

Annex 2

Requirements for yellow fever vaccine

(Requirements for Biological Substances No. 3, revised 1995)

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Introduction

Yellow fever is a viral haemorrhagic fever that, each year, strikes an estimated 200 000 persons worldwide and causes an estimated 30 000 deaths. The case-fatality rate can reach as high as 20% to 80% in severely ill patients who are hospitalized (1). However, the overall case-fatality rate is lower.

The yellow fever virus is small (35–45 nm) and consists of a core containing single-stranded RNA surrounded by a lipoprotein envelope. The yellow fever virus genome has been completely sequenced and has been found to contain 10 862 nucleotides (2). The lipoprotein envelope contains a single glycoprotein with type- and group-specific antigenic determinants.

Yellow fever was first distinguished from malaria, dengue and other tropical diseases during a series of epidemics between 1647 and 1649 in Barbados, Cuba, Guadeloupe and Mexico. Recognized yellow fever epidemics have periodically raged since then in parts of the Americas and Africa. In 1900, a commission headed by the American physician Walter Reed confirmed that the disease was transmitted from human to human by the mosquito *Aedes aegypti*, a hypothesis proposed earlier by the Cuban physician Carlos Finlay in 1881 (3).

There are two epidemiological patterns of yellow fever transmission: the urban cycle and the forest cycle (also known as the jungle or sylvan cycle). The two patterns of transmission lead to a clinically identical disease, since the same virus is responsible. In the Americas, the yellow fever virus circulates today by means of an endemic, forest cycle that results in up to several hundred reports of infection in non-immune forest workers per year. In Africa, the virus circulates by means of both urban and forest cycles and periodically breaks out of its endemic pattern to infect large numbers of non-immune persons in the course of major epidemics.

Two live attenuated yellow fever vaccines were developed in the 1930s; the French neurotropic vaccine (FNV), prepared from human virus passaged in mouse brain, and the “17D” vaccine, prepared from the 17D strain of human virus passaged in embryonated chicken eggs.

Today, 17D vaccine is the only type of yellow fever vaccine produced, as the use of FNV was found to be associated with a high incidence of encephalitic reactions in children (4). The 17D vaccine was developed by Theiler and Smith in 1937.

More than 200 million doses of 17D vaccine have been administered, and it has proved to be one of the safest vaccines ever developed. This

vaccine has also been shown to be very effective for the control of yellow fever during outbreaks and between epidemics. In 1990, the Global Advisory Group of the Expanded Programme on Immunization recommended that all countries at risk of yellow fever should incorporate the vaccine in their routine immunization programmes. As of 1992, 16 of 33 such countries in Africa included yellow fever vaccine in their national immunization programmes.

Requirements for yellow fever vaccine (Requirements for Biological Substances No. 3) were first formulated by a WHO Study Group in 1958 (5). The Requirements embodied recommendations made by the first WHO Expert Committee on Yellow Fever Vaccine (6), and they applied to vaccine prepared from a suitable strain of yellow fever virus. The vaccine was intended to be given by subcutaneous injection. Conformity with these Requirements has been the basis for WHO approval of yellow fever vaccine used for vaccination and revaccination against yellow fever in connection with certification for the purposes of international travel (7), and such approval has been given only to vaccine prepared using seed derived from the 17D strain of yellow fever virus. The Requirements have been used also by national authorities for the control and approval of yellow fever vaccine used in national immunization programmes. In 1969 the twenty-second meeting of the WHO Expert Committee on Biological Standardization agreed that developments in virology in general and in the manufacture and control of yellow fever vaccine in particular warranted a revision of the existing Requirements, with due consideration of both their national and international application (8). In 1975 the twenty-seventh meeting of the WHO Expert Committee on Biological Standardization formulated revised Requirements for Yellow Fever Vaccine (9). Much experience has been gained with the preparation of yellow fever vaccine since 1975, and a further revision of the Requirements has therefore been prepared.

