Annex 1

Recommendations for Japanese encephalitis vaccine (inactivated) for human use (Revised 2007)

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Recommendations published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in type of normal size have been written in such a form that, should a national regulatory authority desire, they may be adopted as they stand as definitive national requirements or used as the basis of such requirements. Those parts of each section printed in small type are comments and additional guidance. It is recommended that modifications be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. To facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol for the recording of results of the tests is given in Appendix 1.
Introduction

These Recommendations are intended to provide national regulatory authorities and vaccine manufacturers with background and guidance on the production, quality control and evaluation of the safety and efficacy of inactivated Japanese encephalitis (JE) vaccines to facilitate their international licensure and use.

Since the adoption of the World Health Organization (WHO) Requirements (now termed Recommendations) for inactivated JE vaccines for human use in 1987 (1) by the WHO Expert Committee on Biological Standardization, alternative modes of production have been introduced that use continuous cell lines as a substrate for production instead of mouse brain.

The Committee, at its fifty-sixth meeting in 2005, recommended that the guidance on inactivated JE vaccines be revised and that sections on nonclinical and clinical evaluation should be added. To facilitate this process, WHO convened two meetings (in Geneva, 1–2 June 2006 (2) and in Bangkok, 7–9 February 2007) at which scientific experts, regulatory professionals and other stakeholders met to develop revised recommendations on inactivated JE vaccines for human use.

The scope of the present Recommendations encompasses inactivated JE vaccines produced in mouse brain and in cell substrates (e.g. primary hamster kidney cells and a continuous Vero cell line).

This document sets out the recommendations for manufacture and quality assessment in Part A. Guidance specific to the nonclinical and clinical evaluation of inactivated JE vaccines is provided in Parts B and C, respectively. Part D provides recommendations for national regulatory agencies. This document should be read in conjunction with all relevant WHO guidelines including those on nonclinical (3) and clinical evaluation (4) of vaccines.

These Recommendations are based on experience gained from the inactivated JE vaccines that have been developed so far, as described below, and may need to be updated to reflect important future developments.

General considerations

JE virus belongs to the family Flaviviridae and is included in the genus Flavivirus. The flaviviruses are enveloped RNA viruses and include yellow fever and dengue viruses, among others, which are serologically related to JE virus. JE viruses are grouped into five genotypes, based on the nucleotide sequence of the envelope (E) gene, but there is only one known serotype.

The principal vectors of JE virus are mosquitoes of the genus Culex. In Asia transmission is mainly via the bites of Culex tritaeniorhynchus. In endemic regions, JE virus is maintained in nature between vector mosquitoes and vertebrate animals, especially pigs. Pigs are also considered to act as an amplifier
for JE virus. Infected humans do not transmit virus to biting mosquitoes (i.e. humans are considered as dead-end hosts) because viraemia is transient with low virus concentrations.

Based on serological studies in endemic areas and on medical histories, JE virus infection may be asymptomatic in humans. It has been estimated that between 1 and 3 per 1000 infected humans may have a clinically manifest illness that includes evidence of virus-induced inflammation in the cerebrum, cerebellum and spinal cord. The incubation period for JE is 5–15 days and the illness usually starts with fever and headache, with or without vomiting, diarrhoea and myalgia. If meningeal irritation occurs it becomes apparent on the second day, after which other cerebral symptoms may develop rapidly, including altered consciousness, apathy or coma. The case fatality rate ranges from 5–30% but approximately 30–50% of the surviving patients have permanent neuropsychiatric sequelae and complete recovery occurs in only one third of patients.

In public health terms, JE is the most important viral encephalitis encountered in the South-East Asian and the Western Pacific countries where it is endemic or occurs in epidemics (5). During the past 25 years, incidence of JE has increased in certain countries. The disease has also extended its geographical borders to previously unaffected areas of Asia and to northern Australia, where cases were reported in the Torres Strait in 1995 and in the York peninsula of the subcontinent in 1998. There is year-round transmission in tropical countries but the transmission pattern in temperate and subtropical zones is seasonally defined.

Nearly 3 billion people are believed to be at risk of JE and approximately 20,000 clinical cases resulting in 6000 deaths are reported annually (5). However, implementation of a surveillance system specifically for JE is incomplete as the etiology of encephalitis is not differentiated in many Asian countries. In the countries where JE virus is hyper-endemic, those most affected are children under 4 years of age and almost all are less than 10 years of age. However, in some countries where routine childhood immunization has been implemented for many years, JE now occurs mainly in adults and especially in the elderly.

Vaccination of humans is the most effective means of preventing JE. There are three types of inactivated vaccines currently used in the world:

- mouse brain-derived, purified vaccine, which is based on either the Nakayama-NIH or Beijing-1 [P-1] strains;
- primary hamster kidney cell-derived, purified vaccine, based on the Beijing-3 [P-3] strain; and
- Vero-cell-derived purified vaccine based on the Beijing-3 [P-3] strain.
Newer Vero-cell-derived inactivated JE vaccines under development use either Beijing-1 or SA14-14-2 strains as virus seeds. All these strains belong to genotype 3.

A mouse brain derived inactivated JE vaccine was first licensed in Japan in 1954. This type of vaccine is manufactured in a similar fashion using the Nakayama-NIH strain in Japan (for export only) and also in India, Republic of Korea, Taiwan Province of China, Thailand, and Viet Nam. Since 1989, the JE vaccine that is actually used in Japan has contained the Beijing-1 strain. The efficacy of mouse brain-derived, inactivated JE vaccines was evaluated in two field trials in endemic areas. A study in Taiwan Province of China, in 1966, showed that the efficacy of the Nakayama-NIH strain vaccine was 80% after two doses. A later study in Thailand demonstrated that the efficacy of both a monovalent vaccine containing the Nakayama-NIH strain and a bivalent vaccine containing the Nakayama-NIH strain and the Beijing-1 strain was 91% over two transmission seasons. The Centers for Disease Control and Prevention (CDC), USA later pointed out that the level of protective efficacy observed in this study might in part reflect past exposure to JE virus and/or other flaviviruses. It was considered that the regimen of two doses given 7 days apart could not be assumed to give similar results in non-immune travellers. Therefore, the US licence for the Nakayama-NIH strain vaccine (approved in the USA in 1992) recommends a three-dose schedule based on immunogenicity data from non-immune US soldiers who received two or three doses. The duration of protection in non-immune people after a three-dose primary regimen remains unknown. Booster doses of the US-licensed Nakayama-NIH strain vaccine are recommended after 2 years although some studies done in Japan indicate that protective antibody levels persist for at least 4 years.

There is considerable information available on the adverse events associated with use of mouse brain-derived inactivated JE vaccines. Local reactions at the injection site and fever each occur in approximately 10% of vaccinated children in Japan. Severe allergic reactions characterized by generalized urticaria, respiratory symptoms and cardiovascular symptoms have been reported. Severe neurological disorders including acute disseminated encephalomyelitis (ADEM) have been reported following vaccination with mouse brain-derived JE vaccine. Eighteen cases of ADEM were reported in Japan after vaccination with mouse brain-derived inactivated JE vaccines from 1996 to 2005, which corresponds to approximately two cases per year following about 3 million inoculations. It is, however, assumed that there are 60–120 cases of ADEM per year in children in Japan whatever the cause (2).

Inactivated JE vaccine prepared from the Beijing-3 strain in primary hamster kidney cells has been produced exclusively in China since 1968. Approximately 75 million doses were distributed annually in China up to 1988.
Randomized field trials in China estimated that protection against JE was about 85% (5).

A Vero-cell-derived inactivated JE vaccine using the Beijing-3 strain has been licensed in China since 1998 where approximately 10 million doses had been distributed by 2006 and a clinical trial showed that the seroconversion rate (based on measurement of neutralizing antibody to JE virus) in school-age children was 92% (data presented at a WHO informal consultation held in Geneva, 1–2 June 2006).

The mouse brain-derived, inactivated vaccine has been used successfully to reduce the incidence of JE in a number of countries and is likely to be used nationally and internationally for several years to come. Because of the high benefit-to-risk ratio of routine vaccination, immunization against JE in public health programmes should continue using available vaccines (5). Nevertheless, the desire to reduce the numbers of animals used for production of vaccines, potential risks relating to residual neural substances in mouse brain-derived vaccines and technological advances in vaccine production are major driving forces in a move away from the conventional mouse brain-derived vaccines towards cell culture-derived vaccines.

In addition, a primary hamster kidney cell-derived, live attenuated vaccine based on the SA14-14-2 strain has been produced in China since 1988 where it has been reported that more than 100 million children have received the vaccine in a regimen of two doses given 1 year apart. This vaccine has been also licensed in Nepal, India, Republic of Korea and Sri Lanka.

Other JE vaccines in various stages of development include a chimeric live yellow fever–JE vaccine, DNA vaccines, poxvirus-based vaccines and virus-like particle vaccines. These products, as well as any other live attenuated JE vaccines, are outside the scope of these Recommendations.

The presence of neutralizing antibody provides the best evidence available that protective immunity is likely to have been established. Epitope mapping studies have indicated that there are at least eight functional epitopes on JE virus although not all of them elicit neutralizing antibody. There are several methods for determining functional antibody responses to the virus (see section C.2.1). The neutralizing antibody assay methodology most often used is the plaque reduction neutralization test (PRNT). The cut-off for seroprotection is defined as a PRNT$_{50}$ of at least 1:10 based on studies in mice that led to a conclusion that a titre of at least 1:10 protected against challenge with a dose of JE virus higher than the maximum titre estimated to be transmitted by a mosquito.

Data obtained in studies on mice have indicated that immune responses against the JE strain in a vaccine can result in cross-neutralizing antibody against different strains of JE virus (6–8). However, neutralizing antibody titres are usually higher against homologous virus strains than against the strains belonging to other genotypes. Recent studies with a candidate JE vaccine and
with the US-licensed Nakayama-NIH strain vaccine showed different results in neutralizing antibody and passive protection tests in mice according to the viral genotype used in the assays and for challenge (9). The degree of clinical cross-protection that might be afforded by vaccine strains against a range of wild-type viruses merits further investigation.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name should be *Japanese encephalitis vaccine (inactivated) 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

*Japanese encephalitis vaccine (inactivated) for human use* is a liquid or freeze-dried preparation of virus grown in mouse brains or in cell cultures and inactivated by a suitable method. The preparations for human use should satisfy all the recommendations formulated below.

