system for laboratory strains of SA14-14-2 virus, and may be further evaluated and considered as an alternative to Kunming Swiss mice (33). Outbred NIH mice have also been used as an alternative to Kunming Swiss mice.

B.4.3 New, live JE vaccines derived by recombinant DNA technology

The established model for vaccine neurovirulence is the nonhuman primate, which has historically been used to evaluate new seeds of YF vaccines (17D substrains 17D-204-derived or 17DD-derived) and live poliovirus vaccines. New, live JE vaccines derived by recombinant DNA technology or by serial passage in cell culture should be tested once for neurovirulence in nonhuman primates. If any vaccine-virus strain is determined to be neurovirulent in nonhuman primates on the basis of neurovirulence testing, neuroinvasiveness in nonhuman primates should also be evaluated via the clinical or peripheral inoculation route as part of the nonclinical safety study.

In the case of a recombinant JE vaccine that uses YF vaccines as viral vector, testing for neurovirulence in nonhuman primates via the intracerebral inoculation route should follow WHO recommendations for the neurovirulence testing of YF vaccines (40–42), as appropriate (see the brief description of the procedure below).

Groups of at least 10 monkeys that have been determined to be nonimmune to JEV, YF virus and other flaviviruses prior to inoculation with the JE vaccine master seed, should be inoculated intracerebrally into the frontal lobe. An active comparator group of 10 monkeys that also have been demonstrated to be nonimmune to JEV, YF virus and other flaviviruses should receive WHO yellow fever reference virus 168-73 or an appropriate YF-17D vaccine. All monkeys should be observed for 30 days for signs of encephalitis prior to necropsy. If the number of monkeys, the observation period or the time-points for necropsy for histopathological examination are different from these recommendations, they should be justified and agreed with the NRA. Clinical scores and scores of the histological lesions in the central nervous system should be recorded (43). Advanced methods of histopathological examination and automated image analysis (44) may be implemented to provide a quantitative assessment of virus-induced histopathology in the central nervous systems of nonhuman primates if the methods have been properly validated by and are acceptable to the NRA. The overall mean clinical and histological scores of the test group should not exceed the scores of the YF-17D vaccine control group. The method of statistical analysis and the significance level of the statistical difference between the test group and the control group should be agreed with the NRA.
8.4.4 Growth characteristics in vector mosquitoes

Flaviviruses exhibit a high degree of specificity in their ability to infect and be transmitted by arthropod vectors. Vector competence is under genetic control, with the susceptibility of the midgut epithelium being the primary determinant (45–48). Biological transmission of a flavivirus depends on the ingestion by the vector of a blood meal that contains virus that can infect the epithelial cells lining the midgut; this allows the virus to escape and disseminate into the haemocoel to infect the salivary glands, from where the virus is secreted into saliva during refeeding on a susceptible host.

JE-CV vaccine has been evaluated for its ability to replicate and to be transmitted by vector mosquitoes. In studies, Culex tritaeniorhynchus, Aedes albopictus and Aedes aegypti mosquitoes ingested a virus-laden blood meal or were inoculated intrathoracically. JE-CV did not replicate following oral feeding in any of the three mosquito species. In Cx. tritaeniorhynchus, replication was not detected after intrathoracic inoculation with the attenuated JE-CV vaccine (45, 47). None of three additional mosquitoes (Cx. annulirostris, Cx. gelidus and Ae. vigilax) became infected after being fed orally with 6.1 log_{10} PFU/ml of JE-CV vaccine (46). Viraemias in individuals immunized with the JE-CV vaccine were of short duration and of low titre; 64% of subjects receiving the vaccine developed detectable viraemia on at least one day after inoculation (49).

Studies on the replication of the attenuated SA-14-14-2 JE vaccine virus in Cx. tritaeniorhynchus mosquitoes indicate that the virus does not replicate in mosquitoes through oral feeding on virus-infected blood solutions, and replicates very poorly in mosquitoes inoculated by the intrathoracic route. The SA14-14-2 attenuated virus was not transmitted to suckling mice bitten by infected Cx. tritaeniorhynchus mosquitoes (41, 50–52).

Even though vector mosquitoes can be infected with JE vaccine virus, if the virus does not replicate effectively in the mosquitoes and does not spread to the salivary glands to facilitate transmission, it cannot infect vertebrate hosts. For these reasons, mosquitoes that are able to transmit wild-type JEV from infected birds or pigs to humans are unable to transmit the attenuated vaccine viruses to wildlife, domestic animals and humans. As a measure of attenuation and safety, all live-attenuated JE vaccines should be shown in a laboratory setting to replicate poorly in cells of the mosquito midgut, and should fail to disseminate to the mosquito salivary glands (41, 45–48, 50–56).

8.5 Characterization of vaccine virus in vector mosquitoes

For live JE vaccines, the primary environmental risks relate to their capacity to spread from human to human by vector mosquitoes, and to the potential for prolonged or repeated cycles of multiplication in the mosquito, facilitating reversion of the virus to virulence. It has been suggested that the currently
licensed live-attenuated JE vaccine viruses replicate poorly in human vaccinees, as has been demonstrated by viraemia studies. It has been suggested that they do not replicate in mosquitoes, so that the risk of transmission by mosquitoes is very low or non-existent \( (37, 45, 46) \). These factors markedly reduce the chance that JE vaccines will revert in mosquitoes to a virulent phenotype. In addition, genetic stability after passage in mosquitoes has been reported for both SA14-14-2 \( (41) \) and JE-CV \( (45, 46) \). Similar studies will need to be performed for future candidate vaccines.

Some investigators have raised a concern that live flavivirus vaccines could revert to virulence in mosquitoes via intragenic recombination with wild-type flaviviruses. Such a phenomenon would seem to be highly unlikely because of the factors noted above, and it is questionable whether flaviviruses are able to undergo recombination at all, even under ideal conditions in vitro \( (48) \).

Guidelines for assessing the environmental risk of live JE vaccines derived by recombinant DNA technology are described in Part D of these Recommendations.

**Part C. Clinical evaluation of Japanese encephalitis vaccines (live, attenuated) for human use**

**C.1 General considerations for clinical studies**

Clinical trials should adhere to the principles described in the Guidelines for good clinical practice (GCP) for trials on pharmaceutical products \( (57) \) and to the Guidelines on clinical evaluation of vaccines: regulatory expectations \( (58) \). All clinical trials should be approved by the relevant local ethics authorities and the NRA.

Some issues that are specific to the clinical evaluation of new, live attenuated JE vaccines are discussed in the following sections. These sections should be read in conjunction with the general guidance mentioned above. It is also recommended that manufacturers consult with the relevant NRA regarding their overall clinical development programme and their plans for assessing immune responses.