General considerations

Yellow fever vaccine has been proved to be safe. Only 19 cases of encephalitis temporally associated with yellow fever vaccine prepared from the 17D virus strain have been reported, mostly in children, despite over 200 million doses having been delivered since 1945. Since all but six of these occurred in children immunized at 4 months of age or younger, a review by a panel of experts recommended that yellow fever vaccine should not be routinely given before 6 months of age (10, 11).

Formerly, there were some problems associated with under- or over-attenuation of the 17D strain on passage. These problems were resolved by the establishment of a virus seed-lot system. There are a number of substrains in use today for the manufacture of 17D vaccine (12). A detailed study using oligonucleotide mapping and monoclonal antibody analysis of the genetic and antigenic variation among these substrains showed an RNA-sequence homology of 98–100%, which demonstrates a high degree of genetic and antigenic similarity among substrains (13).

Although all countries now use seed virus free from avian leukosis viruses (ALV), some permit the production of vaccine in embryonated chicken eggs not proved to be free from ALV. This practice may be justified where the cost and difficulty of obtaining ALV-free embryonated eggs would restrict the availability of yellow fever vaccine, particularly in view of epidemiological evidence that shows no relationship between yellow fever vaccination and leukaemia, lymphoma or other cancers (14). For this reason, the revised Requirements do not specify ALV-free eggs.

It is important to ensure that new pools of virus seed, whether master or working seed, have levels of neurotropism and viscerotropism within safe limits. The relevant safety test, performed on monkeys, has therefore been retained in the revised Requirements.

One important amendment to the Requirements concerns the inclusion of a test of the thermal stability of the vaccine as a requirement instead of as a recommendation (15). Each batch of yellow fever vaccine should accordingly be evaluated by testing for potency before and after storage at 37°C for 2 weeks.

The National Institute for Biological Standards and Control, Potters Bar, distributes the International Reference Preparation of Anti-Yellow-Fever Serum. Such a preparation is needed as a basis for comparison of antibody responses in the monkey neurovirulence test. WHO can provide primary seed virus for the production of yellow fever vaccine (16).

Each of the following sections constitutes a recommendation. The parts of each section that are printed in normal type have been written in the form of requirements so that, if a national control authority so desires, they may be adopted as they stand as definitive national requirements. The parts of each section printed in small type are comments or recommendations for guidance. To facilitate the licensing and international distribution of vaccines made in accordance with these Requirements, a summary protocol for recording the results of tests is provided as Appendix 1. A model summary protocol

for the routine lot release of virus vaccines is provided as Annex 4 of this volume of the WHO Technical Report Series.

Should individual countries wish to adopt these Requirements as the basis for their national regulations concerning yellow fever vaccine, it is recommended that modifications be made only on condition that the modified requirements ensure at least an equal degree of safety and potency of the vaccine. It is desirable that the World Health Organization should be informed of any such changes.

Part A. Manufacturing requirements

A.1 Definitions

A.1.1 *International name and proper name*

The international name shall be “Vaccinum febris flavae”. The proper name shall 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 requirements formulated below.

A.1.2 *Descriptive definition*

Yellow fever vaccine shall consist of a freeze-dried preparation of viable, attenuated yellow fever virus (*Flavivirus hominis*). The preparation shall satisfy all the requirements formulated below.

A.1.3 *International reference materials*

An International Reference Preparation of Anti-Yellow-Fever Serum is available from the National Institute for Biological Standards and Control, Potters Bar, England. A nonimmune control serum is also available. Samples are distributed free of charge, on request, to national control laboratories.

A.1.4 *Terminology*

The following definitions are given for the purpose of these Requirements only.

Primary or master seed lot. A quantity of virus suspension that has been processed in a single production run and has a uniform composition. It is used for the preparation of secondary (working) seed lots.

Secondary or working seed lot. A quantity of virus suspension that has been processed in a single production run, and is uniform with respect to composition, fully characterized and only one passage from a master seed lot. Material drawn from secondary seed lots is used for inoculating embryonated eggs in the preparation of vaccine.

Single harvest. A quantity of virus suspension, derived from tissues of the same origin that were inoculated with the same working seed lot, that has been collected and processed in a single production run.