A.1.3 International standards and reference reagents

At the time that these Recommendations were prepared, no international reference standard preparations were available.

A.1.4 Terminology

The following definitions are given for the purposes of these Recommendations only and may have other meanings in other contexts.

**Adjuvant**: a component that potentiates the immune response to an antigen and/or modulates it towards the desired immune responses.

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

**Cell bank**: a collection of ampoules containing aliquots of a suspension of cells from a single pool of cells of uniform composition, stored frozen under defined conditions (ideally in liquid nitrogen for mammalian cell lines).

**Final bulk**: the formulated bulk present in the container from which the final containers are filled. The final bulk may be prepared from one or
more purified bulks, which may or may not be adsorbed on an aluminium-containing adjuvant.

**Final lot**: a collection of sealed final containers, filled from the same final bulk, which are homogeneous with respect to the risk of contamination during filling or drying. A final lot should therefore consist of containers that have been filled in one working session and, if freeze-dried, have been freeze-dried together in the same chamber at the same time.

**Master cell bank (MCB)**: a quantity of fully characterized cells of human or animal origin stored frozen under defined conditions in aliquots of uniform composition derived from the cell seed, one or more of which may be used for the production of a manufacturer’s working cell bank.

**Master virus seed lot**: a quantity of virus of uniform composition, processed at one time, and distributed into a number of containers. Seed lots are derived from a virus seed used in the preparation of inactivated vaccines shown to be immunogenic in humans, and no more passages removed from it than the number approved by the national regulatory authority. The master virus seed lot is used for the preparation of working virus seed lots.

**Production cell culture**: a cell culture derived from one or more containers of the WCB used for the production of vaccines.

**Purified bulk**: a pool of purified and inactivated single harvests before preparation of the final bulk. It may be prepared from one single harvest or a number of single harvests and may yield one or more final bulks.

**Single harvest**: a virus suspension derived from one cell substrate lot, all the cultures having been inoculated at the same time with the same inoculum and harvested at the same time.

**Working cell bank (WCB)**: a quantity of cells of uniform composition derived from one or more ampoules of the master cell bank, which may be used for the production cell culture. In normal practice, a cell bank is expanded by serial subculture up to passage number (or population doubling, as appropriate) selected by the manufacturer, at which point the cells are combined to give a single pool and preserved cryogenically to form the WCB. One or more of the cryotubes from such a pool may be used for the production of cell culture.

**Working virus seed lot**: a quantity of virus suspension that has been processed together, is of uniform composition, and is no more passages removed from the master virus seed lot than the number approved by the national regulatory authority. Material is drawn from working virus seed lots for inoculating cell cultures or mouse brain for the production of vaccine.

### A.2 General manufacturing recommendations

The general manufacturing recommendations for manufacturing establishments contained in the *Good manufacturing practices for pharmaceutical products: main principles* (10) and the *Good manufacturing practices for biological products*
Annex 1

(11) should apply to establishments manufacturing JE vaccine for human use, with the addition of the following recommendation.

The assignment of a virus to a biosafety level for production and quality control facilities should be based on a risk assessment. Such an assessment will take the risk group, as well as other factors, into consideration in establishing the appropriate biosafety level. For example, a virus assigned to risk group 2 generally requires biosafety level 2 facilities, equipment, practices and procedures for safe conduct of work. The biosafety level assigned for the specific work is based on a risk assessment rather than by automatic assignment of a laboratory biosafety level according to the particular risk group designation of the pathogenic agent to be used. Further guidance on the risk assessment and assignment of appropriate biosafety levels is available in the WHO laboratory biosafety manual (12). However, countries should draw up a national policy for the manufacture of JE vaccines based on risk assessment and by risk group.

All personnel working in the production and control areas should have a serum neutralizing antibody titre of at least 1:10 against JE virus.

Only mouse brain tissue suspensions and cell cultures approved by the national regulatory authority for the production of JE vaccine should be introduced into the production area.

A.3 Control of source materials
A.3.1 Animals and cells for vaccine production
A.3.1.1 Mice

When mice are used for the propagation of JE vaccine virus in the brain, only animals less than 5 weeks of age should be used, and they should be free from any signs of disease.

Animal colonies should be shown to be healthy. Only animal stocks approved by the national regulatory authority should be used for virus propagation.

When an animal colony is established, animals should be screened for ectoparasites, endoparasites, fungi, protozoa, bacteria, and viruses either for which evidence exists of a capacity to infect humans or primates, or for which there is no evidence of infection in humans but which could nevertheless pose a potential danger, for example in immunocompromised individuals. These may include hantavirus (haemorrhagic fever with renal syndrome), lymphocytic choriomeningitis virus (LCMV), reovirus type 3, Sendai virus, ectromelia virus, K virus, lactate dehydrogenase-elevating virus (LDV), minute virus of mice (MVM), mouse adenovirus (MAV), mouse cytomegalovirus (MCMV), Theiler's
mouse encephalomyelitis virus (TMEV, GDVII strain), mouse hepatitis virus (MHV), mouse rotavirus (EDIM), pneumonia virus of mice (PVM), polyoma virus, retrovirus and thymic virus.

The colony should be monitored for zoonotic viruses and markers for contamination at regular intervals. Sera from the animals should be screened for antibodies against viruses. The choice of tests and testing procedures as well as the appropriate number of animals should be approved by the national regulatory authority. For instance, enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition (HAI), indirect fluorescent antibody (IFA) assay or any other suitable method can be used for estimation of these antibodies. For validity of these tests a suitable positive and negative control should always be included.

After the colony is established, it should be monitored by testing a representative group of animals. The choice of tests and testing procedures for monitoring as well as the appropriate number of animals should be approved by the national regulatory authority. In addition, the colony should be screened for the presence of pathogenic bacteria, including mycobacteria, fungi and mycoplasma. This screening should be performed in all of the animals over a defined period of time. The screening programme should be approved by the national regulatory authority.

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 national regulatory authority should be informed and the manufacture of vaccine should be discontinued. In this case, manufacture should not be resumed until a thorough investigation has been completed and precautions have been taken against the infectious agent being present in the product, and only then with the approval of the national regulatory authority.

If the vaccine is produced in mouse brain, methods for intracerebral inoculation and harvesting should be approved by the national regulatory authority.

A3.1.2 Primary hamster kidney cells

When primary hamster kidney cells are used for the propagation of JE vaccine virus, animals and the primary cells should be approved by the national regulatory authority.
A.3.1.2.1 Hamsters

Hamsters, 10–14 days old, may be used as the source of kidneys for cell culture. Only hamster stock approved by the national regulatory authority should be used as the source of tissue and should be derived from a closed, healthy colony. A closed colony is a group of animals sharing a common environment and having their own caretakers who have no contact with other animal colonies. The animals are tested according to a defined programme to ensure freedom from specified pathogens, including the absence of antibodies to these pathogens. When new animals are introduced into the colony, they should be kept in quarantine in vermin proof quarters for a minimum of 2 months and shown to be free from these specified pathogens. The parents of animals to be used as a source of tissue should be kept in vermin proof quarters. Neither parent hamsters nor their progeny should previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored for zoonotic viruses and markers for contamination at regular intervals.

At the time the colony is established, all founder animals should be tested to determine freedom from antibodies to the following pathogens: microorganisms pathogenic for hamsters (e.g. Mycobacterium tuberculosis, lymphoma virus, papilloma virus, polyomavirus, adenoviruses and retroviruses), lymphocytic choriomeningitis virus, pneumonia virus of mice, reovirus type 3, minute virus of mice, Sendai virus, hantavirus, SV 5, Toolans H a virus, mouse poliovirus, mouse hepatitis virus, lactate dehydrogenase-elevating virus, and Kilham rat virus. Antibody production tests in mouse (MAP), hamster (HAP), and rat (RAP) should also be performed. A test for retroviruses using a sensitive polymerase chain reaction (PCR) based reverse transcriptase (Rtase) assay also should be included. The results of such assays need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus like elements that do not encode a complete genome (13). Nucleic acid amplification tests for retrovirus may also be used. A PCR test for hamster polyoma virus should be used on a selected number of hamster tissues, especially kidneys, to qualify the colony, and at intervals thereafter. Once the colony has been established, it should be monitored by testing a representative group of animals at specified intervals. The choice of tests and testing procedures for monitoring as well as the appropriate number of animals should be approved by the national regulatory authority. In addition, the colony should be screened for the presence of pathogenic bacteria, including mycobacteria, fungi and mycoplasma. This screening should be performed in all of the animals over a defined period. The screening programme should be approved by the national regulatory authority.

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
national regulatory authority should be informed and the manufacture of vaccine should be discontinued. In this case, manufacture should not be resumed until a thorough investigation has been completed and precautions have been taken against the infectious agent being present in the product, and only then with the approval of the national regulatory authority.

At the time of kidney harvest, the animals should be examined for the presence of any abnormalities and if kidney abnormalities or other evidence of pathology is found, the animals affected are not to be used for production of JE vaccine.

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, is completed.

A.3.1.2.2 Primary hamster kidney cell cultures
Kidneys derived from animals which comply with the guidelines set out in section A.3.1.2.1 should be dissected and minced under conditions approved by the national regulatory authority. A primary cell suspension is obtained after trypsin digestion and this is distributed into cell culture vessels with growth medium.

A.3.1.3 Continuous cell lines
The use of a continuous cell line for the propagation of JE vaccine virus should be based on a cell bank system and tests on master and manufacturer’s working cell banks should conform with the Requirements for use of animal cells as in vitro substrates for the production of biologicals (13, 14) where appropriate, and should be approved by the national regulatory authority.

WHO has established a cell bank of Vero cells characterized in accordance with the recommendations in the report of the WHO Expert Committee on Biological Standardization (13, 14), which is available to manufacturers as a well characterized starting material for preparation of their own master and working cell bank on request to the Coordinator, Quality, Safety and Standards Team, WHO, Geneva, Switzerland.