The availability and widespread deployment of effective vaccines in areas where JE is endemic makes it unethical to conduct protective efficacy studies (i.e. those that have as their end-point the prevention of clinically apparent illness) that compare a group given a new JE vaccine with an unvaccinated group. In addition, the use of available JE vaccines has reduced the incidence of clinically apparent infections to the extent that a study with sufficient power to estimate the relative protective efficacy of a new vaccine compared with a licensed JE vaccine would require such large sample sizes that it would not be feasible.
As a result, the evaluation of the likely protective efficacy of new, live JE vaccines should be based on evidence derived from active and passive protection in animal models (see Part B), and on the vaccine’s noninferiority to a licensed vaccine as assessed by using an immunological parameter that is a suitable correlate for clinical protection in humans.

C.2 **Assessment of immunogenicity in humans**

C.2.1 **Assessment of immune response**

It is recommended that the primary assessment of the immunogenicity of a new, live JE vaccine should be based on measurement of serum neutralizing antibody in pre-vaccination and post-vaccination serum samples. The plaque-reduction neutralization test (PRNT) is the most commonly used method for measuring neutralizing antibody. However, the PRNT is technically demanding, and test methods vary among laboratories, especially regarding the choice of cell substrate, incubation conditions, exogenous complement, the size of wells and the definition of end-points. Therefore, it is essential that the methods employed for determining PRNT titres in clinical studies are fully validated. It is also preferable to use a single laboratory to perform these assays throughout the duration of a clinical development programme. If this is not possible, cross-validation data should be exchanged between different laboratories.

Expressing neutralizing antibody titres in terms of the highest dilutions of serum that accomplish at least a 50% reduction in viral plaques after mixing with virus preparation (i.e. PRNT$_{50}$) is preferable to the use of a 90% reduction in plaques (i.e. PRNT$_{90}$). The PRNT$_{50}$ end-point has been claimed to provide better differentiation among antibody specific to JE as opposed to antibody directed against closely related co-circulating flaviviruses. However, when assessing immune responses to vaccination, the PRNT$_{50}$ is generally considered to provide more accurate results from the linear portion of the titration curve (59).

Initial studies should seek to establish whether vaccination elicits adequate immune responses to the vaccine strain (i.e. whether it is a homologous virus), and should evaluate antibody kinetics. Further studies should evaluate post-vaccination PRNT$_{50}$ titres against other (i.e. heterologous) strains of JEV in randomly chosen subsets of serum samples. Heterologous strains representing the five JEV genotypes should be tested using PRNTs under conditions agreed with the NRA.

Consideration may also be given to the assessment of vaccine-induced cell-mediated immunity. Studies in mice have shown that the adoptive transfer of T lymphocytes can confer passive protection against viral challenge. Also, peripheral blood mononuclear cells harvested from vaccinated persons can be stimulated to demonstrate CD4 or CD8 responses. However, uncertainties regarding the interpretation of assays of cell-mediated immunity mean that they would be considered secondary immunogenicity parameters.
C.2.2 End-points and analyses

The primary assessment of immune responses should be based on the proportions of previously seronegative subjects who reach a PRNT₉₀ titre against homologous virus of at least 1:10 after vaccination.

The primary population should be predefined in the protocol and should be selected in accordance with the study’s objectives. The population to be used in the primary analysis of immune responses should usually be confined to those subjects who are seronegative for JEV before vaccination (i.e. they have PRNT₉₀ titres < 1:10). Therefore, before commencement of a study in a particular geographical area, an estimate should be made of the likely percentage of subjects who will have pre-vaccination PRNT₉₀ titres ≥ 1:10. In some instances, it may be appropriate to actively exclude those with a history of prior vaccination against JE in order to reduce the likelihood that they will already be seropositive. Alternatively, or additionally, studies could include a screening visit so that a subject’s pre-vaccination serostatus can be determined before they are enrolled in the trial and the vaccine is administered.

For people who are seronegative before vaccination, the most appropriate primary parameter for assessing the immune response will be the proportion reaching PRNT₉₀ titres ≥ 1:10 after vaccination; this will equal the seroconversion rate. Other parameters to be examined should include increases in titres after sequential doses, GMTs and the reverse cumulative distributions of titres. Variability among subjects’ immune responses should also be reported.

In endemic areas it will be important to obtain data on the safety and immunogenicity of the new, live JE vaccine in subjects who are seropositive owing to previous administration of other JE vaccines or to naturally acquired infection with JEV, or both. This is critical because routine or emergency (i.e. outbreak control) vaccination programmes do not determine the serostatus of individuals before vaccinating them. Therefore, some studies should enrol and vaccinate subjects who are already seropositive for JEV. Analyses that include data from all vaccinated persons regardless of baseline serostatus, and that compare responses between previously seronegative and previously seropositive cohorts, should be planned. Depending on the study design and its objectives, immune responses may also be compared among subjects of various ages or with certain demographic characteristics, or some combination of these.

In people who are seropositive at baseline (i.e. who have PRNT₉₀ titres ≥ 1:10), the primary assessment of immune responses to vaccination would usually be based on the proportions that achieve substantial increases (e.g. at least a four-fold rise) in titre after one or more doses of JE vaccine.

After completion of what is considered to constitute a primary course of vaccination, it is essential that studies of vaccinees are conducted to determine the persistence of antibodies specific to JEV. Protocols should
include appropriate long-term serological follow-up in a significant number of subjects in each cohort. It would generally be expected that subjects would be followed for a minimum of two years, and ideally for up to five years, after completion of the primary series. In endemic areas, antibody persistence may reflect past vaccination as well as natural boosting due to exposure to JEV or other flaviviruses, or both. Therefore, data on antibody persistence should not be extrapolated to non-endemic areas or to other endemic areas with much lower or higher risks of exposure to flaviviruses.

Data on antibody persistence should be used to guide the need for and response to booster doses. However, it may also be useful to plan in advance to administer a booster dose to selected cohorts at specified times after the primary series. The timing of the booster doses may be based on currently approved vaccines. It is important to assess antibody responses before and after the booster dose and to follow up after the booster since doing so will provide evidence of past priming with the new JE vaccine.

C.2.3 Dose and schedule

It is essential that sufficient immunogenicity data are generated to support the use of the dose of the chosen vaccine antigen, the number of doses and the dose intervals. However, it is recognized that there are limitations to the number of regimens that can realistically be explored. Therefore, it is essential to justify the choice of regimen by using preliminary data from animal or human studies, and the potency of the vaccine available. As a minimum, an appropriate schedule should be identified for children in endemic areas, taking into account the recommended age at which vaccination to prevent JEV infection should commence.

Across the entire clinical programme, sufficient safety and immunogenicity data should be generated to support the range of viral titres expected to be administered so that the clinical data will help to provide evidence for setting upper and lower specifications for the vaccine virus titre at lot release and at the end of the shelf-life.

If the vaccine is proposed to be used in travellers from non-endemic areas, who are likely to be nonimmune, different primary vaccination schedules may need to be explored. For example, it may be appropriate to investigate the efficacy of an accelerated immunization schedule for people who have to travel at very short notice.