Final bulk. The material prepared from one or more single harvests in the vessel from which the final containers are filled.

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

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

Median mouse lethal dose (mouse LD_{50}). The quantity of a virus suspension that will kill 50% of the mice injected with it.

A.2 Certification of the substrain of 17D virus for use in vaccine production

The seed lot of the substrain of 17D yellow fever virus used in the production of yellow fever vaccine shall be identified by historical records that include information on the origin of the substrain, its method of attenuation and the passage level at which attenuation and immunogenicity were demonstrated by clinical evaluation.

The master and working seed lots of yellow fever virus used in the production of vaccine shall have been shown to be safe and immunogenic by appropriate laboratory tests (see section A.4 of these Requirements), and the master seed lot shall be shown to be immunogenic by clinical trials in susceptible humans. Only seed lots that are approved by the national control authority shall be used.

Seed lots that have been certified previously can be used.

A suitable primary seed lot (213–77) is available from WHO (16).

A.3 General manufacturing requirements

The general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (17) and Biological (18) Products shall apply to establishments manufacturing yellow fever vaccine, with the following additions: those staff directly involved with the production and testing of yellow fever vaccine shall be shown to be immune to yellow fever.

A.4 Production control

A.4.1 Control of source materials

A.4.1.1 Tissues for virus production

Virus for the preparation of master and working seed lots shall be grown in the tissue of chicken embryos obtained from a closed, specific-pathogen-free, healthy flock. This flock shall be monitored at regular intervals for agents pathogenic to birds. However, the national control authority may vary these requirements where the cost and difficulty of obtaining such tissue may restrict the availability of the vaccine and where the vaccine produced has been demonstrated to be both safe and effective.

Examples of agents that it may be necessary to monitor include *Mycobacterium avium*, fowl pox virus, ALV and other avian retroviruses, Newcastle disease virus and other avian parainfluenza viruses, avian encephalomyelitis virus, infectious laryngotracheitis virus, avian reticulo-endotheliosis virus, Marek's disease virus, infectious bursal disease virus, *Haemophilus paragallinarum*, *Salmonella gallinarum*, *Salmonella pullorum*, *Mycoplasma gallisepticum* and *Mycoplasma synoviae*.

In some countries, all birds are bled when a colony is established, and thereafter 5% of the birds are bled each month. The resulting serum samples are screened for antibodies to the relevant pathogens. Any bird that dies is investigated to determine the cause of death.

Virus for the preparation of all vaccine production lots shall be grown in the tissue of chicken embryos obtained from a healthy flock. Monitoring of the flock or embryos shall include at least tests for exclusion of infection by *Salmonella* spp., *Mycobacterium avium* and fowl pox virus. The flock must not have been vaccinated with live Newcastle disease virus vaccine.

In some countries, the national control authority requires that the tissue used for production of vaccine should be shown by suitable tests to be free from ALV.

It is recommended that eggs should be obtained from young birds.

A.4.1.2 Seed-lot system

The production of vaccine shall be based on the master- and working-seed-lot system. All seed lots shall be stored at a temperature of -60°C or below. Primary and secondary seed lots shall not contain any human protein or added serum or antibiotics.

In some laboratories, the master and working seed lots are stored in more than one location.

The master and working seed lots shall be free from ALV, mycoplasmas or other adventitious agents as shown by suitable tests (A.4.1.1 and A.4.1.5).

The inoculum for infecting tissues used in the production of vaccine shall be a working seed lot without intervening passage, in order to ensure that no vaccine shall be manufactured that is more than one passage removed from a seed lot that has passed all safety tests.

A.4.1.3 Monkey safety test

New master and working seed lots shall be tested for viscerotropism, immunogenicity and neurotropism in a group of 10 test monkeys. For the neurotropism test, the test monkeys inoculated with the virus seed lot shall be compared with a similar group of 10 monkeys injected with a reference virus. The reference virus shall be approved by the national control authority.

