WHO Technical Report Series, No. 910, 2002

Annex 3
Guidelines for the production and control of Japanese encephalitis vaccine (live) for human use

This document provides information and guidance concerning the history, characteristics, production and control of live attenuated Japanese encephalitis (JE) vaccine to facilitate progress towards the eventual international licensure of the vaccine. The text is therefore written in the form of guidelines instead of Recommendations in view of the fact that further work is needed to develop and standardize appropriate methods and criteria for certain tests, such as the neurovirulence test. Guidelines allow greater flexibility than Recommendations with respect to expected future developments in the field.

1. General considerations[1]

1.1 Japanese encephalitis vaccine

Japanese encephalitis (JE) virus is the most important cause of viral encephalitis in the Asia–Pacific region, accounting for more than 16 000 reported cases and 5000 deaths annually. JE virus is a mosquito-borne flaviviral infection. In the last 25 years, JE virus transmission has intensified in certain countries and the disease has extended its geographical range to previously unaffected areas of Asia and to northern Australia. The high fatality rate and frequent residual neuropsychiatric sequelae in survivors make JE a considerable health problem. For example, in a placebo-controlled study in Thailand in which patients with JE received a high standard of supportive care that included treatment with dexamethasone, 25% of the patients died and 45% demonstrated neurological sequelae 3 months after diagnosis. Furthermore, in the best-documented study of long-term disability due to JE, which was conducted 10 years after the 1947 outbreak of the disease in Guam, neurological sequelae were reported in 40% of surviving patients, 11% of which were considered severe. Neither of these studies evaluated the proportion of cases with psychomotor retardation, fine motor deficits or behavioural disorders.

JE virus is amplified in nature in a cycle involving *Culex* mosquitoes and vertebrate animals, especially pigs. Humans of all ages are susceptible unless immunized by natural infection or vaccination. Evidence shows that effective vaccines will protect animals and humans against illness and will remove the vaccinated animals from the pool of potential amplifying hosts of the virus. Although the control of mosquitoes and the vaccination of pigs are effective in certain circumstances, these measures are not practical as a means of preventing human illness. It is also important to recognize that humans are incidental hosts, and that, for vaccination to be effective, coverage must be maintained indefinitely in all persons who may be exposed to the virus.

The virus replicates in a variety of cultured cells of vertebrate and arthropod origin. Since the 1960s, both live and inactivated vaccines have been developed that provide active immunity against JE virus. The development of these vaccines represents a major advance in the ability to control JE virus infection and reduce the burden of disease. Viruses isolated from human patients in Japan in 1935 and in China in 1949 provided the prototype Nakayama and Beijing and P3 strains, respectively, that are in principal use in the production of inactivated

[1] Parts of the text of this section are abstracted from the minutes of a WHO/(Children's Vaccine Initiative (CVI)) meeting on new initiatives for the control of Japanese encephalitis by vaccination, held in Bangkok, Thailand, 13–15 October 1998 (*1*).

JE vaccine today. National vaccination programmes in China (Province of Taiwan), Japan and the Republic of Korea, using an inactivated mouse-brain-derived vaccine that meets international requirements (*2*), have controlled the disease to the point of elimination, but in other countries, the expense and complexity of producing the vaccine and the need for repeated doses have limited vaccine use. In addition to the problems posed by multiple doses, use of the vaccine to protect travellers has led to hypersensitivity events, including demyelination sequelae, such events have been reported in North America, Australia and Europe.

As an alternative to inactivated vaccines, a live-attenuated JE vaccine (the SA 14-14-2-PHK strain) was developed in China. Since its licensure in China in 1988, more than 300 million doses of JE vaccine (live) have been produced for administration to children in annual vaccination programmes. The vaccine is of considerable interest to countries where JE virus is endemic but, as of the year 2000, is not yet licensed elsewhere.

1.2 History of development of the live attenuated SA 14-14-2 vaccine and characteristics of the vaccine strain[1]

The wild-type parental virus, SA 14, was isolated from a pool of *Culex pipiens* larvae from Xian, China, by 11 passages in mouse brain. The derivation of the SA 14-14-2 strain was through an empirical process of serial passage, principally in primary hamster kidney (PHK) cells, and demonstrated that a fine balance exists between safety through stable neuroattenuation and immunogenicity, with sufficient viral replication to stimulate immunity. Properties of viral clones in the pedigree from which the vaccine strain was eventually derived illustrate the inherent subtleties of JE viral attenuation. The early 12-1-7 strain exhibited a low degree of pathogenicity; however, it was unstable and reverted to neurovirulence after one passage in mouse brain or several passages in PHK cells. The 9-7 strain derived from the 12-1-7 clone was attenuated in mice and monkeys and did not revert with passage in either mouse brain or PHK cells, but it produced seroconversions in less than 10% of vaccinated children. To increase its immunogenicity, the strain was passaged orally in hamsters and plaque purified in primary chick embryo (PCE) cells to derive the 5-3 strain. This clone was attenuated, but its immunogenicity was only 65% of vaccinated children. Further passages in suckling mice and plaque purifications in PHK cells led to the 14-14-2 strain, which was stably attenuated and immunogenic in 85–100% of vaccinated children.

[1] Further details have been published (*1*).

In contrast to its parent strain, the SA 14-14-2 strain is avirulent when administered by the intracerebral and intraperitoneal routes in weanling mice, Syrian hamsters and in mice given immunosuppressive treatment with cytoxan. The virus is virulent for nu/nu mice only when administered by intracerebral inoculation, but with a longer incubation period than its parent. Monkeys inoculated by both the intrathalamic and intraspinal routes develop asymptomatic infections and, on neuropathological examination, exhibit a minor degree of inflammatory reaction only along the needle track, with minimal neuronal infection or neuronal death. Small plaque morphology and neuroattenuation in mice is retained through at least 23 further PHK cell passages, using conditions of infection (e.g. multiplicity of infection and incubation temperature) identical to those employed in production. Molecular studies indicated that the sequence of the neurovirulent parent SA 14 differs from that of SA 14-14-2 in 57 nucleotides and results in 24 amino acid changes. Studies also showed that eight amino acid substitutions in the E protein genome of SA 14-14-2 vaccine virus were unchanged after additional passage in PHK cells. The points mentioned above provide strong evidence for the genetic stability of the SA 14-14-2 vaccine virus in the PHK cell substrate.

An initial attempt to adapt the SA 14-14-2 strain to primary dog kidney cells found that only nine additional passages led to further attenuation and a reduction in its immunogenicity to only 40% of vaccinated children.

The complete passage history of the SA 14-14-2 strain is given in Appendix 1. Because the passage history of this strain included passage in both hamsters and mice, special attention is required to demonstrate the absence of detectable adventitious agents of these two species in the seed stocks of the vaccine. As with any vaccine, tests for adventitious agents may need to be extended as new infectious agents or test procedures become known and available.