An assessment of the need and optimal timing for booster doses should be built into the overall clinical development plan. However, as with other vaccines, it may be possible to gain an initial marketing authorization without having specific data on antibody persistence and responses to booster doses; when sufficient data are available the prescribing information may need to be modified.
C.2.4 Comparative immunogenicity studies

The clinical development programme for a novel, live-attenuated JE vaccine should include at least one study in which the immune response of a candidate vaccine is compared with that of a licensed and widely used JE vaccine, which may be an inactivated JE vaccine. The comparisons should preferably be made in seronegative persons since such studies would be more sensitive and thus would be better able to detect differences between the vaccines.

In some instances it may be useful or necessary to perform studies to compare a new, live JE vaccine against more than one licensed product, depending on the regions where subjects are enrolled and on the JE vaccines available. If more than one comparative vaccine is used in the same study, the protocol should predetermine whether the primary analysis will compare the new vaccine with pooled comparative vaccines or with individual comparative vaccines. Each of these study designs raises some potentially complex statistical issues, and expert advice should be sought before finalizing the plans for the protocol and analysis.

The comparison of immune responses to the candidate and licensed vaccines should be assessed against their respective vaccine strains if the vaccine strains are different. In such a case, if a common virus strain is used to assess immune responses, the relevance and validity of its selection should be justified because antibody measurements vary widely depending on the virus strain used in neutralization assays, and strains closely related in phylogeny tend to produce higher neutralizing antibody measurements. Immune responses against virus strains that are heterologous to both vaccine strains or to circulating virus isolates of epidemiological significance should also be assessed, using a subset of serum samples from study subjects. The selection of the primary immune parameter should take into consideration the points made in section C.2.2. Whatever is chosen as the primary parameter, the margin of noninferiority will need careful justification; published guidance should be consulted, and expert statistical input should be obtained. In addition, protocols should plan for secondary analyses based on the examination of a full range of immune-response parameters.

Although provision of at least one comparative study during the process of vaccine licensure would be expected, it is recognized that in some countries there is no licensed JE vaccine and in others the comparative vaccine or vaccines that are chosen for study may not be licensed. In these countries the regulatory approach to the data from comparative studies may not be the same as in countries in which at least one of the selected comparative vaccines is licensed. As a result, regulators may place less emphasis on the demonstration of noninferiority and relatively more reliance on immune responses to the new vaccine (especially PRNT50 titres).
C.2.5 Concomitant vaccinations

As with all vaccines, a specific endorsement in the prescribing information for co-administration with another vaccine should be supported by clinical data (see the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations) (58).

Additional considerations will arise if it is proposed that a new, live JE vaccine could be co-administered with a vaccine against another flavivirus. YF vaccines are widely available and widely used, and vaccines against dengue are in development. Data on the co-administration of flavivirus vaccines could be especially useful in regions where JE co-circulates with either YF or dengue, and for vaccinating travellers during a single clinic visit. However, the effects of co-administering vaccines of closely related flaviviruses on safety and immunogenicity cannot be predicted. It is suggested that if co-administration studies are planned, they should be initiated with some degree of caution in seronegative adults (for instance, perhaps by delivering the injections a few days apart before proceeding to same-day co-administration).

C.3 Safety

The general approach to the assessment of safety of an experimental live JE vaccine during preapproval clinical studies should be in accordance with the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (58). The routine monitoring of safety during all prelicensure clinical studies should follow the usual principles, taking into account issues relevant to live-attenuated vaccines.

In the specific case of live-attenuated JE vaccines, adverse events of special interest (AESIs) primarily pertain to neurological disorders that have first onset following vaccination. These AESIs could include episodes of acute encephalitis that cannot be attributed to any other cause, as well as the first appearance of disorders that could represent sequelae of vaccination (e.g., movement disorders, seizures). It is recommended that subjects should be followed up for the occurrence of AESIs for at least six months after vaccination or for a duration that has been agreed between the manufacturer and the NRA. However, establishing whether vaccination had a role in an adverse event is difficult since there are many possible causative factors.

For a novel type of vaccine it is usually expected that a minimum of approximately 3000 subjects will be exposed during prelicensure studies, but this number provides only an indication of adverse events that occur at least uncommonly (i.e. in <1/100 but >1/1000 vaccinated persons). If no AESIs are observed in a dataset of this size it becomes more likely that if they do occur as a result of vaccination, the rate is <1/1000 vaccinated persons, but it cannot be ruled out that the actual rate may be higher.
Many more than 3000 vaccinated subjects would have to be evaluated for safety pre-approval to obtain a more precise estimate of the frequency of AESIs. In addition, rates of AESIs observed following administration of the live-attenuated JE vaccine would have to be compared with rates observed in a setting where no JE vaccines are being used (i.e. they would be compared with background rates) or in which inactivated JE vaccines considered to have an acceptable safety profile are already in use (i.e. the relative risk would be compared with a licensed vaccine).

Individual NRAs may have different opinions regarding the precision of the estimated rates of AESIs that they require before initially approving a live-attenuated JE vaccine. These considerations will direct the possible need to conduct prelicensure studies with predefined safety end-points as opposed to agreeing on a protocol for a post-approval safety study.

C.4  Post-licensure investigations
C.4.1  Effectiveness

Because it is not feasible to study the protective efficacy of a new, live JE vaccine, it is highly desirable that plans should be made to assess its effectiveness using disease surveillance after its introduction into a vaccination programme. However, the issues described below need to be considered.

- Unless a specific, live JE vaccine is to be the only such product used in a country or region, the overall effectiveness measured will not be product-specific but campaign-specific.
- The effectiveness of JE vaccines in a country or region may be heavily influenced by pre-existing immunity in the population, whether this occurred as the result of natural exposure or previous vaccination. Therefore it may not be possible to extrapolate findings from one area to another.
- It is not likely to be possible or appropriate for manufacturers to conduct studies to estimate vaccine effectiveness since coordinated national or regional public health networks and infrastructures are necessary to ensure that cases are reliably detected. However, in countries that have reliable disease surveillance systems, manufacturers should discuss with the NRA arrangements for continual disease surveillance and the potential for estimating effectiveness.
- Effectiveness data should be used in conjunction with data on antibody persistence to identify the need for and timing of booster doses.
Because JE vaccine may be administered during periods when the virus is actively transmitted, it may be challenging to differentiate cases of vaccine failure (i.e. illness caused by wild-type JE infection) from cases resulting from possible loss of attenuation of the vaccine virus. Every attempt should be made to isolate and fully characterize viruses from any suspected case of vaccine failure in order to differentiate wild-type from vaccine-derived viruses, and hence determine the etiology of the illness.

C.4.2 Post-licensure safety
The general considerations for safety surveillance and for development of a pharmacovigilance plan are the same as for all other types of vaccines (58).