The monkeys shall be *Macaca mulatta* (i.e. rhesus monkeys) or *Macaca fascicularis* (i.e. cynomolgus monkeys) and shall have been demonstrated to be non-immune to yellow fever immediately prior to injection of the seed virus. They shall be healthy and shall not have been previously subjected to intracerebral or intraspinal inoculation. Furthermore, they shall not have been inoculated by any route with neurotropic viruses or antigens related to yellow fever.

The test dose shall consist of 0.25 ml containing the equivalent of not less than 5000 and not more than 50000 mouse LD₅₀, as shown by a titration conducted by the method described in Appendix 3. The test dose shall be injected into one frontal lobe of each monkey under anaesthetic, and the monkeys shall be observed for a minimum of 30 days.

Viscerotropism test. The criterion of viscerotropism (indicated by the amount of circulating virus) shall be fulfilled as follows. Sera obtained from each of the test monkeys on the second, fourth and sixth days after injection of the test dose shall be inoculated at dilutions of 1:10, 1:100 and 1:1000 into at least 4 cell-culture vessels (or intracerebrally in 0.03 ml aliquots into at least 6 mice) per dilution, as specified in Appendix 3. In no case shall 0.03 ml of serum contain more than 500 mouse LD₅₀ or the equivalent in PFU (see section A.6.2) and in no more than one case shall 0.03 ml of serum contain more than the equivalent of 100 mouse LD₅₀ (appropriate techniques for potency testing are given in Appendix 3).

Immunogenicity test. The criterion of sufficient virus-neutralizing antibody in the sera (immunogenicity) shall be fulfilled as follows. At

least 90% of the test monkeys shall be shown to have become immune within 30 days following injection of the test dose, as determined by examining their sera in the test for neutralization of yellow fever virus described below.

In some countries, it has been shown that, at low dilutions, some sera contain non-specific inhibitors that interfere with this test. The national control authority may require sera to be treated to remove such substances.

Dilutions of 1:10, 1:40 and 1:160 of serum from each test monkey shall be mixed with an equal volume of strain 17D vaccine virus at a dilution that has been shown to yield an optimum number of plaques when assayed according to one of the cell-culture methods given in Appendix 3. These serum-virus mixtures shall be incubated in a water bath at 37°C for 1 hour and then chilled in an ice-water bath before inoculation of 0.2ml aliquots of each mixture into each of 4 separate cell-culture vessels. The vessels shall be handled according to one of the cell-culture techniques described in Appendix 3. In addition, 10 vessels shall be similarly inoculated with virus as above and an equal volume of a 1:10 dilution of monkey serum known to contain no neutralizing antibodies to yellow fever virus. At the end of the observation period, the mean number of plaques in the vessels receiving virus and non-immune serum shall be compared with the mean number of plaques in the vessels receiving virus and serum from test monkeys. For the immunogenicity test to be satisfied, serum at the 1:10 dilution from no more than 10% of the test monkeys shall fail to reduce the mean number of plaques by 50% as compared with the vessels containing non-immune serum.

Neurotropism test. Monkeys in the test group shall be compared with 10 monkeys injected with the reference virus with respect to both the clinical evidence of encephalitis and the severity of histological lesions of the nervous system (19, 20).

The onset and duration of the febrile reaction should not differ between monkeys injected with the test or reference virus.

The monkeys shall be examined daily for 30 days by personnel familiar with the clinical signs of encephalitis in primates.

If necessary, the monkeys may be removed from their cages and examined for signs of motor weakness or spasticity as described elsewhere (20).

Signs of encephalitis, such as paresis, incoordination, lethargy, tremors or spasticity, shall be assigned numerical values for severity by the following grading method. Each day each monkey shall be given a numerical score based on the scale:

- 1: rough coat, not eating;
- 2: high-pitched voice, inactive, slow moving;

- 3: shaky movements, tremors, incoordination, limb weakness;
- 4: inability to stand, limb paralysis or death.

A monkey that dies receives the score “4” from the day of death until day 30.