The SA 14-14-2 strain grows to a titre of $>10^7$ in PHK cells and produces a cytopathic effect and small plaques under overlay. The use of PHK cells for routine production of JE vaccine (live) is clearly effective, but raises particular cell substrate issues (see section 1.4).

An alternative strategy to the current vaccine production system, which includes extensive testing as described below, is to re-derive a virus master seed from the current seed. This approach would require considerable effort to characterize the new seed and to assess its clinical safety and efficacy, but would result in a significant reduction in testing during manufacture.

1.3 Preclinical studies

In guinea-pigs, a single dose of SA 14-14-2 virus elicits immunity that significantly reduces viraemia levels produced by viral challenge. Either spleen cells or serum could passively transfer immunity. Evidence that the live vaccine elicited a stronger cellular immune response than inactivated vaccine was seen in challenge experiments comparing mice immunized with the respective vaccines. Despite equal titres of circulating neutralizing antibody, survival after intracerebral challenge was significantly higher in animals previously immunized with live vaccine than in those immunized with inactivated vaccine. Cytoxan immunosuppression of mice vaccinated with SA 14-14-2 did not alter their resistance to lethal viral challenge in contrast to mice vaccinated with inactivated vaccine, in whom survival was reduced by 90% after immunosuppression.

In vaccination/challenge studies, mouse survival was significantly greater after vaccination with one dose of live vaccine compared with two doses of inactivated vaccine derived from mouse brain or PHK cells, and challenge with the P3, Nakayama or 12 field strains isolated in China. Experiments also showed that one dose of the live vaccine protected 90–100% of mice against challenge with field strains isolated in countries such as India, Indonesia, Japan, the Philippines, Thailand and Viet Nam (*3*).

Although the growth of SA 14-14-2 virus in *Culex tritaeniorhynchus* has not so far been evaluated, the attenuated SA 14-1-8 clone, derived from the same pedigree with a similar phenotype, showed no transmission in experimental studies (*4*). In contrast, the SA 14 parent was transmitted in mosquitoes at rates of 75–78%. Recent studies to assess the extent of detectable viraemia following vaccination with the SA 14-14-2 strain showed that JE virus is undetectable from the time of inoculation through day 9. These results are consistent with previous reports showing that wild-type JE virus is undetectable in infected symptomatic persons and that there is no amplification of JE virus in humans. In view of the above, and the estimated requirement for 100 000–1 000 000 virions per ml of plasma for mosquitoes to access sufficient virus from a human to transmit to others (*5*), it is highly unlikely that the SA 14-14-2 strain would be transmitted via a mosquito bite of a recent vaccinee. Nevertheless, further consideration should be given to additional transmission studies with the SA 14-14-2 vaccine strain.

1.4 Cell substrate issues

Prevention of transmission of adventitious infections from the virus seed, the cell substrate, and the serum or trypsin used in the manufac-

turing process, is a general concern with all live-virus vaccines. For the SA 14-14-2 vaccine, the lack of precedence for a PHK cell substrate in live attenuated vaccine is a special issue. However, PHK is recognized as an acceptable substrate for inactivated JE vaccine and hantavirus vaccine (Haemorhogic Fever with Renal Syndrome (HFRS) vaccine). Current controls cover a broad range of potential rodent virus contaminants. State-of-the-art developments should be introduced as appropriate. Validation issues with respect to the assays used, e.g. the application of assays for retroviruses to PHK cells, need to be considered. In addition, the principle of reducing the risk of adventitious agents entering the manufacturing process should be encouraged by using healthy animals, preferably from a closed specific pathogen-free colony that is monitored regularly, as a source material for preparation of PHK cells. In common with all live-virus vaccines, steps to exclude potential contaminants of serum and trypsin employed in manufacturing, including specific bovine and porcine viruses and transmissible spongiform encephalopathy agents, would be expected.

The demonstration of consistency of vaccine production in PHK cell culture is also important. As of 2000, three manufacturers in China have 12, 6 and 1 years of experience, respectively, of vaccine production in PHK cells. Criteria that may be monitored include aspects of cell growth such as morphological characteristics and days-to-plating confluency. Consistency of viral yield is evaluated by precise and reproducible titration procedures and possibly by monitoring genotypic and phenotypic markers. For new manufacturers, consistency measures under conditions of scale-up and large-scale production are required.

The creation of a well-characterized master PHK cell bank at the secondary or tertiary level is a potential way to improve lot-to-lot consistency and simplify quality control. Preliminary experience at one manufacturer showed that plating efficiency declined by 40% after primary cells were frozen and thawed, but this does not mean that this approach would not be practicable in the future. Attempts to adapt the SA 14-14-2 strain to other cell cultures would be expected to result in fundamental changes in biological characteristics, and attempts using MRC-5, primary chick and duck embryo cells, and primary dog kidney (PDK) cells were unsuccessful (see above). Preliminary research indicates that the strain can be adapted to Vero cells with the preservation of neuroattenuation in mice but preclinical and clinical equivalence with the PHK-produced SA 14-14-2 vaccine remains to be proven.

1.5 Neurovirulence testing

During development of the SA 14-14-2 strain, it was demonstrated that attenuation and a lack of reversion to neurovirulence of some JE

virus clones could be achieved. This was on the basis of extensive testing in mice, using three different types of test: a test for neurovirulence, a test for reversion to neurovirulence; and a test for neurotropism. It is therefore necessary to demonstrate that master and working seeds, and vaccine lots, have retained the characteristics of attenuation of the original SA 14-14-2 strain. The tests that were used in the development of the vaccine are described later in these Guidelines. It will be necessary to demonstrate that the tests will reliably discriminate suitable from unsuitable materials. Participation in collaborative studies and inclusion of a common reference preparation that is tested in parallel using the same procedures would be extremely useful and is strongly encouraged.

1.6 Clinical safety[1]

Vaccine safety has been evaluated in several small-scale studies and in two large-scale postmarketing studies in China. Studies of 588 512 children aged between 1 and 15 years inoculated with vaccine from one manufacturer and of 60 000 children given vaccine from another manufacturer reported no cases of temporally associated encephalitis. The most common adverse effect associated with vaccination was fever, which was reported in less than 0.2% of vaccinated children, with lower rates for rash and other systemic symptoms.

Daily examination of 867 vaccinated children for fever (>38 °C) disclosed low rates with onset distributed evenly over the 21-day observation period, without clustering as might have been expected if onset were associated with a specific incubation period. Temperature elevations were limited to a single day in most cases. In uncontrolled observations of 1946 children vaccinated between the ages of 1 and 6 years, short-term fever was reported in only two, who had concurrent respiratory infections. Local reactions, occurring in 6.2% of vaccinees overall, were more common in older children who had received previous doses of inactivated JE vaccine.