See section C.3 for more information about preapproval and post-approval safety studies that aim at estimating the risk of vaccine-associated AESIs.

Part D. Environmental risk assessment of Japanese encephalitis vaccines (live, attenuated) for human use derived by recombinant DNA technology

D.1 Introduction

D.1.1 Scope
Some countries have legislation covering environmental and other issues related to the use of live vaccines derived by recombinant DNA technology because those countries consider that the vaccines use genetically modified organisms (GMOs).

This section of the Guidelines considers the environmental risk assessment (ERA) that may be performed during the development of a JE vaccine. An ERA assesses the risk to public health and the environment. It does not assess the risk to the intended recipient of the vaccine because that is assessed through clinical studies of the vaccine. Nor does it assess the risk to laboratory workers.

The NRA is not usually responsible for assessing the environmental impact of a vaccine. Nonetheless, the NRA should receive a copy of the ERA and of any associated decisions taken to ensure that the appropriate procedures have been followed.

D.1.2 Principles and objectives
Live JE vaccines in which the genome has been genetically modified by recombinant DNA technology may be considered GMOs. The manufacture, use and transboundary shipping of such live, recombinant vaccines, for research or commercial use, should comply with any relevant legislation or regulations regarding GMOs in the producing and recipient countries. In some regulatory
regimens, in order to comply with environmental regulations, an ERA should be undertaken if the live vaccine is being tested in a clinical trial or if it is placed on the market. It should be noted that this guidance on ERAs for live, recombinant JE vaccines is not intended to replace existing GMO legislation that countries already have in place.

As explained in detail in WHO Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated) (60), the objective of an ERA is to identify and evaluate, on a case by case basis, the potential adverse effects of a GMO on public health and the environment, regardless of whether these effects might be direct or indirect, immediate or delayed. This means that for each different live, recombinant JE vaccine, a specific ERA should be performed. The data needed for an ERA do not have to be derived solely from experiments performed by the applicant; data available in the scientific literature can also be used in the assessment. An ERA can be based on data from experiments performed previously for other purposes, such as product characterization tests, and nonclinical safety and toxicity studies. Regardless of the source, data should be both relevant and of an acceptable scientific quality.

D.1.3 Procedure for an environmental risk assessment

The principles and methods of an ERA should be applicable irrespective of the geographical location of the intended release of the GMO. An ERA should take into account the specificities associated with the mosquito vector, and whether virus-amplifying hosts are enzoonic or non-enzoonic in the region in which the vaccine trials will be carried out or where licensure is being requested. Depending on local regulatory requirements, an ERA may be undertaken by the applicant or by the appropriate local authority. In all cases, the appropriate local authority should use an ERA as a basis for deciding whether any of the identified environmental risks are acceptable. However, this decision may vary from country to country. Several national and international documents address issues associated with ERAs (61–63).

D.2 Example taken from live-recombinant Japanese encephalitis vaccines

ERAs for live JE vaccines should be conducted according to the general principles described above, particularly taking into consideration the vector responsible for disease transmission. Important issues to consider include the genetic stability of the live-recombinant virus, including reversion and recombination; the potential for transmission of the vaccine virus among hosts by the vector; and the immune status of the population. These issues are further outlined below.
D.2.1 Genetic stability

A live-attenuated recombinant JE vaccine based on strain SA14-14-2 has been licensed, as has a recombinant virus vaccine based on the YF-17D vaccine strain. In this live, recombinant vaccine, the prM/E structural genes of the JEV are cloned into the backbone of the YF-17D vaccine to replace the corresponding structural YF-17D genes (64) (see also Introduction, General considerations above).

D.2.1.1 Reversion

After vaccination, there is the potential for live-attenuated JE vaccine viruses to revert to a virulent form of JEV, although this has not been seen in clinical trials. The potential for reversion is based on the number, stability and nature of the attenuating mutation(s). An attenuating mutation that depends on a single base change may be more susceptible to reversion than a mutation that is stabilized by multiple base substitutions. In addition, attenuating mutations that have been derived by deletions of segments of RNA are generally more stable against reversion. Changes in virus genotype have the potential to influence disease transmission, the tropism of vector vaccine, virulence, or patterns of disease, or some combination of these, resulting in a virus with a previously unknown combination of properties. However, the likelihood of such a reversion depends on the number of attenuation mutations present and the genes involved in the vaccine virus (65).

The likelihood of reversions should be taken into account in an ERA.

Reversion to virulence in the case of JE-CV is unlikely because the attenuation of the YF-17D genome is multigenic, and the genome is known to be relatively stable since analyses of different vaccine lots over the years have revealed identical genome sequences (37, 66). In the case of JE-CV, it has been shown that at least three simultaneous reversions are necessary to increase virulence (39).

The attenuated SA14-14-2 strain of JE differs in 45 nucleotides from its virulent parent SA14 (67, 68). In the E protein of the SA14-14-2 strain are four conserved amino acid changes that are thought to be important to the attenuation of this strain (69). Although the likelihood of reversion is considered to be low, it should be taken into account in an ERA.

D.2.1.2 Recombination

Whether recombination takes place among flaviviruses is debatable. Theoretically, recombination between a live JE vaccine virus and a wild-type flavivirus could produce a virus with an altered phenotype, but there is no evidence to support this for flaviviruses (37, 55, 56, 70, 71).
The potential for recombination within and between flaviviruses has been widely discussed and challenged in the literature (37, 53–55, 70, 72, 73) and as a result of data obtained from specific experiments. In particular, a “recombination trap” has been designed to allow the products of rare recombination events to be selected and amplified in the case of West Nile virus, tickborne encephalitis and JEV (55). Intergenomic but aberrant recombination was observed only in the case of JEV, and not in the West Nile or tickborne encephalitis viruses. Moreover, its frequency appeared to be low, and it generated viruses with impaired growth properties (68). Similarly, no homologous recombination was seen using YF-17D replicons (56).

Nevertheless, while the likelihood of a recombined JEV is low, the potential adverse effects of these viruses should be evaluated in an ERA. In this respect, a worst-case scenario for chimeras has been constructed to address this risk (72, 73).

Different studies have shown that recombinants constructed artificially from a wild-type flavivirus and a recombinant vaccine (70), or from two wild-type viruses – including JEV, Kunjin and the highly virulent YF Asibi virus (37, 73) – were largely attenuated when compared with their parental viruses. The constructed viruses were nonpathogenic in cell culture, mosquito vectors and animal models, including monkeys. These data provide experimental evidence that the ability of these particular recombinant viruses to cause disease or to spread, should they ever emerge, would probably be low.