The clinical score for a monkey is the average of its daily scores; the clinical score for a group is the arithmetic mean of the individual scores. For the clinical criterion of the neurotropism test to be satisfied, the clinical score of the monkeys injected with the virus being tested shall not exceed the clinical score of the monkeys injected with the reference virus.

The cervical and lumbar enlargements of the spinal cord and specific structures at five levels of the brain shall be examined (20) (see Appendix 1). The cervical and lumbar enlargements shall each be divided equally into six blocks. The blocks shall be dehydrated and embedded in paraffin wax; 15-µm sections shall be cut and stained with gallocyenin. One section, consisting of two hemisections, shall be cut from each block.

Tissue blocks 3–4mm thick shall be taken from the brain by making the following frontal cuts:

- Block I: the corpus striatum at the level of the optic chiasma;
- Block II: the thalamus at the level of the mamillary bodies;
- Block III: the mesencephalon at the level of the superior colliculi;
- Block IV: the pons and cerebellum at the level of the superior olives;
- Block V: the medulla oblongata at the mid-level of the inferior olives.

These blocks shall be dehydrated and embedded in paraffin wax and 15-µm sections shall be cut and stained with gallocyenin. A single section, consisting of two hemisections, shall be cut from each block.

Sections shall be examined microscopically and numerical scores given to each hemisection of the lumbar and cervical cord enlargements and to each anatomical structure (see Appendix 1) within each hemisection of the brain blocks according to the following grading system:

- 1 (minimal): 1–3 small, focal inflammatory infiltrates. A few neurons may be changed or lost;
- 2 (moderate): more extensive focal inflammatory infiltrates. Neuronal changes or loss affects not more than one-third of neurons;
- 3 (severe): neuronal changes or loss of 33–90% of neurons, with moderate focal or diffuse inflammatory infiltration;
- 4 (overwhelming): more than 90% of neurons are changed or lost, with variable, but frequently severe, inflammatory infiltration.

Each brain block contains several anatomical structures which contribute in different ways to the assessment of a test sample. For

example, certain structures differentiate more reproducibly than others between acceptable and unacceptable yellow fever seed lots and vaccines (20). These are called discriminator areas, whereas structures that are more susceptible to yellow fever virus replication are called target areas. Though either rhesus or cynomolgus monkeys are acceptable, the discriminator and target areas are different for the two species. The major difference is that in cynomolgus monkeys the cervical and lumbar cord are target areas whereas in rhesus monkeys they are discriminator areas. The footnotes to the worksheets (Appendix 1) indicate in more detail discriminator and target areas for the two species. The worksheets also lists other anatomical structures that will be present in the brain sections but are not included in the evaluation of a test sample because they are rarely affected (spared areas).

Three separate scores shall be calculated for each monkey: discriminator areas only, target areas only, and discriminator plus target areas. These scores shall be calculated as shown in the example worksheets provided in Appendix 1 (pp. 55–57). Overall mean scores shall also be calculated for each group of monkeys as the arithmetic mean of individual monkey scores for discriminator areas only and for discriminator plus target areas. Both overall mean scores shall be considered in determining virus seed lot acceptability. For the histological criterion of the neurotropism test to be satisfied both overall mean scores for the test monkeys shall not be significantly greater (at the 5% significance level) than the overall mean scores for the monkeys injected with reference virus.

Both the clinical and histological criteria of the neurotropism test shall be satisfied for the virus seed lot to satisfy the requirement for neurotropism.

A.4.1.4 Identity test

An identity test shall be performed on at least one container from each master and working seed lot, using one of the techniques described in section A.6.1.

A.4.1.5 Sterility tests on primary and secondary seed lots

Each lot of seed virus in its final containers shall pass the tests described in Part A, sections 5.2 and 5.3, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (21).¹

¹ See the 1995 amendment to the General Requirements for the Sterility of Biological Substances, section 5.3, *Sterility test for mycoplasmas* (Annex 3, p. 70 of this report).

A.4.2 Tests on uninoculated eggs

If monitoring of the flocks supplying embryonated eggs is not under the direct responsibility of the vaccine manufacturer, the following tests shall be performed.