The maximum number of passages (or population doublings) allowable between the MCB, the WCB and the production cells should be approved by the national regulatory authority. Additionally, the MCB or WCB cells should be propagated up to or beyond the maximum production level and be examined for tumorigenicity in an animal test system and for the presence of bacteria, fungi, mycoplasmas, retroviruses and other adventitious agents.
The MCB is made in sufficient quantities and stored in a secure environment and is used as the source material to make the manufacturer’s WCB. In normal practice, an MCB is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer and approved by the national regulatory authority, at which point the cells are combined to give a single pool distributed into ampoules and preserved cryogenically to form the WCB.

Tests on the MCB and WCB are performed in accordance with the Requirements for use of animal cells as in vitro substrates for the production of biologicals \((13, 14)\).

Full characterization may be performed on either the MCB or on the WCB.

The manufacturer’s WCB is used for the preparation of production cell culture, and thus for production of batches of JE vaccine virus.

The manufacturer’s WCB should be identified by means of, for example, biochemical (e.g. isoenzyme analysis), immunological and cytogenetic marker tests, approved by the national regulatory authority.

### A.3.1.4 Cell culture medium

If serum is used for the propagation of cells, it should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the recommendations given in Part A, sections 5.2 and 5.3 of the revised Requirements for biological substances no. 6 \((15, 16)\), and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the Recommendations for production and control of poliomyelitis vaccine (oral) \((17)\).

Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera. As an additional monitor of quality, sera may be examined for freedom from phage, endotoxin and antibodies to JE virus. Gamma-irradiation may be used to inactivate potential contaminant viruses.

The acceptability of the source(s) of any components used which originate from cattle, pigs, sheep or goats should be approved by the national regulatory authority. These components should comply with the Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products \((18)\) and WHO guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies \((19)\).

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

Human serum should not be used. However, human serum albumin may be used. If used, it should meet the revised Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (requirements for biological substances no. 27) (20), as well as WHO guidelines on transmissible spongiform encephalopathies (18, 19).

Penicillin and other beta-lactams should not be used at any stage of the manufacture because they are highly sensitizing substances. Other antibiotics may be used in the manufacture provided that the quantity present in the final product is acceptable to the national regulatory authority.

Minimal concentrations of suitable antibiotics such as kanamycin and neomycin may be used if approved by the national regulatory authority.

Any other substances added should be approved by the national regulatory authority.

Non-toxic pH indicators may be added, e.g. phenol red at a concentration of 0.002%.

A.3.2 **Virus seed**

Different virus seed strains are used for production of inactivated JE vaccine. A seed lot system should be followed during the preparation of master and working seed. Passage number of the working seed and final product is similar to that of the batch which has been found effective in clinical efficacy studies.

A.3.2.1 **Strain of virus**

The strains of virus used in the production of all seed lots should be approved by the national regulatory authority and should yield safe and immunogenic vaccines when the virus has been inactivated. They should be identified by historical records which include passage history. They should be shown to be free of adventitious agents by infectivity tests, serological or molecular biological tests, and animal inoculation.

A.3.2.2 **Virus seed lot system**

The preparation of JE vaccine should be based on the use of a virus seed lot system. The national regulatory authority should determine the acceptable
number of passages from the master virus seed lot to produce working virus seed lots. If mice are used for the passages, suckling mice are preferred. Vaccines should be made from a working virus seed lot without further intervening passage. Virus seed lots should be freeze-dried or frozen. The dried seed should be kept at or below −20 °C, whereas the frozen seed should be kept at or below −60 °C.

Seed lots should have been shown, to the satisfaction of the national regulatory authority, to be capable of yielding vaccine that meets all these Recommendations.

A.3.2.3 Tests on the master virus seed lots
A.3.2.3.1 Test for identity
The master virus seed lot should be identified as JE virus strain by methods approved by the national regulatory authority.

A.3.2.3.2 Tests for bacteria, fungi and mycoplasmas
Each master virus seed lot should be tested for bacterial, fungal and mycoplasmal contamination by appropriate tests according to Part A, sections 5.2 and 5.3 of the revised Requirements for biological substances no. 6 (General requirements for sterility of biological substances) (15, 16).

A.3.2.3.3 Tests for adventitious agents
The master virus seed lot should be tested for adventitious agents. For these tests the virus should be neutralized by a specific anti-Japanese-encephalitis serum. The specificity and sensitivity of assays should be defined and approved by the national regulatory authority.

A.3.2.3.4 Additional tests
Tests should be carried out to characterize the virus strain. Such tests should include the titration of virus. Additional tests should also take into account the passages of the virus in different animal species.

A.3.2.4 Tests on the working virus seed lots
A.3.2.4.1 Test for identity
The working virus seed lot should be identified as JE virus strain as in A.3.2.3.1.

A.3.2.4.2 Tests for bacteria, fungi and mycoplasmas
Each working virus seed lot should be tested for bacterial, fungal and mycoplasmal contamination as described in section A.3.2.3.2.
A.3.2.4.3 *Tests for adventitious agents*
If the working virus seed lot is derived from mouse brain or primary cell cultures, it should be tested for adventitious agents as in section A.3.2.3.3. If working virus seed lots are produced in cells derived from a validated cell bank where a master virus seed lot was tested for adventitious agents, these tests do not have to be repeated.

A.3.2.4.4 *Additional tests*
Each time a new working virus seed lot is prepared, tests should be carried out to characterize the virus strain as described in section A.3.2.3.4.

A.4  **Control of vaccine production**

A.4.1  **Mouse brain**
The brains of mice inoculated intracerebrally with the virus strain for production should be harvested when the mice exhibit advanced signs of JE virus infection, such as encephalitis. The harvested mouse brains should be homogenized in a suitable medium and processed to give a uniform virus suspension. The harvested and processed virus suspension should be subjected to the control tests for single virus harvests given in section A.4.3 of these Recommendations.

A.4.2  **Cell cultures**

A.4.2.1  **Preparation of control cell cultures**
At least 5% of the cell suspension (not less than 500 ml) at the concentration employed for inoculating vaccine production cultures should be used to prepare control cultures.

In some countries in which the technology of large-scale production by means of a bioreactor has been developed, the national regulatory authority should determine the size of the cell sample to be examined and the control methods to be applied.

A.4.2.2  **Tests on control cell cultures**
The control cell cultures should be treated in a similar way to the production cell cultures, but they should remain uninoculated to serve as control cultures for the detection of extraneous viruses.

The control cell cultures should be incubated under the same conditions as the inoculated cultures for at least 14 days and should be examined during this period for evidence of cytopathic changes. For the test to be valid, not
more than 20% of the control cell cultures should have had to be discarded for nonspecific, accidental reasons. At the end of the observation period, the control cell cultures should be examined for the presence of adventitious agents as described below (sections A.4.2.2.2 and A.4.2.2.3).

If this examination or any of the tests specified in this section shows evidence of the presence in a control culture of any adventitious agent, the JE virus grown in the corresponding inoculated cultures should not be used for vaccine production.

Samples not tested immediately should be stored at –60 °C or below.

A.4.2.2.1 Identity test if continuous cell lines are used

At the production level, and for vaccines produced in continuous cell lines, the cells should be identified by using one of the methods specified in the Requirements for the use of animal cells as in vitro substrates for production of biologicals (13, 14). The method(s) should be approved by the national regulatory authority.

Methods for identity testing include, but are not limited to, biochemical (e.g. isoenzyme analysis), immunological (e.g. major histocompatibility antigens), cytogenetic tests (e.g. for chromosomal markers), and tests for genetic markers (DNA fingerprinting).

A.4.2.2.2 Tests for haemadsorbing viruses

At the end of the observation periods, haemadsorbing viruses should be tested. If multiple harvest pools are prepared at different times, the cultures should be observed and tested at the time of the collection of each pool.

In some countries, 25% of the control cells are tested for the presence of haemadsorbing viruses by using guinea-pig erythrocytes. If the red blood cells have been stored, the duration of storage should not have exceeded 7 days and the temperature of storage should have been in the range of 2–8 °C.

In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

In some countries the national regulatory authority requires that tests for haemadsorbing viruses should also be done with erythrocytes from other species, including human blood group O, monkeys and chickens (or other avian species).
The results of all tests should be noted after incubation of the erythrocytes with the cultured cells for 30 minutes at 0–4 °C and again after a further incubation for 30 minutes at 20–25 °C. For the test with monkey erythrocytes, the results should be noted a third time after a final incubation for 30 minutes at 34–37 °C.

A.4.2.2.3 Tests for other adventitious agents on supernatant fluids

At the end of the observation period, a sample of the pooled fluids from each group of control cultures should be tested for adventitious agents. At least 10 ml of each pooled supernatant fluid from the control cultures should be tested in the same cell substrate, but not the same batch of cells, as that used for production. Additional samples of at least 10 ml should be tested in human cells and at least one other sensitive cell system.

The samples should be inoculated into bottles of these cell cultures in such a way that the dilution of the supernatant fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm²/ml of supernatant fluid. At least one bottle of each of the cell cultures should remain uninoculated and serve as a control.

The inoculated culture should be incubated at 35–37 °C and should be observed for cytopathic effects for a period of at least 14 days.

For the tests to be valid, at least 80% of the cell cultures should be available and suitable for evaluation at the end of the test period.

If any cytopathic changes due to adventitious agents occur in any of the cultures, the virus harvest produced from the batches of cells from which the control cells were taken should be discarded.

A.4.3 Control of single virus harvests

After inoculation of the production cells with the virus working seed lot, inoculated and control cell cultures should be kept within a temperature range approved by the national regulatory authority for the defined incubation periods. The optimal range for pH, multiplicity of infection, cell density and time of incubation should be established, and be approved by the national regulatory authority.

The appropriate time for harvest should be defined and approved by the national regulatory authority.

It is advisable that the inoculated cell cultures are processed in such a manner that each virus suspension harvested remains identifiable as a single harvest and is kept separate from other harvests until the results of all the tests described in section A.4.2 have been obtained.