0.2.2 Vector transmission

Pigs and various wild birds represent the natural reservoir of JEV, which could be transmitted to new animal hosts and occasionally to humans by mosquitoes. The mosquito vectors play a key role in the transmission of flaviviruses and potentially the transmission of live JEV vaccines from a vaccinated subject to other individuals. JEV does not spread directly from person to person, except via blood transfusion in rare instances in which a donor was JEV viraemic. Therefore, transmission of the JEV vaccine virus in regions where the vector is absent is highly unlikely. JEV is present in almost all Asian countries. As a result of climate change, there is the possibility of a geographical shift in mosquito populations. Conceivably, this could lead to the spread of JEV to areas that are currently non-endemic.

Recombination between a live JEV vaccine virus and a wild-type flavivirus could theoretically occur in a vaccinee (see section D.2.1.2) and possibly also within an infected mosquito or natural reservoir of JEV. A recombined JEV could potentially – in combination with climate change – use new vectors for transmission, leading to previously unknown transmission characteristics. Therefore, the presence of a relevant mosquito vector and a climate that
favours JEV in the vaccination area should be taken into account in ERAs for live JE vaccines.

To assess the likelihood of effective transmission of the vaccine virus from a vaccinated individual, one has to take three parameters into consideration – namely, the level of viraemia in the vaccinated hosts; the ability of the mosquito vectors to transmit the live JE vaccine virus to new hosts; and, for transmission to be sustained, the capacity and presence of amplifying hosts to be fed upon, to be infected, and to sustain viraemia that is adequate to allow other feeding mosquitoes to become infected.

The ability of JE-CV to replicate and be transmitted to mosquitoes has been studied (37, 45, 68). Compared with wild-type JEV, the JE-CV virus cannot infect the oral route and cannot replicate in different mosquito vectors known to transmit members of Flaviviridae. The combination of the low-level replication of the vaccine virus, and the absence of virus replication and dissemination in the mosquito vector, make it unlikely that JE-CV would be transmitted. As a result, it is also unlikely that other amplifying hosts, such as pigs and birds, will become infected. It has been shown that pig infection with JE-CV does not result in any detectable viraemia (37, 68). Thus, it is highly unlikely that vaccinated subjects would ever spread the vaccine virus via mosquito transmission.

The outcome of an ERA for clinical trials in regions where the vector is absent will be that the environmental risk is negligible. The mosquito vector is not present and therefore the vaccine, or theoretical de novo recombinant viruses, cannot be transmitted to other people. However, in endemic areas, NRAs need to decide whether to perform an ERA.

D.2.3 Immune status

Live JE vaccines are able to replicate in vaccinated individuals. The immune status of a vaccinee in relation to the vaccine antigens, the viral vectors or the cross-reacting flaviviruses – or to a combination of these – may be confounding factors in assessing the environmental risk of a live JE vaccine. In general, the presence of pre-existing immunity resulting from earlier exposure to JEV will reduce the extent and duration of vaccine virus replication and dissemination within a vaccinee. The potential for transmission of the vaccine virus is therefore considered to be greater in naive or immunocompromised individuals. Results from clinical studies of individuals who were naive for YF virus might also inform a risk assessment (68). However, the potential for transmission into the environment would still be limited by the virus' lack of ability to replicate in mosquitoes (37, 45, 68).

An unvaccinated population with no pre-existing immunity will respond differently upon exposure to the vaccine when compared with a population in an area where JE is endemic. Moreover, it has been reported that in a case where a person is infected with dengue virus prior to JEV, high antibody titres
are present due to the cross-reactivity of JEV with other flaviviruses (74). The immune status of a population should therefore be taken into account in an ERA because it can influence the environmental impact of vaccines (75).

Part E. Recommendations for NRAs

E.1 General

The general recommendations for NRAs and national control laboratories given in the Guidelines for national authorities on quality assurance for biological products (76) and Guidelines for independent lot release of vaccines by regulatory authorities (77) apply.

The detailed procedures for the production and control of live-attenuated JEV vaccine and any significant changes in them that may affect the quality, safety and efficacy of the vaccine should be discussed with and approved by the NRA. The NRA may obtain the product-specific working reference from the manufacturer and use this for lot release until an international or national standard preparation has been established.

Consistency in production has been recognized as an essential component in the quality assurance of vaccines. In particular, NRAs should carefully monitor production records and results of quality control tests on clinical lots, as well as a series of consecutive lots of the final bulk.

E.2 Release and certification by the NRA

A vaccine should be released only if it fulfills all national requirements or satisfies Part A of these Recommendations, or both (77).

A protocol based on the model given in Appendix 4, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for release of the vaccine for use.

A certificate signed by the appropriate official of the NRA should be provided to the manufacturing establishment, and should certify that the lot of vaccine in question meets all national requirements as well as Part A of these Recommendations. The certificate should provide sufficient information about the product. A model certificate is given in Appendix 5. The official national release certificate should be provided to importers of the vaccines. The purpose of the certificate is to facilitate exchange of vaccines between countries.

Authors

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An improved version of the draft was prepared, taking into consideration proposed changes and comments provided by the working group, and was posted for further consultation on the WHO web site during May and June 2012, before being submitted for consideration by the WHO Expert Committee on Biological Standardization.

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References


Appendix 1

Passage history of Japanese encephalitis SA14-14-2 virus

SA14 virus isolated from pool of *Culex pipiens* larvae by
11 passages in mouse brain (SA14)

↓

100 serial passages in PHK cells, followed by three plaque
purifications in PCE cells (SA14 clone 12-1-7)

↓

Two plaque purifications in PCE cells (SA14 clone 17-4)

↓

One intraperitoneal passage in mice; harvesting of spleen
for plaque purification in PCE cells (SA14 clone 2)

↓

Three plaque purifications in PCE cells (SA14 clone 9)

↓

One passage in mice; harvesting of skin and subcutaneous
tissue for plaque purification in PCE cells (SA14 clone 9-7)

↓

Six oral passages in hamsters; harvesting of spleens for two
plaque purifications in PHK cells (SA14 clone 5-3)

↓

Five passages in suckling mice; harvesting of skin and
subcutaneous tissue for two plaque purifications in PHK cells
(SA14 clone 14-2)*

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PCE = primary chick embryo; PHK = primary hamster kidney.