One longer-term study followed approximately 150 000 children vaccinated between 1 and 6 years of age for 5 years and found no potential late-onset complications such as optic atrophy (*6*). Another recent study (7) included 230 children aged 13–15 years who were evaluated clinically and serologically at 1, 2, 2.5 and 3 months after vaccination. No neurological signs or symptoms, or other significant medical sequelae were found.

The best-characterized study, conducted under the auspices of the Rockefeller Foundation in Chengdu, southwestern China, compared

[1] Further details have been published (*1*).

hospitalizations and specific illnesses and symptoms in block randomized cohorts of more than 13 000 vaccinated children and more than 12 500 children in whom routine JE vaccination was deferred for 1 month (*8*). The 1–2-year-olds were followed prospectively for 1 month after their initial visit for vaccination, and on their return, parents were questioned about hospitalizations and the occurrence of specific illnesses in the intervening month, including encephalitis and meningitis, and the new onset of seizures. No illnesses involving the central nervous system were reported in either group, and the rates of occurrence of recent onset of seizures, hospitalizations, fever lasting >3 days, and various other illnesses such as diarrhoea, respiratory infections and reactions consistent with anaphylaxis were similar in the two groups.

A subset of 266 vaccinated children was examined prospectively for side-effects 1, 2, 3 and 7 days after the vaccination visit. Various minor local and systemic symptoms were observed, but all at a low rate. Fever occurred in 5% of vaccinees. This study provides convincing evidence of the short-term safety of the SA14-14-2 vaccine during the first 30 days after immunization.

Concern that a live vaccine derived from an encephalitogenic virus might lead to vaccine-associated encephalitis could not be addressed to a high level of sensitivity, even with a study of 26 000 children. From the observation of no cases in the month after immunization, an interval expected to encompass the incubation period of infectious encephalitis, the upper 95% confidence interval indicates the rate of encephalitis following vaccination with SA 14-14-2 vaccine is unlikely to exceed 2.3 cases per 10 000.

Since the introduction of SA 14-14-2 vaccine in China in 1988, over 300 million doses have been given to an estimated 120 million children. All doses were administered on a campaign basis in March and April of each year. Vaccine-associated cases of encephalitis would be expected to occur in April and May, thus creating a shoulder on the normal seasonal curve of hospitalization due to JE. No such shoulder has been observed in a preliminary examination of the data. Further monitoring and refinement of these data are continuing in China, and are encouraged. Other epidemiological opportunities to evaluate the safety of the vaccine should also be considered.

An assessment of the benefits and risks related to SA 14-14-2 vaccine is an important element in determining the appropriate use of the vaccine, and should include an evaluation of the health burden attributable to JE before the introduction of the vaccine in another country.

1.7 Immunogenicity in children[1]

Immunogenicity in children follows a dose–response gradient, with antibody responses in >92% of vaccinees receiving 1 000 000 viral plaque-forming units (PFU) from SA 14-14-2 vaccines produced in China after 1988. Antibody responses are seen in all subjects given a second dose. Among the responders to primary immmunization, all retained measurable neutralizing antibodies for at least 3 years. On the basis of data on the immunogenicity and efficacy of the vaccine in children in various ethnic groups in China as well as in children in Nepal and the Republic of Korea, the immune response does not appear to be influenced by the ethnic background of vaccinees.

1.8 Efficacy in clinical studies[1]

The SA 14-14-2 vaccine was shown to provide protection in over 300 000 children aged between 1 and 10 years in four field trials. The efficacy of one dose was >95% in every trial and, in one location where the vaccinated children were followed through five transmission seasons, the efficacy was >98% throughout this interval. In 1999, the SA 14-14-2 vaccine was given as a single dose to over 220 000 residents of the Terai region of Nepal in an effort to reduce the impact of an emerging epidemic of JE. A case–control study demonstrated 99.12% efficacy (*9*).

The above-mentioned trials were conducted in such a way that may have introduced biases in risk of exposure in vaccinated and unvaccinated subjects. However, when a fully effective vaccine is licensed and in actual use, it is not ethically permissible to re-study vaccine efficacy using a placebo-controlled field trial. A reliable, sensitive and accurate alternative that takes advantage of inadvertent vaccine failures, usually due to missed immunizations, is a case–control study in which the vaccination histories of cases and matched controls are compared. A study conducted under the auspices of the Rockefeller Foundation in Sichuan province, China, in which all village children of the same age as serologically confirmed JE cases were selected as controls, found an effectiveness of 98% with two doses and 80% after one dose (95% confidence interval (CI), 44–93%). The broad confidence interval of the latter estimate reflects the small number of subjects in that category. The results of this study support the previously cited studies of the effectiveness of SA 14-14-2 vaccine while extending those observations relating to its effectiveness under field conditions of delivery, storage and administration.

[1] Further details have been published (*1*).

2. Scope

These Guidelines relate to the production of JE vaccine (live) in PHK cells using the SA 14-14-2 strain of virus. No provision has been made for vaccines produced with other viral strains or cell lines.

The Guidelines cover control of the following three areas:

— the starting materials;
— the manufacturing process;
— the final product.

The general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (*10*) and Biological (*11*) Products should apply to establishments manufacturing live JE vaccine, with the addition of the following:

All staff directly involved in the production and testing of live JE vaccine should be shown to be immune to JE by appropriate haemagglutination inhibition or neutralizing antibody tests.

Written descriptions of the standard operating procedures used for the preparation and testing of live JE vaccine, together with evidence of appropriate validation of each production step, should be submitted for approval to the national control authority as part of the licensing application. Proposals for any modifications of the manufacturing and/or control methods should be submitted for approval to the national control authority before they are implemented.

2.1 Control of starting materials

2.1.1 *Animals*

Syrian hamsters aged between 10 and 14 days may be used as the source of kidneys for cell culture. Only hamster stock approved by the national control authority and derived from a closed, healthy colony should be used as the source of tissue. 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 should be tested according to a defined programme to ensure freedom from specified pathogens and antibodies to those pathogens. At the time the colony is established, all animals should be tested to show freedom from antibodies to Hantaan virus, Kilham rat virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse hepatitis virus, mouse poliovirus, pneumonia virus of mice, respiratory enteric orphan (REO) virus 3, Sendai virus (parainfluenza virus 1), Simian virus 5 and Toolans H-a virus. Tests for production of hamster, mouse and rat antibodies should also be performed. A test for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse

transcriptase (Rtase) assay should also be included. The results of such assays may need to be interpreted with caution because Rtase activity is not unique to retroviruses and may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (*12*). Nucleic acid amplification tests for retroviruses 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 should be repeated at intervals thereafter. When new animals are introduced into the colony, they should be maintained in quarantine in vermin-proof quarters for a minimum of 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 maintained in vermin-proof quarters. Neither the parent hamsters nor their progeny should previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored for zoonotic viruses and markers of contamination according to a defined programme at regular intervals.