Only the virus harvests satisfying the recommendations below should be pooled and used in the preparation of the inactivated virus harvest.
A.4.3.1 Sterility tests for bacteria and fungi
A sample removed from each virus harvest should be tested for bacterial and fungal contamination by appropriate tests recommended in Part A, section 5.2 of the revised Requirements for biological substances no. 6 (General requirements for the sterility of biological substances) (16). Any single virus harvest in which contamination is detected should be discarded.

A.4.3.2 Identity test for vaccine virus
The single virus harvest should be identified as JE vaccine virus using suitable methods approved by the national regulatory authority.

A.4.3.3 Test of virus content
A sample removed from each virus harvest should be tested for virus content using suitable methods approved by the national regulatory authority.

Both mice and cell culture with defined sensitivity are suitable for testing infectivity. Manufacturers should set an in-house specification for titre of each harvest.

A.4.3.4 Consistency of yield
Virus content as mentioned above is an appropriate parameter for monitoring the consistency of yield. Therefore, internal specifications should be set.

A.4.4 Preparation and control of purified bulk
A.4.4.1 Preparation of purified bulk
Only virus harvests satisfying the recommendations for sterility and virus content in section A.4.3 should be pooled.

One or more single harvests may be purified and/or concentrated by methods demonstrated to yield safe, potent and immunogenic vaccine. The virus harvest or pools should be inactivated by a validated method at a defined stage of the process which may be before or after concentration and purification.

The process should be approved by the national regulatory authority and should be shown to give consistent results.

The bulk suspension derived from mouse brains should be purified by a process designed to reduce the myelin content to the lowest possible level and should have been approved by the national regulatory authority (see section A.4.4.3.1).

A.4.4.2 Inactivation of virus
A.4.4.2.1 Treatment before inactivation
When cell cultures are used, the bulk material should be filtered or clarified by centrifugation prior to inactivation.
The importance of filtration or clarification using centrifugation of the crude virus suspensions as a means of improving the consistency of the inactivation process has been clearly established. Generally, filters are used in series or filtration is performed step-wise through filters of decreasing porosity. Satisfactory results have been reported with several filter types, but a final filtration using a 0.22-μm filter should be done.

A.4.4.2.2 **Inactivation**

The process for the inactivation of the JE virus should be approved by the national regulatory authority.

Inactivation should be commenced immediately after the preparation and sampling of single virus harvests when mouse brain is used, or immediately after filtration when cell cultures are used.

One method that has been successfully used to inactivate JE virus is the treatment of the virus harvest with formalin at a final concentration of 1:2000 for 50–60 days at 4 °C.

A.4.4.2.3 **Test for effective inactivation**

Each bulk suspension should be tested in an appropriate test system for effective inactivation of the virus before the addition of preservatives and other substances. The sensitivity of the assay should be determined according to the JE virus used for production and the most sensitive assay should be used. This test should be performed immediately after inactivation.

If samples are not tested immediately after inactivation they should be stored frozen at −60 °C or below. The conditions of storage should be validated to confirm no loss of virus titre. If the test is performed at a later stage of production, appropriate biosafety levels should be maintained.

The test should be approved by the national regulatory authority and should be performed with the undiluted bulk suspension. A test sample corresponding to no less than 25 human doses of the final bulk should be used.

In some countries the test involves direct inoculation intracerebrally into mice followed by three blind passages.
The total volume of the test sample should be inoculated into the primary culture of hamster kidney cells, or any other cell cultures with no less susceptibility to the virus than hamster-kidney cells, and incubated at 35 ± 1 °C for a period of 14 days. A cell culture sheet not less than 3 cm² should be used for 1 ml of the test material.

During the incubation period, no cytopathic change should be detected. On completion of the observation, the cultured fluid should be collected and inoculated intracerebrally at a dose of 0.03 ml into at least 10 mice of about 4 weeks of age. The animals should be observed for 14 days. The bulk passes the test if the product has been shown to be free from residual live virus.

A.4.4.3 Tests on purified bulk
A.4.4.3.1 Test for myelin basic protein if mouse brain was used for production

Each purified bulk should be tested for myelin basic protein. The method and specification for myelin basic protein content should be approved by the national regulatory authority.

Some licensed JE vaccines have been reported to contain myelin basic protein at concentrations lower than 2 ng per human dose.

A.4.4.3.2 Protein content

Each purified bulk should be tested for the total protein content using a suitable method such as the micro-Kjeldahl method or the Lowry technique.

A.4.4.3.3 Antigen content

The test for viral antigen content should be done on each bulk suspension. The method used should be approved by the national regulatory authority.

A.4.4.3.4 Test for residual DNA if continuous cell lines are used for production

For viruses grown in continuous cell lines, purified bulk should be tested for residual cellular DNA. If this test has not been carried out at this stage, it should be done on final bulk or final lot.

The removal process should be shown to consistently reduce the amount of cell DNA. It is expected that the levels of residual host cell DNA in a final dosage form will meet the maximum levels cited in the Requirements for use of animal cells as in vitro substrates for the production of biologicals (13).
A.4.4.3.5 *Test for residual animal serum*

If animal serum is used for production of cell culture vaccines, residual bovine serum albumin (BSA) content should be measured as an indicator of animal serum in the purified bulk. This should result in a level of no greater than 50 ng per human dose or its equivalent.

In some countries, tests are carried out to estimate the amount of residual animal serum in the final vaccine. Other serum proteins may also be measured.

A.4.4.3.6 *Test for residual chemicals*

The concentration of chemicals such as inactivating agent remaining in the final vaccine should be determined using methods approved by the national regulatory authority. These concentrations should not exceed the upper limits specified by the national regulatory authority. For preservatives, both the method of testing and the concentration should be approved by the national regulatory authority.

Alternatively, tests for residual chemicals may be performed on the final bulk.

A.4.5 *Preparation and control of final bulk*

A.4.5.1 *Preservatives and other substances including adjuvants added*

In the preparation of the final bulk, only adjuvant, preservatives or other substances such as human albumin approved by the national regulatory authority should be added. Such substances should have been shown by appropriate tests not to impair the safety or effectiveness of the product in the amounts used.

If formalin has been used for inactivation, the procedure should be such that the amount of formaldehyde in the final bulk is no greater than 0.01%. The test method used should be approved by the national regulatory authority.

Additional antibiotics should not be added to the final bulk of JE vaccine for human use.

Antigen produced in cell cultures may be adsorbed onto an adjuvant such as aluminium. In that case, the mineral vehicle and its concentration should be approved by the national regulatory authority. Antigen produced in the mouse brain should not be adsorbed onto any adjuvant. Until the bulk is formulated into the final bulk, the suspension should be stored under conditions shown by the manufacturer to retain the desired biological activity.
A.4.5.2 Tests on final bulk

A.4.5.2.1 Sterility tests for bacteria and fungi

Each final bulk should be tested for sterility according to the recommendations in Part A, section 5.2 of the revised Requirements for biological substances no. 6 (General requirements for the sterility of biological substances) (16).

A.4.5.2.2 Adjuvant content and degree of adsorption (where appropriate)

If an adjuvant has been added to the vaccine, its content should be determined by a method approved by the national regulatory authority. The amount and nature of the adjuvant should be within the range shown to be clinically effective and should be approved by the national regulatory authority. When aluminium compounds are used, the content of aluminium should not be greater than 1.25 mg per single human dose.

The formulation of adjuvant and antigen should be stable and consistent. The purity of the adjuvant should be demonstrated to be within the range found for vaccine lots shown to be clinically effective.

Adsorbed bulk may be assayed for the content of the adjuvant until production consistency is demonstrated.

The degree of adsorption (completeness of adsorption) of each adsorbed bulk should be assessed. This test may be omitted upon demonstration of process consistency.

A.4.5.2.3 Preservative content

If a preservative has been added to the vaccine, the content of preservative should be determined by a method approved by the national regulatory authority. The amount of preservative in the vaccine dose should be shown neither to have any deleterious effect on the antigen nor to impair the safety of the product in humans. The preservative and its use at different stages of the manufacturing process as well as the residual amount present in the product should be approved by the national regulatory authority.

A.4.5.2.4 Potency

This test may be performed on the final bulk. The method for detection of neutralizing antibody and the analysis of data should be approved by the national regulatory authority. The vaccine potency should be compared with that of a reference preparation and the national regulatory authority should determine
limits of potency. The national regulatory authority should approve the reference preparation used.

This test may be conducted on each final lot derived from the final bulk.

A.5 **Filling and containers**

The recommendations concerning filling and containers in the *Good manufacturing practices for biological products* (11) should apply, with the addition of the following recommendations.

Containers of freeze-dried vaccine should be hermetically sealed under vacuum or after filling with pure, dry, oxygen-free nitrogen or any other gas not deleterious to the vaccine. All containers sealed under vacuum should be tested for leaks and all defective containers should be discarded.

Care should be taken to ensure that the materials of which the container and, if applicable, transference devices and closure, are made do not adversely affect the quality of vaccine.

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

A.6 **Control tests on final lot**

A.6.1 **Inspection of final containers**

Every container in each final lot should be inspected visually, and those that show abnormality should be discarded.

A.6.2 **Identity test**

An identity test should be performed on at least one labelled container from each final lot by methods approved by the national regulatory authority.

The test for potency, as described in section A.6.6 of these Recommendations may serve as an identity test.

A.6.3 **Sterility tests for bacteria and fungi**

Each final lot should be tested for bacterial and fungal sterility according to the recommendations in Part A, section 5.2 of the revised *Requirements for biological substances no. 6* (*General requirements for the sterility of biological substances*) (16).

A.6.4 **Tests of pH**

The pH value of a pool of final containers should be tested. The freeze-dried vaccine is dissolved in the approved diluent. The pH value should be approved
by the national regulatory authority, and be within the range of values found for vaccine lots shown to be clinically safe and effective.

**A.6.5 Test of osmolarity**

The osmolarity of a pool of final containers should be tested. The freeze-dried vaccine is dissolved in the approved diluent. The osmolarity should be approved by the national regulatory authority, and be within the range of values found for vaccine lots shown to be clinically safe and effective.