* The notation SA14 clone 14-2 is abbreviated to SA14-14-2.
Appendix 2

Derivation of Japanese encephalitis–yellow fever chimeric virus (CV) vaccine

Appendix 3

Production and passage level of live-attenuated Japanese encephalitis vaccine

SA14-14-2 vaccine
Premaster seed (P 6)
Master seed (P 7)
Working seed (P 8)
Single harvest (P 9)
Virus pool
Final bulk
Final lot

Japanese encephalitis chimeric virus vaccine
Premaster seed (P 10)
Master seed (P 11)
Working seed (P 12)
Single harvest (P 13)
 Bulk material
Final bulk
Final lot
Appendix 4

Model protocol for the manufacturing and control of Japanese encephalitis vaccines (live, attenuated) for human use

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

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

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

1. Summary information on finished product (final lot)

International name: ____________________________
Trade name/commercial name: _______________________
Product licence (marketing authorization) number: ________________
Country: __________________________
Name and address of manufacturer: ______________________
Name and address of product licence holder, if different: ____________________

Virus strains: ___________________________________________
Origin and short history: ________________________________
Batch number(s): _______________________________________
Finished product (final lot): _____________________________
Final bulk: ___________________________________________
Type of container: ______________________________________
Number of filled containers in this final lot: _________________
Number of doses per container: ___________________________
Composition (antigen concentration)/volume of single human dose: __________________________

Target group: ________________________________________________________________

Expiry date: _________________________________________________________________

Storage conditions: ___________________________________________________________

2. Summary information on manufacture

Batch number of each monovalent bulk: __________________________________________

Site of manufacture of each monovalent bulk: ______________________________________

Date of manufacture of each monovalent bulk: ________________________________

Batch number of final bulk: _________________________________________________

Site of manufacture of final bulk: _____________________________________________

Date of manufacture of final bulk: _____________________________________________

Date of manufacture (filling or lyophilizing) of finished product (final vaccine lot):
                                                                                   __________________________

Date on which last determination of virus concentration was started: ________________

Shelf-life approved (months): _________________________________________________

Storage conditions: __________________________________________________________

Volume of single dose: _______________________________________________________

Prescribed virus concentration per human dose: ________________________________

A genealogy of the lot numbers of all vaccine components used in the formulation of the final product should be provided.

The following sections are intended for reporting the results of the tests performed during production of the vaccine.

3. Control of source materials

3.1 Cell cultures

Where primary hamster kidney cells are used

*Animals*

Information on how animal colonies were established and maintained should be provided at the time of licensing.

Information on the test programme to ensure freedom from specified pathogens and detectable antibodies to those pathogens described in Part A, section A.3.1.3.1, and test results should be provided at the time of licensing or on the establishment of new breeding colonies.

A summary table and details for hamster antibody production, mouse antibody production and rat antibody production tests and results should be provided at the time of licensing or on the establishment of new breeding colonies.
A regular health-monitoring programme should be provided, and test results should be updated.

**Primary hamster kidney cells**

Date of preparation: 
Methods of preparation: 

Gross examination of kidneys
Specification: 
Date: 
Result: 

Microscopic observation of prepared cells
Specification: 
Date: 
Result: 

Where continuous cell cultures are used

**General information on the cell banking system**

Information and results in relation to characterization tests on the cell banking system from cell seed – if applicable, the master cell bank, working cell bank, end of production cells, or the extended cell bank – should be provided according to WHO Recommendations for the evaluation of animal-cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks.

Name and identification of cell substrate: 
Origin and short history (attach a flowchart if necessary): 
Lot number and date of preparation of each bank: 
Date each bank was established: 
Date of approval by the NRA: 
Total number of ampoules stored for each bank: 
Passage level or population-doubling level of each bank: 
Maximum passage level or population-doubling level approved for each bank: 
Storage conditions: 
Date of approval of protocols indicating compliance with the requirements of the relevant monographs and with the marketing authorization: 
Characterization tests on cell seed (if applicable), the master cell bank, working cell bank, end of production cells, or extended cell banks

A summary table for characterization tests on each bank should be provided.

Characterization tests performed on each bank

Methods: 
Specification: 
Date of test: 
Results: 

Cell-culture medium

Serum used in cell-culture medium

Animal origin of serum: 
Batch number: 
Vendor: 
Country of origin: 
TSE-free certificate reference number: 
Tests performed on serum

Methods: 
Specification: 
Date of test: 
Results: 

Trypsin used for preparation of cell cultures

Animal origin of trypsin: 
Batch number: 
Vendor: 
Country of origin: 
TSE-free certificate reference number: 
Tests performed on trypsin

Methods: 
Specification: 
Date of test: 
Results: 

Antibiotics

Nature and concentration of antibiotics or selecting agent(s) used in the production cell culture's maintenance medium: 

Other source material

Identification and source of starting materials used in preparing production cells, including excipients and preservatives (particularly any materials of human or animal origin – e.g. albumin, serum):

__________________________________________________________________________

3.2 Virus seeds

Vaccine virus strain(s) and serotype(s):

__________________________________________________________________________

Substrate used for preparing seed lots:

__________________________________________________________________________

Origin and short history of virus seeds:

__________________________________________________________________________

Authority that approved virus strain(s):

__________________________________________________________________________

Date approved:

__________________________________________________________________________

Information on seed lot preparation

Virus master seed

Source of virus master seed lot:

__________________________________________________________________________

Virus master seed lot number:

__________________________________________________________________________

Name and address of manufacturer:

__________________________________________________________________________

Passage level:

__________________________________________________________________________

Date of inoculation:

__________________________________________________________________________

Date of harvest:

__________________________________________________________________________

Number of containers:

__________________________________________________________________________

Conditions of storage:

__________________________________________________________________________

Date of establishment:

__________________________________________________________________________

Maximum passage level approved for virus master seed:

__________________________________________________________________________

Date approved by the NRA:

__________________________________________________________________________

Virus working seed

Virus working seed lot number:

__________________________________________________________________________

Name and address of manufacturer:

__________________________________________________________________________

Passage level from virus master seed lot:

__________________________________________________________________________

Date of inoculation:

__________________________________________________________________________

Date of harvest:

__________________________________________________________________________

Number of containers:

__________________________________________________________________________

Conditions of storage:

__________________________________________________________________________

Date of establishment:

__________________________________________________________________________

Date approved by the NRA:

__________________________________________________________________________
Tests on virus seeds

Identity test
Method: ________________________________
Specification: ___________________________
Lot number of reference reagents: ________________
Dates of test (start, end): ______________________
Result: ________________________________

Virus titration for infectivity
Method: ________________________________
Specification: ___________________________
Dates of test (start, end): ______________________
Result: ________________________________

Genetic and/or phenotypic characterizations
Method: ________________________________
Reference reagents: _______________________
Specification: ___________________________
Dates of test (start, end): ______________________
Result: ________________________________

Tests for bacteria and fungi
Method: ________________________________
Specification: ___________________________
Media: ________________________________
Number of containers tested: ________________
Volume of inoculum per container: ________________
Volume of medium per container: ________________
Temperatures of incubation: ________________
Dates of test (start, end): ______________________
Result: ________________________________

Test for mycoplasmas
Method: ________________________________
Specification: ___________________________
Media: ________________________________
Volume tested: ___________________________
Temperature of incubation: ________________
Positive controls: _________________________
Dates of test (start, end): ______________________
Result: ________________________________
Test for mycobacteria

Method: ____________________________
Specification: _______________________
Media: ____________________________
Volume tested: ______________________
Temperature of incubation: __________
Dates of test (start, end): ____________
Result: _____________________________