Once the colony is established, it should be monitored by testing a representative group of animals consisting of at least 5% of the animals that are bled at intervals acceptable to the national control authority. For example, birds used in the production of chick embryo fibroblast cells for measles vaccine (*13*) are bled at monthly intervals. In addition, the colony should be screened for pathogenic bacteria, including mycobacteria, fungi and mycoplasmas, as agreed with the national control authority. The screening programme should test 100% of the animals over a defined period of time, as agreed with the national control 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 control authority should be informed and the manufacture of live JE vaccine may 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 then only with the approval of the national control authority.

At the time of harvest of the kidneys, the animals should be examined for the presence of any abnormalities. If any kidney abnormalities or other evidence of pathology is found, those animals should not be used for JE vaccine production.

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

2.1.2 *Cell cultures for virus propagation*

2.1.2.1 Primary hamster kidney cells

Kidneys derived from animals which comply with section 2.1.1 should be dissected and homogenized under conditions approved by the national control authority. A primary cell suspension is obtained after digestion with trypsin and this is distributed, together with growth medium, into cell culture vessels.

Penicillin and other β-lactam antibiotics should not be used at any stage of manufacture.

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

2.1.2.2 Serum used in cell culture medium

Serum used for the propagation of cells for JE vaccine production should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in Part A, sections A.5.2 and A.5.3 of the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*), and to demonstrate freedom from infectious viruses.

> In some countries, sera used for other vaccines are also examined for freedom from certain phages.

Serum of bovine origin should be approved by the national control authority and should come from countries or herds certified to be free of bovine spongiform encephalopathy and should comply with the requirements given in the Requirements for the Use of Animal Cells as in vitro Substrates for the Production of Biologicals (*15*) and the Report of a WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Encephalopathies (*16*).

> For other vaccines, some countries require that bovine serum should come from herds that have not been given feed derived from ruminant protein.

Suitable tests for detecting bovine viruses in serum are given in Appendix 1 of the 1999 Recommendations for the Production and Control of Poliomyelitis Vaccine (Oral) (*17*).

If human albumin is used, it should meet the 1992 Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives (*18*), as well as current guidelines in relation to human transmissible encephalopathies (*16*). In addition, human albumin, if used, should be tested by PCR to demonstrate freedom from human immunodeficiency virus (HIV) and hepatitis C virus nucleic acids.

2.1.2.3 Trypsin used for preparing cell cultures

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

The source(s) of bovine trypsin, if used, should be approved by the national control authority. Bovine trypsin, if used, should comply with the Requirements for the Use of Animal Cells as in vitro Substrates for the Production of Biologicals (*15*) and the Report of a WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Encephalopathies (*16*).

2.1.3 ***Virus seeds***

The virus master and working seed lots used to produce JE vaccine (live) should be shown to be safe and immunogenic by appropriate laboratory tests (sections 2.1.3.2.1, 2.1.3.2.2 and 2.2).

2.1.3.1 Virus strain certification

Only SA 14-14-2 PHK strains of JE virus that are approved by the national control authority should be used in the production of vaccine. Each strain should be identified by historical records, which should include information on its origin, its method of attenuation, and the passage level(s) at which attenuation, immunogenicity, safety and efficacy were demonstrated by clinical studies.

2.1.3.2 Virus seed lot system

The production of vaccine should be based on the virus seed lot system that includes a master seed and a working seed. All virus seed lots should be stored in a suitable manner.

2.1.3.2.1 Tests on virus master seed lots

The virus master seed should be produced under the conditions described in section 2.2, and should be free from detectable adventitious agents.

(1) Identity test

Each virus master seed lot should be identified as JE virus by serological methods approved by the national control authority.

Appropriate serological methods include enzyme immunoassays using a JE virus-specific monoclonal antibody, immunofluoresence, or JE neutralization assays using a reference serum or monoclonal antibody specific to JE virus. A test for genetic identity should also be performed.

(2) Virus titration and infectivity

The infectivity of each virus master seed lot should be established in an assay approved by the national control authority, such as the PFU method in the baby hamster kidney (BHK)-21 cell line. Alternative cell lines may also be acceptable to the national control authority. Manufacturers should determine the appropriate titre to reliably produce vaccine.

In the PFU method in BHK-21 cells used in some laboratories, samples from the virus suspension are diluted 10-fold serially, and the titre is estimated after plaques have developed.

The titration should be made in parallel with a reference vaccine that is approved by the national control authority.

Alternative assays such as that to determine the cell-culture infective dose 50% ($CCID_{50}$) may be used with the approval of the national control authority, provided they have been calibrated against the PFU assay.

(3) Freedom from bacteria, fungi and mycoplasmas

Each virus master seed lot should be shown to be free from bacterial, mycotic and mycoplasmal contamination by appropriate tests as specified the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*).

(4) Tests for adventitious agents

Each virus master seed lot should be tested in cell cultures for adventitious agents appropriate to the passage history of the seed virus. Neutralization of JE virus is necessary for many tests because the virus is generally cytopathogenic. JE virus immune serum, produced in a heterologous system, or a JE virus-specific monoclonal antibody, should itself be shown to be free of adventitious agents.

A volume of each virus master seed lot of at least 10 ml should be tested for adventitious agents by inoculation onto cultures of human cells, mouse cells, simian cells, mosquito cells (e.g. C6/36), BHK-21 cells and PHK cells. The cell culture should not be from the same batch as that used in the preparation of the virus seed. Uninoculated control cell cultures should be included in the tests. All cell cultures (except mosquito cells) should be incubated at 35–37 °C and observed for at least 14 days. At least one subculture of one cell culture fluid should be made and observed for 14 days in order to enhance the opportunity to detect adventitious agents.

The cells should be observed microscopically for cytopathic changes. At the end of the observation period, the cells or fluids should be tested for haemadsorbing viruses as specified in section 2.2.1.1. For the test to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period. For the seed virus to be satisfactory, no cytopathic changes or adventitious agents should be detected.

Tests for the following agents should be conducted on the virus master seed lot to rule out the presence of adventitious agents associated with any primary cell cultures that were used in the adaptation of the JE virus. Such agents include mouse viruses using the mouse antibody production (MAP) assay; hamster viruses using the hamster antibody production (HAP) assay; human, porcine and bovine viruses by PCR; endogenous hamster, avian and other retroviruses using a sensitive assay such as product-enhanced reverse transcriptase (PERT) (*12*).