This test may be discontinued when consistency of production has been demonstrated.

**A.6.6 General safety (innocuity) tests**

Each final lot should be tested for the absence of abnormal toxicity using a general safety (innocuity) test approved by the national regulatory authority.

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

**A.6.7 Test for protein content**

For mouse brain vaccine, the maximum protein content should not be greater than 80 µg/ml.

Experience from production by some manufacturers indicates that that levels of 10–40 µg/ml are obtained.

The protein content of cell culture-derived vaccines should be approved by the national regulatory authority.

If protein stabilizers such as gelatin are added to vaccine, the total protein content should reflect such additions.

**A.6.8 Test for residual cellular DNA**

When continuous cell lines are used for production, the cellular DNA content in the final dosage form should be determined. As recommended in the WHO Requirements for the use of animal cells as in vitro substrates for the production of biologicals (13), the amount of residual cell DNA should be less than 10 ng per purified human dose. The assay for determination of residual cell DNA
with defined sensitivity for detection of specified levels should be approved by
the national regulatory authority. If this test has already been carried out at an
earlier stage of production, e.g. purified or final bulk, an estimate of the level of
residual cellular DNA retained in the final lot should be presented and justified.
The specification set for the level of residual DNA should comply with current
WHO requirements for cell substrates (13). This test may be discontinued once
consistency has been demonstrated.

A.6.9 Potency test

The potency should be determined by titration of the neutralizing antibody
produced in immunized mice by the plaque-reduction neutralization test.
Neutralization antibody titres should be calculated as 50% plaque-reduction
neutralization titre. The test should be done in parallel with a reference vaccine
(standard) derived from a homologous virus strain. The challenge strain should
be the virus strain homologous to that in the test vaccine. The mouse strain used
should have been shown to give adequate responses following immunization
with the JE vaccine being tested.

Appropriate vaccine and challenge virus are approved by the national
regulatory authority and should be homologous to the JE virus strain used for
production.

The test procedure used, including the reference vaccine, should be
approved by the national regulatory authority (see section D.1).

The reference vaccine should be well characterized in respect of its
immunogenic potential. The reference vaccine should either have been
included and shown to be efficacious in clinical trials, or be traceable to such a
batch of vaccine.

In some countries, a single dilution assay has been used. However,
a multi-dose assay has been implemented in at least one country,
which facilitates statistical evaluation.

Briefly, the multi-dose test is as follows: the test vaccine and a
reference are diluted to make appropriate serial dilutions. Five
hundred microlitres of each dilution is injected intraperitoneally
into at least ten mice, of the same sex, approximately 4 weeks
of age and typically having a body weight of 14–18 g, on two
occasions 7 days apart. Seven days after the second injection,
each animal is bled. An equal volume of separated serum is
pooled and heat-inactivated at 56 °C for 30 minutes. The pooled
serum may be stored at −20 °C or below.
If pooled serum is tested for virus neutralizing antibodies, the following procedures are used:

A series of dilutions are prepared in Eagle's minimum essential medium (MEM) containing fetal bovine serum, or another appropriate medium. Equal volumes of the diluted serum and the challenge virus are mixed. The mixture is kept at 36 ± 1 °C for 90 minutes with intermittent shaking every 15–30 minutes. One hundred microlitres of the serum–virus mixture is inoculated on to at least three wells of appropriate cells, such as Vero cells or chick embryo fibroblast cells, in 6-well plates. The challenge virus is mixed with an equal volume of the medium used for dilution to serve as the virus control. All the inoculated cells are incubated at 36 ± 1 °C for 90 minutes in 5% carbon dioxide. The infected cells are overlaid with the overlaying agar medium containing 1% agar or methyl cellulose.

After incubation for an appropriate time (5–8 days), the cells are stained and the number of plaques counted. The mean number of plaques in the control should be 50–150 per dish. Neutralizing antibody titres (based on initial serum dilutions before mixing with virus preparation) are calculated as 50% PRNT.

The 50% PRNTs induced by the vaccine being tested are compared with those induced by the reference, by the appropriate statistical methods approved by the national regulatory authority. The potency of the test sample should be no less than that of the reference vaccine.

A.6.10 Accelerated thermal stability test

The performance of accelerated thermal stability tests should be considered in the context of the overall stability evaluation of JE vaccine (21) (section A.11.1).

Previous experience indicates that the potency of liquid vaccine may be determined after storage of samples at 37 °C for 1 week and for freeze-dried vaccines after storage at 37 °C for 4 weeks.

If accelerated stability data consistently meet requirements for potency and other stability indicating parameters, a thermal stability test for the purpose of lot release could be performed at regular intervals instead of testing each lot as part of ongoing stability studies following licensing.
A.6.11  **Residual moisture tests on freeze-dried vaccine**

The residual moisture in a representative sample of each freeze-dried lot should be determined by a method approved by the national regulatory authority. The upper limit for the moisture content should be specified by the national regulatory authority.

Moisture levels of 3% or less are generally considered satisfactory.

A.6.12  **Test for pyrogenic substances**

Each final lot should be tested for pyrogenic substances. The test procedures should be approved by the national regulatory authority.

A.6.13  **Test for residual animal serum**

For cell culture-derived vaccines, the final lot should be tested to verify that the level of residual bovine serum albumin as an indicator of residual serum protein in the final vaccine is less than 50 ng per human dose.

This test may be performed on the purified bulk or on the final bulk. Tests for other residual serum proteins may also be used. (See section A.4.4.3.5).

A.6.14  **Test for preservatives**

Each final lot should be tested for the presence of preservative, if added. The concentrations of preservatives should be approved by the national regulatory authority. Such substances should have been shown by appropriate tests not to impair the safety or immunogenicity of the vaccine.

If any modification of preservative content in an already licensed vaccine is made, general principles for vaccine evaluation described in the WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines (22), should be followed.

A.6.15  **Adjuvant content and degree of adsorption (where appropriate)**

If vaccines are adjuvanted, each final lot should be assayed for the adjuvant content. When aluminium compounds are used, the content of aluminium should not be greater than 1.25 mg per single human dose.

The degree of adsorption (completeness of adsorption) of the antigen in each final lot should be assessed and the limits should be approved by the national regulatory authority. This test may be omitted for routine lot release upon demonstration of product consistency.
A.7 **Records**  
The recommendations in the *Good manufacturing practices for biological products* (11) should apply.

A.8 **Retained samples**  
The recommendations in the *Good manufacturing practices for biological products* (11) should apply.

A.9 **Labelling**  
The recommendations given in section 7 of the *Good manufacturing practices for biological products* (11) should apply, with the addition of the following information.

The label on the carton, the container or the leaflet accompanying the container should state:

- that the vaccine has been prepared from mouse brains, primary hamster kidney cells, or Vero cells;
- the strain of the vaccine virus present in the preparation;
- the number of doses, if the product is issued in a multiple dose container;
- the name and maximum quantity of any antibiotic present in the vaccine;
- the name and concentration of any preservative added;
- the name and concentration of any adjuvant added;
- the temperature recommended during storage and transport;
- the expiry date; and
- any special dosing schedules.

A.10 **Distribution and shipping**  
The recommendations in the *Good manufacturing practices for biological products* (11) should apply.

A.11 **Stability, storage and expiry date**

A.11.1 **Stability testing**  
Stability testing should be performed at different stages of production, namely on single harvests, purified bulk, final bulk and final lot. Stability-indicating parameters should be defined or selected appropriately according to the stage of
production. It is advisable to assign a shelf-life to all in-process materials during vaccine production, in particular 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 national regulatory authorities on at least three lots of final product. Accelerated thermal stability tests may be undertaken on each final lot to give additional information on the overall stability of a vaccine (see section A.6.10).

The formulation of vaccine and adjuvant (if used) should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with national authorities.

A.11.2 Storage conditions
The vaccine in its final container should be kept at +2 to +8 °C. If other storage conditions are used, they should be fully validated and approved by the national regulatory authority. The vaccine should have been shown to meet the release specifications for a period equal to that between the date of release and the expiry date. During storage, liquid vaccines should not be frozen.

A.11.3 Expiry date
The expiry date should be fixed with the approval of the national regulatory authority, and should take account of the experimental data on stability of the vaccine.

In one country, the expiry dating period of a liquid JE vaccine was set no less than 3 months after the potency test and for a freeze-dried vaccine no less than 2 years.

For freeze-dried vaccines, the expiry date for the vaccine and the diluent may be different.

Part B. Nonclinical evaluation of new Japanese encephalitis vaccines (inactivated) for human use
Nonclinical evaluation of new JE vaccines should be based on the Guidelines on nonclinical evaluation of vaccines (3). The following specific issues should be considered in the context of the development of an inactivated JE vaccine. In any event, the nonclinical experiments should be discussed with the national regulatory authorities.
B.1 Immunogenicity studies
For JE virus the role of antibody in protection is well studied and neutralization assays are considered more appropriate than the virus binding assays such as ELISA (see General considerations and section C.2). Nonclinical studies should normally be undertaken using the same formulation of the vaccine as that intended for use in clinical trials unless otherwise justified.

The first studies should involve immunization of animals with various doses of the candidate vaccine given at various regimens and evaluation of the kinetics of the neutralizing antibody response. The inclusion of at least one licensed vaccine as a comparator may provide useful supporting data, but is optional. Studies are usually undertaken in mice as this species demonstrates an adequate immune response.

B.2 Active protection studies
The protective efficacy of the vaccine may be evaluated in challenge studies. The focus of these studies should be to demonstrate that prior vaccination protects against disease due to the homologous virus strain. The studies should be performed before the commencement of clinical studies. Protection studies that employ challenge should be undertaken with at least one other genotype 3 virus (e.g. another strain used for vaccine production). Similar studies using a non-genotype 3 virus are encouraged. These studies may be performed later on in the development programme. Issues regarding biocontainment should be taken into consideration.

The optimal concentration of challenge virus and the route of inoculation which consistently result in disease and/or death in unvaccinated mice should be established. The intracerebral route is generally used for challenge but the intraperitoneal route may be appropriate for some virus strains. Mice are generally challenged with virus at the time of the maximum immune response.