Tests for adventitious agents

Volume of virus seed samples for neutralization and testing: __________
Batch number(s) of antisera or antiserum used for neutralization of virus seeds: ______________________

Tests in tissue cultures for adventitious agents

Test in human cells

Type of human cells: _______________________
Quantity of neutralized sample inoculated: __________
Incubation conditions: _______________________
Method: ______________________________
Specification: __________________________
Dates of test (start, end): _____________
Proportion of cultures viable at end of test: __________
Result: _______________________________

Test in simian cells

Type of simian cells: _______________________
Quantity of neutralized sample inoculated: __________
Incubation conditions: _______________________
Method: ______________________________
Specification: __________________________
Dates of test (start, end): _____________
Proportion of cultures viable at end of test: __________
Result: _______________________________

Other cell types (if appropriate)

Type of cells: __________________________
Quantity of neutralized sample inoculated: __________
Incubation conditions: ______________________
Method: 
Specification: 
Dates of test (start, end): 
Proportion of cultures viable at end of test: 
Result: 

Test in animals for adventitious agents
Method: 
Specification: 
Dates of test (start, end): 
Result: 

Tests by molecular methods for adventitious agents
Method: 
Specification: 
Dates of test (start, end): 
Result: 

Tests for retroviruses
Method: 
Specification: 
Dates of test (start, end): 
Result: 

Tests for attenuation
The items tested and a detailed protocol should be provided.

Tests in mice for neurovirulence, reversion and neuroinvasiveness, when appropriate
Method: 
Specification: 
Dates of test (start, end): 
Result: 

Tests in nonhuman primates for neurovirulence
Method: 
Specification: 
Dates of test (start, end): 
Result:
4. Control of vaccine production

4.1 Control of production cell cultures

Information on preparation

Lot number of master cell bank: ________________________________
Lot number of working cell bank: ______________________________
Date of thawing of ampoule of working cell bank: ________________
Passage number of production cells: __________________________
Date of preparation of control cell cultures: ____________________
Result of microscopic examination: ____________________________

Tests on control cell cultures

Number of control cultures, or ratio of control cultures to production cell cultures: ________________________________

Incubation conditions: ____________________________
Period of observation of cultures: ____________________________
Dates started and ended: ________________________________
Proportion of cultures discarded, and reason: ____________________
Results of observation: ________________________________
Date supernatant fluid collected: ____________________________

Test for haemadsorbing viruses

Quantity of cells tested: ________________________________
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Result: ________________________________

Test for adventitious agents on supernatant culture fluids

Test in simian cells

Type of simian cells: ________________________________
Quantity of pooled sample inoculated: ________________________________
Incubation conditions: ________________________________
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Proportion of cultures viable at end of test: ________________________________
Result: ________________________________

Test in human cells

Type of human cells: ________________________________
Quantity of pooled sample inoculated: ________________________________
Incubation conditions: __________________________________________________________
Method: _______________________________________________________________________
Specification: __________________________________________________________________
Dates of test (start, end): _______________________________________________________
Proportion of cultures viable at end of test: ________________________________________
Result: _______________________________________________________________________

Other cell types (if appropriate)
Type of cells: __________________________________________________________________
Quantity of pooled sample inoculated: ____________________________________________
Incubation conditions: _________________________________________________________
Method: _______________________________________________________________________
Specification: __________________________________________________________________
Dates of test (start, end): _______________________________________________________
Proportion of cultures viable at end of test: ________________________________________
Result: _______________________________________________________________________

Test for identity of cells, if appropriate
Method: _______________________________________________________________________
Specification: __________________________________________________________________
Dates of test (start, end): _______________________________________________________
Result: _______________________________________________________________________

Cells used for vaccine production
Observation of cells used for production (if appropriate)
Specification: __________________________________________________________________
Date: ________________________________________________________________________
Result: _______________________________________________________________________

4.2 Single harvests
Information on manufacture
Batch number(s): __________________________________________________________________
Date of inoculation: __________________________________________________________________
Date of harvesting: __________________________________________________________________
Lot number of virus master seed lot: ________________________________________________
Lot number of virus working seed lot: ______________________________________________
Passage level from virus working seed lot: __________________________________________
Methods and date of purification, if relevant: _________________________________________
Volume(s), storage temperature, storage time and duration
of approved storage period: ______________________________________________________

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Tests on single harvests

Test for identity
Method:
Specification:
Lot number of reference reagents:
Specification:
Date of test:
Result:

Virus titration for infectivity, if appropriate
Method:
Specification:
Dates of test (start, end):
Result:

Test for bacteria and fungi
Method:
Specification:
Media:
Number of containers tested:
Volume of inoculum per container:
Volume of medium per container:
Temperatures of incubation:
Dates of test (start, end):
Result:

Test for mycoplasmas
Method:
Specification:
Media:
Volume tested:
Temperature of incubation:
Positive controls:
Dates of test (start, end):
Result:

Test for mycobacteria
Method:
Specification:
Media:
Volume tested:
Temperature of incubation: ________________________________
Dates of test (start, end): ________________________________
Result: _______________________________________________

Test for adventitious agents

Test in simian cells
Type of simian cells: ________________________________
Quantity of neutralized sample inoculated: ________________________________
Incubation conditions: ________________________________
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Proportion of cultures viable at end of test: ________________________________
Result: _______________________________________________

Test in human cells
Type of human cells: ________________________________
Quantity of neutralized sample inoculated: ________________________________
Incubation conditions: ________________________________
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Proportion of cultures viable at end of test: ________________________________
Result: _______________________________________________

Other cell types (if appropriate)
Type of cells: ________________________________
Quantity of neutralized sample inoculated: ________________________________
Incubation conditions: ________________________________
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Proportion of cultures viable at end of test: ________________________________
Result: _______________________________________________

4.3 Virus pool or bulk material

Information on manufacture
Batch number(s): ________________________________
Date of preparation: ________________________________
Methods and date of purification, if relevant: ________________________________
Volume(s), storage temperature, storage time and duration of approved storage period: ________________________________
Tests on virus pool or bulk material

Test for identity, if appropriate
Method: 
Specification: 
Lot number of reference reagents: 
Specification: 
Dates of test (start, end): 
Result: 

Virus titration for infectivity
Method: 
Specification: 
Dates of test (start, end): 
Result: 

Test for bacteria and fungi
Method: 
Specification: 
Media: 
Number of containers tested: 
Volume of inoculum per container: 
Volume of medium per container: 
Temperatures of incubation: 
Dates of test (start, end): 
Result: 

Test for adventitious agents, if not performed on the single harvest
Test in simian cells
Type of simian cells: 
Quantity of neutralized sample inoculated: 
Incubation conditions: 
Method: 
Specification: 
Dates of test (start, end): 
Proportion of cultures viable at end of test: 
Result: 