Consideration should be given to electron microscopic investigation using the negative stain technique in order to obtain additional information on extraneous agents that may not be detected by other methods.

(5) Tests for attenuation

Neurovirulence tests in monkeys
New virus master seed lots should be tested for neurovirulence in monkeys (see Appendix 2). To avoid the unnecessary use of monkeys, virus master seed lots should be prepared in large quantities. A reference preparation should be included in each test. An alternative test in monkeys may be used, with agreement of the national control authority, if equal or greater sensitivity has been demonstrated.

Neurovirulence tests in mice
The virus master seed lot should be shown to be free from neurovirulence by a test in mice. A reference preparation should be prepared and included as a positive control to validate each test. The selection of one or more reference preparations is a matter of high priority and should be made in consultation with experts in neurovirulence testing who also should advise on the development and implementation of a collaborative study to validate the ability of the test system to reliably distinguish suitable from unsuitable vaccine preparations.

During development of the vaccine the following test was used:

Each of at least 10 mice of the Kunming strain of Swiss mice, 17–19 days old, were inoculated intracerebrally with 0.03 ml of the undiluted virus master seed lot. Mice were observed daily for 14 days after intracerebral inoculation. Mice that died within 3 days of inoculation were considered to have died from brain trauma, and were not included in the evaluation of the test. If more than 20% of mice died within 3 days, the test was considered invalid. From any mouse that showed signs of any illness more than 3 days after inoculation, the brain was removed immediately for further testing. An approximately 10% mouse brain suspension was made and 0.03 ml of –1, –2, –3 and –4 log dilutions were each separately inoculated into four 17–19-day-old mice (Kunming strain of Swiss mice). If the intracerebral titre of any mouse brain suspension exceeded 3.0 log LD_{50}/0.03 ml in 17–19-day-old mice the test material was considered unsuitable.

Modifications to this test may be used to evaluate a new virus master seed lot if approved by the national control authority.

Female ICR mice aged between 28 and 32 days provide a neurovirulence test system for laboratory strains of SA 14-14-2 virus, and may be further evaluated and considered as an alternative to Kunming Swiss mice (*19*). NIH mice have also been used in one country as an alternative to Kunming Swiss mice.

Test for reversion to neurovirulence in mice

The virus master seed lot should be shown to be free from reversion to neurovirulence by a test in mice that will reliably distinguish suitable from unsuitable preparations. A reference vaccine should be included to validate each test.

During development of the vaccine the following test was used:

Each of at least 10 suckling mice of the Kunming strain of Swiss mice 3–5 days old were inoculated intracerebrally with 0.02 ml of the undiluted virus master seed lot. Experience had shown that most mice showed signs of illness after 6–8 days. The brains of 3–5 of the mice with the most severe signs (of convulsions and irritation) were removed and then emulsified together into a 10% suspension. The suspension was assessed in two ways. Firstly by the intracerebral inoculation of 17–19-day-old mice (Kunming strain of Swiss mice) with suspension prepared as follows. Serial 10-fold dilutions of the 10% suspension, from –1 to –4 inclusive, were made in medium that contained 0.5% lactalbumin hydrolysate and 2% calf serum and 0.03 ml of each dilution was inoculated into four mice. If the intracerebral LD_{50} titre exceeded 3.0 log LD_{50}/0.03 ml the preparation was considered unsuitable.

The parental SA14 virus had an LD_{50}/0.03 ml of 8.5 log in this test. In the second assessment the 10% brain suspension was inoculated by the subcutaneous route into at least ten 15–17-day-old mice (Kunming strain of Swiss mice) (0.1 ml per mouse). If any mouse showed signs of JE viral infection (such as convulsions or irritation) during the 14-day observation period the preparation was considered unsuitable. The parental SA14 strain had an LD_{50}/0.1 ml of 5.0 log in this test.

Alternative strains of mice may also be suitable for this test and the incubation period may be extended beyond 14 days.

Modifications to this test may be used to evaluate a new virus master seed lot if approved by the national control authority.

Test for neurotropism in mice
During development of the vaccine the following test was used:

Each of at least 10 mice of the Kunming strain of Swiss mice 15–17 days old were conditioned by injecting a sterile needle into the head to destroy the blood–brain barrier, and were then inoculated subcutaneously with 0.1ml of the master virus seed. If any mice showed signs of infection with JE virus (such as convulsions or irritability) during the 14-day observation period, the preparation was considered unsuitable.

Omission of this test on a new virus master seed lot may be considered with the approval of the national control authority.

2.1.3.2.2 Tests on virus working seed lots

The virus working seed lot used for the production of vaccine batches should be prepared from a qualified virus master seed lot and by a method approved by the national control authority. The virus working seed lot should be limited to three passages in PHK cells beyond the master seed lot. If the virus working seed is in liquid form, it should be stored at –60 °C or below for a period agreed with the national control authority. If the virus working seed is in freeze–dried form, it should be stored at –20 °C or below for a period agreed with the national control authority.

(1) Identity

Each virus working seed lot should be identified as JE virus by serological methods approved by the national control authority. Appropriate serological methods include enzyme immunoassays using a JE virus-specific monoclonal antibody, immunofluorescence, or neutralization assays using a reference serum or monoclonal antibody specific to JE virus. A test for genetic identity

should also be performed, such as determination of the sequence of the E protein.

(2) Virus titration and infectivity

The infectivity of each working seed lot should be established in an assay approved by the national control authority, such as the PFU method in the BHK-21 cell line. Alternative cell lines may also be acceptable to the national control authority. Manufacturers should determine the appropriate titre to reliably produce vaccine.

In the PFU method in BHK-21 cells used in some laboratories, samples from the virus suspension are 10-fold serially diluted and the titre is estimated after plaques have developed.

The titration should be made in parallel with a reference vaccine that is approved by the national control authority.

Alternative assays such as those to determine the $CCID_{50}$, may be used with the approval of the national control authority.

(3) Freedom from bacteria, fungi and mycoplasmas

Each virus working seed lot should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in Part A, sections A.5.2 and A.5.3 of the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*).

(4) Tests for adventitious agents

Each virus working seed lot should be tested in cell cultures for adventitious agents. Neutralization of JE virus is necessary for most tests because the virus is generally cytopathogenic.

A volume of each seed lot of at least 10 ml should be tested for adventitious agents by inoculation into a cercopithecid cell culture. Similar volumes should be tested in cultures of human cells, BHK-21 cells and cells of the same type but not the same batch as those used in the preparation of the virus seed. Uninoculated control cell cultures should be included in the tests. All cell cultures should be incubated at 35–37 °C and should be observed for at least 14 days.