B.3 Passive protection studies
Passive protection studies involve the administration of sera from vaccinated animals or humans to unvaccinated animals followed by virus challenge as described for active protection studies. By this means it may be possible to estimate titres of neutralizing antibody raised in response to vaccination which correlate with protection. While not necessarily mandatory, such studies could be undertaken in conjunction with phase I clinical studies when post-vaccination human sera become available.
B.4  Toxicology

Toxicology studies on vaccines should reflect the maximum clinical dose anticipated for use in humans, the route of administration and the anticipated schedule.

If a vaccine is to be indicated for use in women of childbearing age, reproductive and developmental toxicology studies are recommended. However, these are not required if the vaccine is to be recommended only for use in children under the age of 12 years.

The addition of any preservative and novel adjuvant requires additional toxicological analysis. The absence of detailed toxicology studies should be justified. Changes in the manufacturing procedures might require a nonclinical assessment.

Part C. Clinical evaluation of new Japanese encephalitis vaccines (inactivated) for human use

C.1  General considerations for clinical studies

C.1.1  Clinical development programme

Clinical trials should adhere to the principles described in the Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (23) and to the Guidelines on clinical evaluation of vaccines: regulatory expectations (4). All clinical trials should be approved by the relevant national regulatory authorities.

Some of the issues that are specific to the clinical evaluation of 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 should consult with the relevant national regulatory authorities regarding the overall clinical development programme and the plans for assessment of immune responses.

This guidance is intended to be applicable to all novel inactivated JE vaccines whatever the mode of production (i.e. including use of vectors to express viral antigens).

C.1.2  Range of clinical studies

The availability and widespread deployment of effective vaccines in areas where JE is endemic makes it unethical to conduct protective efficacy studies (i.e. with the end-point of prevention of clinically apparent illness) that compare a group given a new JE vaccine with an unvaccinated group. In addition, the use of the available JE vaccines has reduced the incidence of clinically apparent infections to such an 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 JE vaccines should be based on evidence derived from active and passive protection in animal models (see section B) and on an immunological parameter that is a suitable correlate for clinical protection in humans (see General considerations and section C.2).

It is important that the immunogenicity of a new JE vaccine should be assessed in accordance with the intended mode of use. Ideally, the clinical development programme should assess the safety and immunogenicity of the new vaccine in cohorts resident in non-endemic, endemic and hyper-endemic areas in order to enrol subjects with no pre-existing immunity and subjects with varying degrees of pre-existing immunity to JE as a result of previous vaccination and/or natural exposure. Some important considerations include the following:

- The focus for use of JE vaccines in endemic and hyperendemic areas is most likely to be the vaccination of residents from an early age. Therefore an adequate assessment of the immunogenicity of a new vaccine in children in various age groups is important. Studies in the youngest children should generally follow once satisfactory assessments of safety and immunogenicity have been obtained in adults and older children.

- In endemic and hyperendemic areas a substantial proportion of residents may have received JE vaccines in the past. Therefore it may be useful to evaluate the ability of a new JE vaccine to boost immunity in people who were previously vaccinated with other types of JE vaccines.

- Study design and location should take into consideration the existence of cross-reactive immunity between flaviviruses, which can influence pre-vaccination and post-vaccination levels of antibody to JE virus. For example, past natural infection with dengue or West Nile viruses (which may have been subclinical or not diagnosed) and/or past exposure to, or vaccination against, yellow fever may result in detectable antibody to JE virus before any vaccine is administered.

- In contrast, the use of JE vaccine in non-endemic areas is mainly intended to protect those who are travelling to endemic regions. Thus, although there is a potential for use across all age groups, most recipients of JE vaccines in non-endemic areas are likely to be non-immune adults.
C.2 Immunogenicity

C.2.1 Methodology

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

Expression of neutralizing antibody titres in terms of the highest dilutions of sera before mixing with virus preparation that accomplish a 50% reduction in viral plaques (i.e. PRNT$_{50}$) is preferred over the use of 90% reduction in plaques (i.e. PRNT$_{90}$).

Initial studies should seek to establish whether vaccination elicits adequate immune responses to the vaccine strain (i.e. homologous virus) and should evaluate antibody kinetics. Further studies should evaluate post-vaccination PRNT$_{50}$ titres against other (i.e. heterologous) strains of JE virus in randomly chosen subsets of sera. There are five JE genotypes. Therefore use of heterologous strains of various genotypes of JE virus in PRNT assays is encouraged.

Methods that assess total (i.e. including non-functional) antibody may also be used during the clinical development programme but the results of these tests should be regarded as secondary immunogenicity parameters. These methods include haemagglutination inhibition (HI), enzyme-linked immunosorbent assay (ELISA) or immunofluorescent antibody (IFA) tests. If such tests are performed, any correlation between the results and those of PRNT$_{50}$ should be explored.

Consideration may also be given to the assessment of vaccine-induced, cell-mediated immunity. Studies in mice have shown that adoptive transfer of T lymphocytes can confer passive protection against viral challenge. Also, human CD4 and CD8 cells harvested from vaccinated people can be stimulated by JE virus to proliferate in vitro. However current uncertainties regarding the interpretation of these data mean that they would also be considered secondary immunogenicity parameters.
C.2.2 End-points and analyses

The primary assessment of immune responses should be based on proportions of previously seronegative subjects who reach a PRNT\(_{50}\) titre of at least 1:10 after vaccination (see also General considerations).

The primary population should be predefined in the protocol and should be selected in accordance with the study objectives. The population to be used in the primary analysis of immune responses should usually be confined to those subjects who are seronegative for JE virus before vaccination (i.e. have PRNT\(_{50}\) titres < 1:10). Therefore, before commencement of a study in a particular geographical area, an estimate should be made of the likely percentage of subjects who will have pre-vaccination PRNT\(_{50}\) titres ≥ 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 subjects will already be seropositive. Alternatively, or in addition, studies could include a screening visit so that a subject’s pre-vaccination serostatus can be determined before enrolment and administration of the vaccine.

In people who are seronegative before vaccination, the most appropriate primary parameter for assessment of the immune response will be the proportion reaching PRNT\(_{50}\) titres ≥ 1:10 after vaccination, which will equal the seroconversion rate. Other immune parameters examined should include increases in titres after sequential doses, geometric mean titres and reverse cumulative distributions of titres. Variability between subjects in the immune response should also be reported.

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

In people who are seropositive at baseline (i.e. have PRNT\(_{50}\) titres ≥ 1:10) the primary assessment of immune responses to vaccination would usually be based on proportions achieving substantial increases (e.g. at least a 4-fold rise) in titre after one or more doses.

After completion of what is considered to constitute a primary course of vaccination, it is vital that assessment of antibody persistence is planned.
Therefore protocols should include appropriate long-term serological follow-up at least in selected cohorts of subjects. Generally it would be expected that subjects should be followed for a minimum of 2 years and ideally for up to 5 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 JE virus and/or other flaviviruses. Therefore, data on antibody persistence should not be extrapolated to non-endemic areas or to other endemic areas with much lower or higher risk 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 pre-plan for administration of a booster dose to selected cohorts at specified times post-primary. The timing of booster doses may be based on currently approved vaccines. Pre-boost and post-boost antibody responses and post-boost follow-up are important elements of the overall assessment and will provide evidence of past priming with the new JE vaccine.

C.2.3 Dose and schedule

Based on past experience with inactivated JE vaccines it is anticipated that more than one dose will be needed to achieve and maintain protection. As with all vaccines it is important that sufficient immunogenicity data are generated to support the dose of antigen chosen, number of doses and dose intervals. However, it is accepted that there are limitations on the number of possible regimens that can realistically be explored and so some degree of justification for the regimen chosen based on available vaccines may be acceptable.

As a minimum, it is important that an appropriate schedule is identified for children in endemic areas taking into account the recommended age from which vaccination should commence. If the vaccine is proposed for travellers from non-endemic areas, who are very likely to be non-immune, different primary vaccination schedules may have to be explored. For example, it may be important to study accelerated immunization schedules for people who have to travel at very short notice.

As mentioned in section C.2.2, the assessment of the need for optimal timing of booster doses should be built into the overall clinical development plan. However, as with other vaccines, it is commonly possible to gain an initial marketing authorization without specific data on antibody persistence and responses to booster doses and to modify the prescribing information at a later date whenever sufficient data become available.

C.2.4 Comparative immunogenicity studies

The clinical development programme for a novel JE vaccine should include at least one study in which the immune response is compared between the candidate and a licensed and widely used JE vaccine. These comparisons should
preferably be made in seronegative people since such studies would be more sensitive and thus better able to detect any real differences between vaccines.

In some instances it may be useful or necessary to perform studies to compare a new JE vaccine against more than one licensed product depending on the regions where subjects are enrolled and the JE vaccines that are available. If more than one comparative vaccine is used in the same study then the protocol should predetermine whether the primary analysis should 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 protocol and analysis plan.

The comparison between immune responses to the candidate and to the licensed vaccine should be assessed against the respective vaccine strains. Immune responses to heterologous strains should also be assessed. 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 non-inferiority will need very careful justification: published guidance should be consulted and expert statistical input obtained. In addition, protocols should plan for secondary analyses based on examination of a full range of immune response parameters.

Although provision of at least one comparative study 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. Therefore in these countries the regulatory approach to the data from such studies may not be the same as in the 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 non-inferiority and relatively more reliance on the immune response to the new vaccine especially in relation to PRNT$_{50}$ titres.

C.2.5 Concomitant vaccinations

As with all vaccines a specific endorsement in the prescribing information for co-administration with another vaccine should be supported by clinical data (see the WHO guidelines on regulatory expectations for the clinical evaluation of vaccines (4)).

However, special considerations would arise if it is proposed that a new JE vaccine could be co-administered with a vaccine against another flavivirus. Yellow fever vaccines are widely available and used and vaccines against dengue and West Nile fever are in development. There are some overlaps in endemic areas between each of these diseases and JE. The effects of co-administration of antigens from closely related flaviviruses on safety and immunogenicity cannot easily be predicted. Therefore special care may be needed when considering such investigations.
C.3  Safety

The general approach to the assessment of safety of a new JE vaccine during clinical studies should be in accordance with the WHO guidelines on regulatory expectations for the clinical evaluation of vaccines (4).