Test in human cells
Type of human cells: 
Quantity of neutralized sample inoculated: 
Incubation conditions: 
Method: ____________________________
Specification: ____________________________
Dates of test (start, end): ____________________________
Proportion of cultures viable at end of test: ____________________________
Result: ____________________________

Other cell types (if appropriate)
Type of cells: ____________________________
Quantity of neutralized sample inoculated: ____________________________
Incubation conditions: ____________________________
Method: ____________________________
Specification: ____________________________
Dates of test (start, end): ____________________________
Proportion of cultures viable at end of test: ____________________________
Result: ____________________________

Test for host-cell proteins, if applicable
Please provide these results as part of the characterization studies submitted for the licensing application.
Method: ____________________________
Specification: ____________________________
Date of test: ____________________________
Result: ____________________________

Test for residual cellular DNA
Method: ____________________________
Specification: ____________________________
Dates of test (start, end): ____________________________
Result: ____________________________

Test for retroviruses, if applicable
Method: ____________________________
Specification: ____________________________
Dates of test (start, end): ____________________________
Result: ____________________________

Test for consistency of virus characteristics
Method: ____________________________
Specification: ____________________________
Dates of test (start, end): ____________________________
Result: ____________________________
4.4 Final bulk

Information on manufacture

Batch number(s): __________________________________________

Date of formulation: ________________________________________

Total volume of final bulk formulated: _________________________

Monovalent virus pools used for formulation (serotype, lot number, volume added, virus concentration): _________________

Name and concentration of added substances, if relevant (e.g. diluent, stabilizer): _________________________________

Volume(s), storage temperature, storage time and duration of approved storage period: ___________________________)

Tests on final-bulk lot

Virus titration for infectivity, if applicable

Method: ___________________________________________________

Specification: _____________________________________________

Dates of test (start, end): _________________________________

Result: __________________________________________________

Test for bacteria and fungi

Method: ___________________________________________________

Specification: _____________________________________________

Media: ___________________________________________________

Number of containers tested: _______________________________

Volume of inoculum per container: __________________________

Volume of medium per container: __________________________

Temperatures of incubation: _______________________________

Dates of test (start, end): _________________________________

Result: __________________________________________________

Test for neurovirulence in mice

Method: ___________________________________________________

Specification: _____________________________________________

Dates of test (start, end): _________________________________

Result: __________________________________________________

5. Filling and containers

Lot number: _____________________________________________

Date of filling: __________________________________________

Type of container: ________________________________________
Volume of final bulk filled: ____________________________________________
Filling volume per container: __________________________________________
Number of containers filled (gross): _____________________________________
Date of lyophilization: _________________________________________________
Number of containers rejected during inspection: ___________________________
Number of containers sampled: __________________________________________
Total number of containers (net): _________________________________________
Maximum approved period of storage: _________________________________
Storage temperature and period: _______________________________________

6. Control tests on final lot

6.1 Tests on final vaccine lot

Inspection of final containers

Appearance:
Specification: _______________________________________________________
Date of test: _________________________________________________________
Results: ____________________________________________________________
Before reconstitution: ________________________________________________
After reconstitution: _________________________________________________
Diluent used: _________________________________________________________
Lot number of diluent used: _____________________________________________

Test for identity

Method: _____________________________________________________________
Specification: _______________________________________________________
Dates of test (start, end): _____________________________________________
Result: ____________________________________________________________

Test for potency

Method: _____________________________________________________________
Batch number of reference vaccine and assigned potency: __________________
Specification: _______________________________________________________
Dates of test (start, end): _____________________________________________
Result: ____________________________________________________________

Thermal stability

Method: _____________________________________________________________
Specification: _______________________________________________________
Dates of test (start, end): _____________________________________________
Result for each serotype: _____________________________________________
Test for bacteria and fungi

Method: 
Specification: 
Media: 
Volume tested: 
Temperatures of incubation: 
Dates of test (start, end): 
Result: 

General safety (unless omission of test authorized)

Tests in mice

Date of inoculation: 
Number of animals tested: 
Volume and route of injection: 
Dates of observation period: 
Specification: 
Results (give details of deaths): 

Tests in guinea-pigs

Date of inoculation: 
Number of animals tested: 
Volume and route of injection: 
Dates of observation period: 
Specification: 
Results (give details of deaths): 

Test for pH

Method: 
Specification: 
Date of test: 
Result: 

Residual moisture, if applicable

Method: 
Specification: 
Dates of test (start, end): 
Result: 

Residual antibiotics, if applicable

Method: 
Specification: 
Dates of test (start, end): ______________________________________________________
Result: ______________________________________________________________________

Endotoxins
Method: ______________________________________________________________________
Specification: __________________________________________________________________
Dates of test (start, end): __________________________________________________________________
Result: ______________________________________________________________________

6.2 Diluent
Name and composition of diluent: ________________________________________________
Lot number: ___________________________________________________________________
Date of filling: __________________________________________________________________
Type of diluent container: __________________________________________________________________
Appearance: _____________________________________________________________________
Filling volume per container: __________________________________________________________________
Maximum approved period of storage: __________________________________________________________________
Storage temperature and period: __________________________________________________________________
Other specifications: ___________________________________________________________________

7. Certification by the manufacturer

Name of the manufacturer __________________________________________________________
Name of head of production (typed) ________________________________

Certification by the person from the control laboratory of the manufacturing company taking responsibility for the production and control of the vaccine

I certify that lot no. __________________ of Japanese encephalitis vaccine (live, attenuated) for human use, whose number appears on the label of the final container, meets all national requirements and/or satisfies Part A of the WHO Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use (2014).²

Signature __________________________________________
Name (typed) __________________________________________
Date ________________________________________________

¹ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
8. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 5), a label from a final container and an instruction leaflet for users.
Appendix 5

Model certificate for the release of Japanese encephalitis vaccines (live, attenuated) for human use by NRAs

Lot-release certificate

Certificate no. ____________________

The following lot(s) of Japanese encephalitis vaccine (live, attenuated) for human use produced by ____________________ in ____________________, whose numbers appear on the labels of the final containers, complies with the relevant national specifications and provisions for the release of biological products and Part A of the WHO Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use (2014), and complies with WHO good manufacturing practices: main principles for pharmaceutical products; Good manufacturing practices for biological products; and Guidelines for independent lot release of vaccines by regulatory authorities.

The release decision is based on ____________________

The certificate may include the following information:

- name and address of manufacturer
- site(s) of manufacturing
- trade name and common name of product
- marketing authorization number
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)

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1 Name of manufacturer.
2 Country of origin.
3 If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.
4 With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
9 Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.
- type of container used
- number of doses per container
- number of containers or lot size
- date of start of period of validity (e.g. manufacturing date) and/or expiry date
- storage conditions
- signature and function of the person authorized to issue the certificate
- date of issue of certificate
- certificate number.

The Director of the NRA (or other appropriate authority)

Name (typed)  
Signature  
Date  