The cells should be examined for cytopathic changes during the observation period. At the end of the observation period, the cell cultures should be tested for haemadsorbing viruses and other adventitious agents as specified in sections 2.2.1.1.1 and 2.2.1.1.2, respectively. One subpassage of one cell culture per cell line should be done and the tests in sections 2.2.1.1.1 and 2.2.1.1.2

repeated. For a test to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period. For the seed virus to be satisfactory, no cytopathic changes or adventitious agents should be detected.

(5) Test for neurovirulence in mice

Comparative experimental studies of 17–19-day-old mice and rhesus monkeys have shown that mice infected by intracerebral inoculation are a more sensitive system to detect JE viral neurovirulence than are intracerebrally inoculated monkeys (*20*).

The virus working seed lot should be shown to be free from neurovirulence by the test for neurovirulence in mice (section 2.1.3.2.1).

2.2 Control of the manufacturing process

2.2.1 *Control of vaccine production*

2.2.1.1 Control cell cultures

At least 5% of the total volume or 500 ml, whichever is greater, of the cell suspension should be used to prepare control cultures. This approach is similar to that taken for measles vaccine produced in chick embryo fibroblast cells (*13*).

The control cells should be maintained under similar conditions of time, temperature and media as the infected cells. They should be observed for cytopathic effects for 14 days from the time of seeding the cells, which may include a subpassage and observation for a further 14 days (i.e. a total of 28 days). At the end of the observation period, the cells should be checked for haemadsorbing viruses as described in section 2.2.1.1.1.

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

If any such tests show evidence of the presence in control cultures of any adventitious agents, the harvest of virus should not be used for vaccine production.

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

2.2.1.1.1 Test for haemadsorbing viruses

At the end of the observation period, 25% of the control cells should be tested for the presence of haemadsorbing viruses, using guinea-pig red blood cells. If the red cells have been stored, the duration of

storage should not have exceeded 7 days, and the temperature of storage should have been in the range of 2–8 °C.

In some countries, the national control authority requires that the control cell cultures described above should be tested for the presence of haemadsorbing viruses at the end of the production culture incubation period(s). If this is the case, the test for haemadsorbing viruses described here may be deleted.

In some countries, the national control authority requires that other types of red cells, including cells from humans (blood group O), monkeys and chickens (or other avian species) should be used in addition to guinea-pig cells. In all tests, readings should be taken after incubation for 30 minutes at 0–4 °C, and again after a further incubation for 30 minutes at 20–25 °C. For the test with monkey red cells, readings should also be taken after a final incubation for 30 minutes at 34–37 °C.

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

2.2.1.1.2 Tests for other adventitious agents

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

Each sample should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm^2 per ml of pooled fluid. At least one bottle of each kind of cell culture should remain uninoculated and should serve as a control.

The cultures should be incubated at a temperature of 35–37 °C and should be observed for a period of at least 14 days. During this observation period the cultures should be examined at least every third day for cytopathic changes.

The tests are satisfactory if no cytopathic changes attributable to adventitious agents in the test sample are detected.

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

2.2.2 *Virus inoculation and incubation*

Cell cultures with a dense single layer should be selected and washed thoroughly, after which a suitable volume of maintenance medium

should be added. The virus working seed inoculum should achieve a multiplicity of infection of approximately 0.001 (some manufacturers use a final titre in the culture vessel of 2.7–3.7 log PFU per ml). The inoculated cultures should be incubated at 35–36 °C, usually for 3 days, but not more than 4 days.

2.2.3 *Control of single virus harvests*

2.2.3.1 Harvest of vaccine virus

The vaccine virus fluid should be harvested when the cytopathic effect (CPE) becomes obvious. This is usually after 3 days, and should not be more than 4 days. The harvests should be stored at an appropriate temperature, as agreed with the national control authority.

2.2.3.2 Tests for sterility

A sample of each single harvest or virus culture supernatant should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*). If contamination is detected, the harvest should be discarded.

2.2.3.3 Virus content

The virus content of each single harvest should be tested using an infectivity assay approved by the national control authority, such as the PFU method in the BHK-21 cell line to determine the acceptability of the material for further processing and to confirm consistency of production. Tests using other cell lines may also be acceptable to the national control authority.

> In the PFU method in the BHK-21 cell line used in some laboratories, samples from the virus suspension are diluted 10-fold serially, and the titre is estimated after plaques have developed.
>
> In one country the virus titre should not be less than 7.2 log PFU per ml.
>
> The titration should be made in parallel with a reference vaccine that is approved by the national control authority.

Alternative assays, such as those to determine the $CCID_{50}$, may be used with the approval of the national control authority.

2.2.3.4 Test for identity

A test for identity (see section 2.1.3.2.2 of these Guidelines) should be performed if not done on the virus pool. It is not necessary to perform a test for genetic identity on single virus harvests.

2.2.3.5 Test for adventitious agents

If the single virus harvests are not pooled on the same day, a test for adventitious agents should be performed on each single virus harvest (see section 2.1.3.2.2).

2.2.4 *Preparation and control of virus pool*

2.2.4.1 Pooling of single virus harvests

Only virus harvests meeting the recommendations for sterility and virus content in sections 2.2.3.2 and 2.2.3.3 of these Guidelines should be pooled.

2.2.4.2 Clarification of vaccine virus pool

The vaccine virus pool should be clarified or filtered by a method that maximizes removal of cells and cell debris.

Samples of the clarified bulk suspension should be taken immediately after clarification to ensure that no microscopically observable cells or cell particles remain. Samples should also be taken to confirm the identity and determine the infectious virus content of the pool. If not tested immediately for virus content, the samples should be stored below –60 °C until testing is done.

2.2.4.3 Virus content

The virus content of the virus pool should be assayed by titration in cell culture against a reference preparation of live JE vaccine as described in section 2.2.3.3 of these Guidelines, and must be approved by the national control authority.

2.2.4.4 Test for identity

A test for identity (see section 2.1.3.2.2 of these Guidelines) should be used if not done on the single virus harvest. However, it is not necessary to perform the genetic identity test on the vaccine virus pool.

2.2.4.5 Tests for sterility

After clarification the virus pool should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*), or by a method approved by the national control authority.

2.2.4.6 Test for adventitious agents

A test for adventitious agents (see section 2.1.3.2.2 of these Guidelines) should be performed if not done on the single virus harvests.

2.2.4.7 Test for residual materials

Each manufacturer should demonstrate, by testing each virus pool, or by validation of the manufacturing process, that any residual materials used in manufacture, such as animal sera, are consistently reduced to a level acceptable to the national control authority.

2.2.4.8 Tests for retroviruses

Samples from the filtered virus pool should be examined for the presence of retroviruses by an assay for reverse transcriptase (Rtase) acceptable to the national control authority. Confirmation that the assays used will detect retroviruses potentially present in PHK cells should be presented.