Matters relating to JE vaccines that require particular attention include:

- reactogenicity with sequential doses in the primary series and with boosters administered at different intervals;
- comparisons of reactogenicity between subpopulations with or without pre-existing antibody to JE virus and/or other flaviviruses as a result of natural exposure; and
- comparisons of reactogenicity between persons who have or have not been exposed to other JE vaccines and/or other flavivirus vaccines in the past.

The second and third items are especially important for actual use since pre-vaccination serostatus and the vaccination history may be unknown or uncertain.

C.4  Post-licensure investigations

C.4.1  Effectiveness

Because it is not feasible to study the protective efficacy of a new JE vaccine before initial licensure, it is highly desirable that plans should be made to assess its effectiveness by disease surveillance after its introduction into a vaccination programme. However, the following issues need to be taken into consideration:

- Unless a specific JE vaccine were 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 from natural exposure or previous vaccination. Therefore it may not be possible to extrapolate the findings from one region 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, manufacturers should discuss arrangements for continuous disease surveillance and the potential for estimating effectiveness with the relevant national regulatory authorities in the countries where the new vaccine is to be used and where reliable surveillance systems are in place.
Effectiveness data should be used in conjunction with data on antibody persistence to identify the need for and timing of booster doses.

C.4.2 Safety
The general considerations for safety surveillance and for development of a pharmacovigilance plan are the same as for all other types of medicinal products. It is particularly important that data are collected on any vaccine failures.

If particular issues arise during pre-licensure studies or during post-licensure safety surveillance it may be necessary to conduct specific post-licensure safety studies and/or to put in place a scheme for enhanced surveillance of specific adverse events.

C.4.3 Studies to support change in manufacturing process
Changes in production methods and/or vaccine formulation may sometimes require the provision of the results of a comparative clinical study. Such studies would usually compare the safety and immunogenicity of the “new” with that of the “previous” vaccine. The need for, and the design of, a clinical study intended to support the proposed change should be evaluated on a case by case basis after a careful assessment of the data provided by a manufacturer. For this reason, it is recommended that relevant national regulatory authorities should be consulted on all changes before they are implemented since this would enable an early appraisal of the likely need for clinical data to be generated. The design of a clinical study to support a change will depend on the primary objective. In most instances it is likely that the primary or co-primary objective would be to demonstrate that the immune responses to the “new” vaccine are non-inferior to those elicited by the “previous” vaccine. Further details on demonstrating non-inferiority are provided in the Guidelines on clinical evaluation of vaccines: regulatory expectations (4).

C.4.4 Studies to support new dosing schedules and a new population
In general any proposed modifications of the mode of use of a vaccine after initial licensure would require provision of suitable clinical data. Examples include endorsements for use in immunosuppressed, elderly and premature populations. In these cases it is usual to perform a comparative safety and immunogenicity study to compare vaccination of the population of interest with vaccination of the population in which the vaccine is already approved. In the case of adding recommendations for booster dose(s), data on antibody persistence and post-licensure effectiveness may indicate the need for and optimal timing of additional doses.
Part D. Recommendations for national regulatory authorities

D.1 General
The general recommendations for national regulatory authorities provided in the *Guidelines for national authorities on quality assurance for biological products* (24) should be followed. These specify that no new biological substance should be licensed until consistency of production has been established.

The detailed production and control procedures as well as any change to them that may affect quality, safety and efficacy of JE vaccine should be discussed with and approved by the national regulatory authority.

D.2 Release and certification
A vaccine lot should be released only if it fulfils Part A of these Recommendations. Before any vaccine lot is released from a manufacturing establishment, the recommendations for consistency of production provided in the *Guidelines for national authorities on quality assurance for biological products* (24) should be met.

A statement signed by the appropriate official of the national regulatory authority or national control laboratory should be provided and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these Recommendations. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory potency test as well as the assigned expiry date on the basis of shelf-life should be stated. A copy of the official national release document should be attached.

The purpose of the certificate is to facilitate the exchange of JE vaccine between countries.

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References


Appendix 1

Model summary protocol for Japanese encephalitis vaccine (inactivated) for human use

The following protocol is intended to provide general guidance, and indicates the information that should be provided as a minimum by the manufacturer to the national regulatory authority. The protocol should be accompanied by a lot release certificate from the licensing authority which may or may not be the country of manufacturing origin. Information and tests may be added or deleted as required by the national regulatory authority of the importing country, if applicable.

It is thus 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 guidance for a particular product should be given in the protocol submitted.

The section concerning the final product should be accompanied by a sample of the label and a copy of the leaflet that accompanies 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 national regulatory authority of the country in which the vaccine was produced stating that the product meets national requirements as well as Part A of these WHO Recommendations.

It is important to note that satisfactory test results do not necessarily imply that the vaccine is safe and effective, since many other factors should be taken into account, including the characteristics of the manufacturing facility.

1. Summary information on the finished product (final lot)

  International name: ____________________________
  Trade name: ____________________________
  Batch number(s): ____________________________
  Type of container: ____________________________
  Total number of containers in this batch: ____________________________
  Number of doses per container: ____________________________
  Composition (antigen concentration)/volume of single human dose: ____________________________
  Target group: ____________________________
  Date of expiry: ____________________________
  Storage temperature: ____________________________
  Product licence number: ____________________________
Name and address of manufacturer: ________________________________
Name and address of product licence holder
(if different): ________________________________

2. Production information

Purified bulk
Site of manufacture: ________________________________
Date of manufacture: ________________________________

Final bulk
Site of manufacture: ________________________________
Date of manufacture: ________________________________

Finished product
Site of manufacture: ________________________________
Date of manufacture: ________________________________

3. Cell banks and virus seeds

The information requested below is to be presented on each submission. Full details on cell banks and virus seed lots should be provided upon first submission only and whenever a change has been introduced.

3.1 Cell banks

Origin of cell substrate ________________________________

Master cell bank (MCB)
Lot number: ________________________________
Date of preparation: ________________________________
Population doubling level: ________________________________

Manufacturer’s working cell bank (MCWB)
Lot number: ________________________________
Date of preparation: ________________________________
Population doubling level: ________________________________

Identification of cell substrate
Method: ________________________________
Specification: ________________________________
Date: ________________________________
Result: ________________________________
Nature and concentration of antibiotics or selecting agent(s) used in production cell culture maintenance medium: ____________________________

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

3.2. Virus seed
Strain name and short description of history: ____________________________

Master seed lot
Lot number: ____________________________
Date of preparation: ____________________________

Working seed lot
Lot number: ____________________________
Date of preparation: ____________________________

Number of passages between master and working virus seed lots: ____________________________

Number of subcultures between working seed lot and production: ____________________________

3.2.1 Test for each seed lot
Identity
Method: ____________________________
Specification: ____________________________
Date: ____________________________
Result: ____________________________

Bacteria and fungi
Method: ____________________________
Media: ____________________________
Volume inoculated: ____________________________
Date (test on – off): ____________________________
Result: ____________________________
Mycoplasmas
Method: ________________________________
Media: ________________________________
Volume inoculated: ____________________
Date (test on – off): ____________________
Result: ________________________________

Adventitious agents
Method: ________________________________
Specification: __________________________
Date: ________________________________
Result: ________________________________

Additional tests e.g. virus titration
Method: ________________________________
Specification: __________________________
Date: ________________________________
Result: ________________________________

4. Control cell cultures
Provide information on control cells corresponding to each single harvest.

Ratio or proportion of control to production cell cultures: ______________________________
Volume of control cells: ______________________________
Period of observation of cultures: ______________________________
Percentage rejected for non-specific reasons: ______________________________
Result: ______________________________

Identity test by DNA fingerprinting (if applicable)
Method: ________________________________
Probe: ________________________________
Reference cells: _________________________
Restriction enzymes: ____________________
Date (test on – off): ____________________
Result: ________________________________

Test for haemadsorbing viruses
Type(s) of red blood cell (RBC): ______________________________
Storage time and temperature of RBC: ______________________________
Incubation time and temperature of RBC: _________________________________
Percentage cultures tested: ____________________________________________
Date (test on – off): _________________________________________________
Result: _____________________________________________________________

Tests on supernatant fluids for other adventitious agents (if relevant)
Date of sampling from production ______________________________________
cell cultures: _________________________________________________________

Name of production cell: _____________________________________________
Quantity of sample inoculated: _________________________________________
Incubation temperature: _____________________________________________
Date (test on – off): _________________________________________________
Percentage of viable culture at the end: _________________________________
Result: _____________________________________________________________

Name of human cells: ________________________________________________
Quantity of sample inoculated: _________________________________________
Incubation temperature: _____________________________________________
Date (test on – off): _________________________________________________
Percentage of viable culture at the end: _________________________________
Result: _____________________________________________________________

Name of other sensitive cells: _________________________________________
Quantity of sample inoculated: _________________________________________
Incubation temperature: _____________________________________________
Date (test on – off): _________________________________________________
Percentage of viable culture at the end: _________________________________
Result: _____________________________________________________________

5. **Single harvests (or pools)**
Batch number(s): _____________________________________________________
Date of inoculation: _________________________________________________
Date of harvesting: _________________________________________________
Volume(s), storage temperature, storage time
and approved storage period: _________________________________________

Pooling of single virus harvest
Number of single harvests pooled: _________________________________
Volume of pooled bulk material: _________________________________
Bacteria and fungi
Method: ________________________________
Media: ________________________________
Volume inoculated: ________________________________
Date (test on – off): ________________________________
Result: ________________________________

Identity of vaccine virus
Method: ________________________________
Specification: ________________________________
Date: ________________________________
Result: ________________________________

Virus content
Method: ________________________________
Specification: ________________________________
Date: ________________________________
Result: ________________________________

Consistency of yield
Method: ________________________________
Reference preparation: ________________________________
Specification: ________________________________
Date: ________________________________
Result: ________________________________