Recently developed highly sensitive PCR-based assays for Rtase may be considered, but the results need to be interpreted with caution because Rtase activity is not unique to retroviruses and may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (*12*). Nucleic acid amplification tests for retroviruses may also be used.

2.3 Preparation and control of the final bulk

2.3.1 *Pooling bulk material*

More than one virus pool satisfying the control tests in section 2.2.4 of these Guidelines may be pooled and diluted to form the final bulk.

2.3.2 *Added substances*

In the preparation of the final bulk, only substances such as diluents or stabilizers approved by the national control authority should be added. The concentration of such substances should be approved by the national control authority.

Such substances should have been shown by appropriate tests not to impair the safety or effectiveness of the vaccine.

2.3.3 *Tests for sterility*

Each final bulk should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in the 1973 General Requirements for the Sterility of Biological Substances as amended in 1995 (*14*), or by a method approved by the national control authority.

2.3.4 *Virus content*

The live virus content of the virus pool should be assayed by titration in cell culture against a reference preparation of live JE vaccine as described in section 2.2.3.3 of these Guidelines, and must be approved by the national control authority.

2.3.5 *Test for neurovirulence in mice*

Each final bulk should be tested for neurovirulence in mice (see section 2.1.3.2.1 of these Guidelines).

The test may be performed before or after the addition of other substances, such as stabilizers, as approved by the national control authority.

2.4 Control of the final lot

The requirements concerning filling and containers in Good Manufacturing Practices for Biological Products (*11*) should apply.

2.4.1 *Control tests on the final lot*

2.4.1.1 Identity test

An identity test, as described in section 2.1.3.2.1 of these Guidelines, should be performed on at least one final labelled container from each filling lot after reconstitution of the vaccine according to the instructions of the manufacturer for preparing the vaccine for human administration. However, it is not necessary to perform the genetic identity test on the final lot.

2.4.1.2 Virus content

The virus content in each of at least three containers selected at random from the final lot should be determined individually against a reference preparation of live JE vaccine (using the method described in section 2.2.3.3 of these Guidelines). The virus content should be determined after reconstitution of the freeze–dried product. Limits for accuracy and precision of the virus titration should be agreed with the national control authority. Since no international reference materials have been established for live JE vaccine, no recommendations for potency based on such materials can be formulated. The national control authority should provide or approve a reference preparation of live JE vaccine for use in tests to determine virus concentration.

The national control authority should specify the minimum amount of vaccine virus that one human dose should contain.

> In one country, the minimum amount of vaccine virus in one human dose is 5.4 log PFU per 0.5 ml at the time of release.

2.4.1.3 Test for residual animal serum protein

A sample of the final lot should be tested to verify that the level of serum in the final reconstituted vaccine is less than 50 ng per human dose. Alternatively, this test may be performed on the clarified virus pool or on the final bulk.

2.4.1.4 Thermostability test

An accelerated stability test should be performed. The detailed procedures for carrying out this test and for interpreting the results

should be approved by the national control authority, which also should specify the acceptable confidence limits.

In one country, samples are incubated at 37 °C for 7 days and the loss of titre should not be more than 1.0 log.

2.4.1.5 Sterility test

Each final lot should be tested for bacterial, mycotic and mycoplasmal sterility according to the requirements given in the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*).

2.4.1.6 General safety (innocuity) tests

If a general safety (innocuity) test is required by the national control authority, each final lot should be tested for the absence of abnormal toxicity by appropriate tests in mice and guinea-pigs, using parenteral injections. The test procedures should be approved by the national control authority.

2.4.1.7 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 control authority. The upper limit for the moisture content should be approved by the national control authority.

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

2.4.1.8 Test for pH values

When freeze–dried vaccine is dissolved by the approved diluent, the pH value should be approved by the national control authority, and be within the range of values found for vaccine lots shown to be clinically safe and effective.

2.4.2 ***Inspection of final containers***

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

2.4.3 ***Records***

The recommendations given in section 8 of Good Manufacturing Practices for Biological Products (*11*) should apply.

2.4.4 ***Samples***

The recommendations given in section 9 of Good Manufacturing Practices for Biological Products (*11*) should apply.

2.4.5 ***Labelling***

The recommendations given in section 7 of Good Manufacturing Practices for Biological Products (*11*) should apply, with the addition of the following.

The leaflet accompanying the package should include the following information:

— the site of inoculation;
— the nature of the cell culture used;
— the strain of Japanese encephalitis virus used in the production of the vaccine;
— a statement that contact of the vaccine with disinfectants is to be avoided;
— the minimum amount of infectious virus contained in one recommended human dose;
— the age range for which the vaccine is recommended;
— a statement that the dose of vaccine is the same for all ages;
— the nature and amount of any residual antibiotic present in the vaccine;
— if the vaccine is in freeze–dried form, the volume and nature of the diluent to be added to reconstitute the vaccine;
— the recommended storage conditions for the vaccine, including after reconstitution;
— state that the reconstituted vaccine should be kept at 2–8 °C and in the dark, and should be used or discarded within 1 hour after the container is opened;
— any contraindications to the use of the vaccine.

2.4.6 *Distribution and shipping*

The recommendations given in section 8 of Good Manufacturing Practices for Biological Products (*11*) should apply.

2.5 Storage and expiry date

The statements concerning storage temperature and expiry date appearing on the label and in the leaflet, as recommended in Good Manufacturing Practices for Biological Products (*11*), should be based on experimental evidence and should be submitted for approval to the national control authority.

2.5.1 *Storage conditions*

JE vaccine (attenuated) should be stored at all times at a temperature between 2 °C and 8 °C. Alternative storage temperatures may be used only if justified and approved by the national control authority.

2.5.2 *Stability of vaccine and expiry date*

Adequate stability studies form an essential part of vaccine development. The stability of the vaccine in its final containers at the

recommended storage temperature should be established. As a guide, at least three consecutive final lots derived from different vaccine virus pools should be tested in the stability programme.

Accelerated degradation studies at 37 °C may provide useful additional information, but should not replace real-time studies at the recommended storage temperature.

The expiry date should be based on experimental evidence and should be submitted to the national control authority for approval.

2.6 Reference materials

No international reference materials for Japanese encephalitis vaccine are available currently. However, work has begun, or is planned, on the following materials:

- a condidate International Standard for Japanese Encephalitis Antisera.
- a condidate International Reference Preparation of Japanese Encephalitis Virus for Potency Testing. A national reference preparation for potency testing has been produced by one national control authority. This reference preparation of live attenuated JE virus should be tested further in an international collaborative study designed to establish an international reference preparation for validating tests to determine virus content.
- a condidate International Reference Preparation of Japanese encephalitis virus for neurovirulence testing. A reference preparation of live attenuated JE virus should be established for use in neurovirulence tests in mice.

2.7 Guidance for national control authorities

2.7.1 *General*

The general recommendations for control laboratories given in Guidelines for National Authorities on Quality Assurance for Biological Products (*21*), which specify that no new biological substance should be released until consistency of production has been established, should apply, with the addition of the following.

The national control authority should approve the virus strains and cell substrate to be used in the production of JE vaccine, and the potency specifiations. The national control authority should take into consideration information on the currently available strains before deciding on those to be permitted for vaccine production. The national control authority must be satisfied that the results of all tests,

including those done on virus pools during the process of manufacture, are satisfactory and that consistency has been established.

The national control authority should give directions to manufacturers concerning the recommended dose for humans.

In addition, the national control authority should provide or approve a reference preparation of live JE virus for tests for potency testing (see section 2.6) and should specify the virus content required to achieve adequate immunization of humans at the recommended dose.

2.7.2 *Release and certification*

A vaccine lot should be released only if it fulfils the national requirements.

A protocol based on the summary protocol for the lot release of virus vaccines (*22*), signed by the responsible official of the manufacturing establishment, should be prepared, and where appropriate, submitted to the national control authority in support of a request for release of JE vaccine for use.

At the request of the manufacturing establishment, the national control authority may provide a certificate that states whether the vaccine meets all national requirements.

The purpose of the certificate is to facilitate the exchange of JE vaccines among countries.

Acknowledgements

The first draft of these Guidelines was prepared by Dr Yu Yongxin, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, and revised by the following: Dr V. Grachev, Chumakov Institute of Poliomyelitis and Viral Encephalitis, Moscow, Russian Federation; Dr S. Halstead, Office of Naval Research, Arlington, VA, USA; Dr J. Petricciani, International Association for Biologicals, Palm Springs, CA, USA; and Dr T. Tsai, Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, USA.

A second draft was formulated by the following participants at an Informal WHO Consultation held at the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, from 7 to 9 July 1999:

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The second draft was reviewed by the WHO Expert Committee on Biological Standardization at its fiftieth meeting in 1999 (WHO Technical Report Series, 904, p. 7).

A third draft was prepared by Dr D. Wood, National Institute of Biological Standards and Control, Potters Bar, Herts., England, and reviewed by the following participants at on Informal WHO Meeting held at the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, from 16 to 17 October 2000:

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For their comments and advice on these Guidelines acknowledgements are also due to:

Dr J. Furesz, Ottawa, Canada; Dr I. Levenbook, Chicago, IL, USA; Dr A. Lewis, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA; Dr L. Markoff, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA; Dr G. Stacey, National Institute of Biological Standards and Control, Potters Bar, Herts., England; Dr Y Yu, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China.

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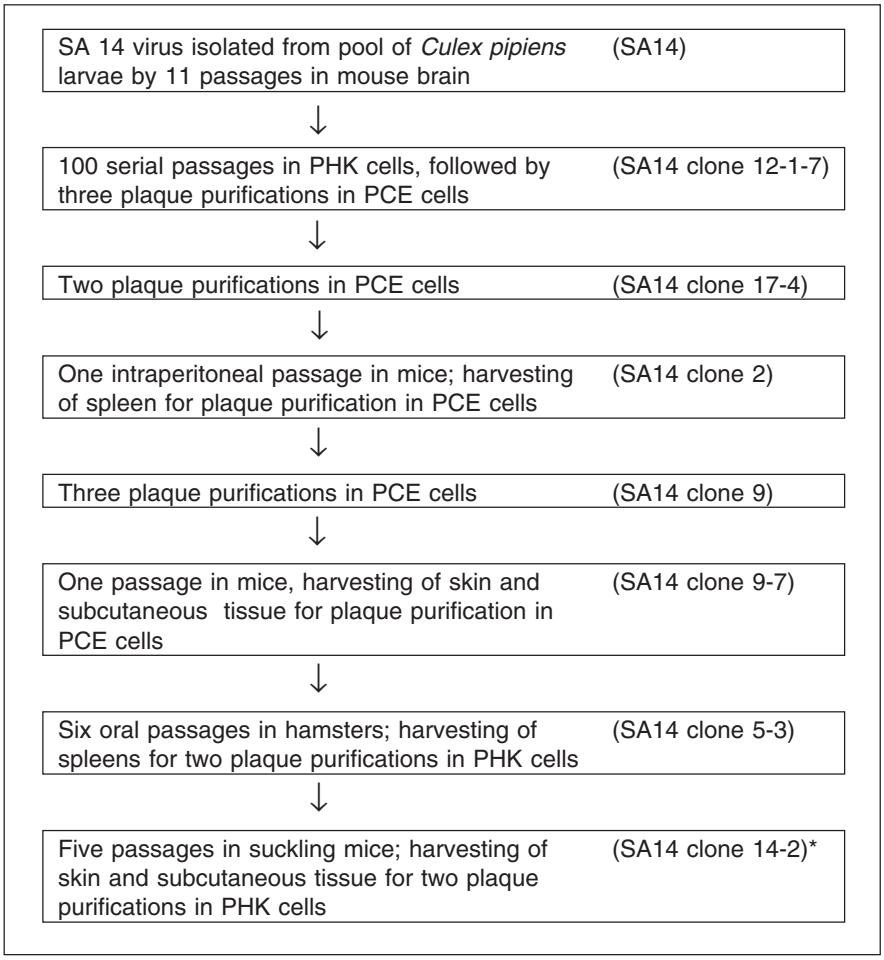
Appendix 1

Passage history of Japanese encephalitis SA 14-14-2 virus



PCE: primary chick embryo; PHK: primary hamster kidney.
* The notation SA14 clone 14-2 is abbreviated to SA14-14-2.

Appendix 2

Test for neurovirulence in monkeys

Each new master seed lot should be tested for neurovirulence in monkeys as described in the neurotropism test specified in the Requirements for Yellow Fever Vaccine (*1*), with the following modifications:

The monkeys should be *Macaca mulatta* (i.e. rhesus monkeys), as insufficient experience has been gained with the use of *Macaca fascicularis* (i.e. cynomolgus monkeys) for tests of live attenuated JE vaccines.

The animals shall have been demonstrated to be non-immune to JE virus immediately prior to inoculation of the samples.

The test dose should consist of the equivalent of not less than one human dose.

For the clinical criteria of the neurotropism test to be satisfied, the animals should not exhibit clinical signs of encephalitis and the clinical score of the monkeys injected with the virus being tested should not exceed the clinical score of those injected with reference virus.

Reference

1. Requirements for yellow fever vaccine. In: *WHO Expert Committee on Biological Standardization. Forty-sixth report.* Geneva, World Health Organization, 1998, Annex 2 (WHO Technical Report Series, No. 872).