6. Purified bulk
Batch number(s) of inactivated, purified bulk: ________________________________
Date(s) of purification(s) and/or inactivation: ________________________________
Volume(s), storage temperature, storage time and approved storage period: ________________________________

6.1 Inactivation
Agent and concentration: ________________________________
Temperature: ________________________________

Period of inactivation
Date of start of inactivation: ________________________________
Date of completion of inactivation: ________________________________
Test for effective inactivation
Method: __________________________________________
Specification: ____________________________________
Date: ___________________________________________________________________________
Result: __________________________________________________________________________

6.2 Purification of virus
Method of purification: __________________________________________
Concentration: _____________________________________________________________________

6.3 Tests on purified bulk
Myelin basic protein content (if applicable)
Method: __________________________________________
Specification: ____________________________________
Date: ___________________________________________________________________________
Result: __________________________________________________________________________

Protein content
Method: __________________________________________
Specification: ____________________________________
Date: ___________________________________________________________________________
Result: __________________________________________________________________________

Antigen content
Method: __________________________________________
Specification: ____________________________________
Date: ___________________________________________________________________________
Result: __________________________________________________________________________

Ratio of antigen: protein content
Specification: ____________________________________
Result: __________________________________________________________________________

Residual DNA (if applicable)
Method: __________________________________________
Specification: ____________________________________
Date: ___________________________________________________________________________
Result: __________________________________________________________________________

Residual animal serum
Method: __________________________________________
7. **Final bulk**

Batch number: ______________________________
Date of manufacture: __________________________

Batch numbers and volumes of purified bulk vaccines used for the formulation of the final bulk vaccine: ______________________________

Batch number(s) and volume(s) of bulk alum diluent (if applicable): ______________________________

Volume, storage temperature, storage time and approved storage period: ______________________________

Preservatives and other substances – name and concentrations: ______________________________

7.1 **Tests on final bulk**

**Bacteria and fungi**
Method: ______________________________
Media: ______________________________
Volume inoculated: ______________________________
Date (test on – off): ______________________________
Result: ______________________________

**Adjuvants (if applicable)**
Method: ______________________________
Specification: ______________________________
Date: ______________________________
Result: ______________________________
Degree of adsorption (if applicable)
Method: ______________________________________
Specification: ______________________________________
Date: ______________________________________
Result: ______________________________________

Preservative content
Method: ______________________________________
Specification: ______________________________________
Date: ______________________________________
Result: ______________________________________

Potency
Species, strain, sex and weight specifications: ______________________________________
Dates of vaccination, bleeding: ______________________________________
Date of assay of each type: ______________________________________
Batch number of reference vaccine and assigned potency: ______________________________________
Vaccine doses (dilutions) and number of animals responding at each dose for each type: ______________________________________
Specification: ______________________________________
PRNT$_{50}$\(^1\) or ED$_{50}$\(^2\) of reference and test vaccine for each type: ______________________________________
Potency of test vaccine versus reference vaccine for each type with 95% fiducial limits of mean: ______________________________________

8. Final lot
Batch number: ______________________________________
Date of filling: ______________________________________
Type of container: ______________________________________
Filling volume: ______________________________________
Number of containers after inspection: ______________________________________

Inspection of final container (appearance)
Method: ______________________________________
Specification: ______________________________________

\(^1\) Plaque reduction neutralization test.
\(^2\) Effective dose (the dosage that produces a desired effect in half the test population).
Date: ____________________________
Result: __________________________

**Identity**
Method: ____________________________
Specification: ______________________
Date: ____________________________
Result: ____________________________

**Bacteria and fungi**
Method: ____________________________
Media: ____________________________
Volume inoculated: __________________
Date (test on – off): __________________
Result: ____________________________

**pH**
Method: ____________________________
Specification: ______________________
Date: ____________________________
Result: ____________________________

**Osmolarity**
Method: ____________________________
Specification: ______________________
Date: ____________________________
Result: ____________________________

**General safety (abnormal toxicity)**
Method: ____________________________
Specification: ______________________
Date: ____________________________
Result: ____________________________

**Protein content**
Method: ____________________________
Specification: ______________________
Date: ____________________________
Result: ____________________________

**Residual DNA (if applicable)**
Method: ____________________________
Annex 1

Specification: __________________________________________
Date: __________________________________________
Result: __________________________________________

**Potency**

Species, strain, sex and weight specifications: __________________________________________
Dates of vaccination, bleeding: __________________________________________
Date of assay of each type: __________________________________________
Batch number of reference vaccine and assigned potency: __________________________________________
Vaccine doses (dilutions) and number of animals responding at each dose for each type: __________________________________________
Specification: __________________________________________
PRNT\textsubscript{50} or ED\textsubscript{50} of reference and test vaccine for each type: __________________________________________
Potency of test vaccine versus reference vaccine for each type with 95% fiducial limits of mean: __________________________________________

**Accelerated thermal stability**

Method: __________________________________________
Specification: __________________________________________
Date: __________________________________________
Result: __________________________________________

**Residual moisture (if applicable)**

Method: __________________________________________
Specification: __________________________________________
Date: __________________________________________
Result: __________________________________________

**Pyrogenic substances**

Method: __________________________________________
Specification: __________________________________________
Date: __________________________________________
Result: __________________________________________

**Residual animal serum albumin**

Method: __________________________________________
Specification: __________________________________________
WHO Expert Committee on Biological Standardization  Fifty-eighth report

Date: ____________________________
Result: __________________________

Preservatives
Method: __________________________
Specification: _______________________ 
Date: __________________________
Result: __________________________

Adjuvant content (if applicable)
Method: __________________________
Specification: _______________________ 
Date: __________________________
Result: __________________________

Degree of adsorption (if applicable)
Method: __________________________
Specification: _______________________ 
Date: __________________________
Result: __________________________

Extractable volume (if applicable)
Method: __________________________
Specification: _______________________ 
Date: __________________________
Result: __________________________

Freezing point (if applicable)
Method: __________________________
Specification: _______________________ 
Date: __________________________
Result: __________________________

Other tests
Additional comments (if any): __________________________
____________________________________________________

A sample of a completed final container label and package insert should be attached.
9. **Certification**

9.1 **Certification by producer**

Certification by head of the quality assurance department taking overall responsibility for production and control of the final vaccine:

I certify that lot no ___________ of Japanese encephalitis vaccine (inactivated) for human use, whose number appears on the label of the final container, meets all national requirements\(^3\) and satisfies Part A of the WHO *Recommendations for Japanese encephalitis vaccine (inactivated) for human use*.

Signature: __________________________________________
Name (typed): __________________________________________
Date: __________________________________________

9.2 **Certification by the national controller**

If the vaccine is to be exported, please provide a copy of the certificate from the national regulatory authority as described in section D.2 and by referring to the model certificate in Appendix 2, together with a label for a final container and a leaflet containing instructions for users.

\(^3\) If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.
Appendix 2

Model certificate for the release of Japanese encephalitis vaccine (inactivated) for human use

Certificate No. ________________

Lot release certificate

The following lot(s) of Japanese encephalitis vaccine (inactivated) for human use produced by ________________\(^1\) in ________________,\(^2\) whose numbers appear on the labels of the final containers, meet all national requirements\(^3\) and Part A\(^4\) of the WHO Recommendations for Japanese encephalitis vaccine (inactivated) for human use (_______),\(^5\) and comply with Good manufacturing practices for pharmaceutical products: main principles\(^6\) and Good manufacturing practices for biological products.\(^7\)

As a minimum, this certificate is based on examination of the summary protocol of manufacturing and control.

<table>
<thead>
<tr>
<th>Final lot no.</th>
<th>No. of released human doses in this final lot</th>
<th>Expiry date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Director of the National Regulatory Authority (or Authority as appropriate):

Name (typed) ___________________________________________________________
Signature __________________________________________________________
Date ________________________________________________________________

---

\(^1\) Name of manufacturer.

\(^2\) Country of origin.

\(^3\) If any national requirements are not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national regulatory authority.

\(^4\) With the exception of provisions on distribution and shipping, which the national regulatory authority may not be in a position to assess.


## Appendix 3

### General scheme for the preparation of Japanese encephalitis vaccines (inactivated) for human use

<table>
<thead>
<tr>
<th>Stage</th>
<th>Procedures</th>
<th>Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single harvest (1 production run)</td>
<td></td>
<td>• Sterility &lt;br&gt; • Identity &lt;br&gt; • Virus content</td>
</tr>
<tr>
<td>• Cell culture-derived vaccine</td>
<td>• Mouse brain-derived vaccine</td>
<td>Inactivation</td>
</tr>
<tr>
<td>Purified bulk (1 or more pooled harvests)</td>
<td>Filtration or continuous centrifugation &lt;br&gt; Inactivation &lt;br&gt; Purification</td>
<td>• Inactivation &lt;br&gt; • Myelin basic protein &lt;br&gt; • Protein content &lt;br&gt; • Antigen content &lt;br&gt; • Residual DNA &lt;br&gt; • Residual animal serum &lt;br&gt; • Residual chemicals</td>
</tr>
<tr>
<td>• Cell culture derived vaccine</td>
<td>• Mouse brain derived vaccine</td>
<td>Purification</td>
</tr>
<tr>
<td>Final bulk (1 or more pooled purified bulks)</td>
<td>Addition of preservatives and stabilizers</td>
<td>• Sterility &lt;br&gt; • Adjuvant content if applicable &lt;br&gt; • Preservative content &lt;br&gt; • Potency</td>
</tr>
<tr>
<td>Final lot</td>
<td>Filling</td>
<td>• Inspection of final containers &lt;br&gt; • Identity &lt;br&gt; • Sterility &lt;br&gt; • pH &lt;br&gt; • Osmolarity &lt;br&gt; • General safety &lt;br&gt; • Protein content &lt;br&gt; • Residual DNA &lt;br&gt; • Potency &lt;br&gt; • Accelerated stability &lt;br&gt; • Residual moisture &lt;br&gt; • Pyrogenicity &lt;br&gt; • Residual animal serum &lt;br&gt; • Preservative content &lt;br&gt; • Adjuvant content if applicable</td>
</tr>
</tbody>
</table>