A sample of 2% of, but in any case not less than 20 and not more than 50 uninoculated embryonated eggs from the batch used for vaccine production shall be incubated under the same conditions as the inoculated eggs. At the time of virus harvest, the uninoculated eggs shall be processed in the same manner as the inoculated eggs, and the extract from the control embryos shall be shown to be free from haemagglutinating agents and other extraneous agents by tests approved by the national control authority.

A.4.3 Single harvests

After inoculation and incubation at a controlled temperature, only living, typical chick embryos shall be harvested. The age of embryos at the time of harvest shall be reckoned from the initial introduction of the eggs into the incubator and shall be no more than 12 days. After homogenization and centrifugation, the embryonic extract shall be kept at -60°C or below until further processing.

A.4.3.1 Sterility tests

The embryonic extract that forms a single harvest shall be tested for the presence of bacteria, fungi and mycoplasmas as described in Part A, section 5.2 and 5.3, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (21).¹

A.4.3.2 Virus titration

Live virus content shall be determined by titration in cell culture against a reference preparation of yellow fever virus as described in Appendix 3.

A.4.4 Final bulk

The final bulk shall be prepared from one or several single harvests and shall be submitted to the following tests, unless these tests have already been performed on each single harvest. The final bulk shall in any case be tested for sterility. Samples that are not tested immediately shall be stored at or below -60°C .

¹ See the 1995 amendment to the General Requirements for the Sterility of Biological Substances, section 5.3, *Sterility test for mycoplasmas* (Annex 3, p. 70 of this report).

A.4.4.1 Sterility tests

The requirements concerning tests for bacteria, fungi and mycoplasmas as described in Part A, sections 5.2 and 5.3, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (21) shall apply to yellow fever vaccine.¹

A.4.4.2 Adventitious agents

Mycobacterium avium shall be tested for by cell-culture methods approved by the national control authority.

In some countries, the national control authority requires that the final bulk should be shown by suitable tests to be free from ALV or other agents.

A.4.4.3 Protein content

The level of protein nitrogen before the addition of any stabilizer shall be not more than 0.25 mg per human dose.

A.4.4.4 Addition of stabilizers and preservatives

No β -lactam antibiotics shall be added to the virus suspension at this or at any other stage during production. Added stabilizing agents shall be approved by the national control authority.

A.4.4.5 Virus titration

The live virus content of each final bulk shall be determined by titration in cell culture against a reference preparation of yellow fever virus as described in Appendix 3.

A.5 Filling and containers

The requirements concerning filling and containers given in Good Manufacturing Practices for Biological Products (18) shall apply to yellow fever vaccine.

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

Single- and multiple-dose containers may be used.

The vaccine shall be freeze-dried.

Failure to achieve adequate drying will result in a product that is liable to rapid deterioration even at 0°C.

¹ See the 1995 amendment to the General Requirements for the Sterility of Biological Substances, section 5.3, *Sterility test for mycoplasmas* (Annex 3, p. 70 of this report).

Since yellow fever virus is extremely labile, unless the container is well sealed variations in virus content may occur during storage. The manufacturer should ensure that the seal is satisfactory.

The manufacturer shall provide the national control authority with adequate data to prove the stability of the vaccine under appropriate conditions of storage and shipping.

A.6 Control tests on final lot

A.6.1 Identity test

An identity test shall be performed on at least one container from each filling lot after reconstitution of the vaccine according to the indications of the manufacturer for preparing the vaccine for human administration. A high-titre, monospecific immune serum known to be free from neutralizing agents that react with other flaviviruses shall be used. Either of two methods may be used for the identity test.

A.6.1.1 Test in mice

Progressive dilutions not greater than fivefold each shall be made of the vaccine in a suitable diluent. Aliquots of each dilution shall be mixed with equal volumes of a reference immune serum and similar aliquots mixed with equal volumes of a reference non-immune serum. The serum-virus mixtures shall be incubated at 37°C and each shall be injected into not less than 6 mice per dilution as described in Appendix 3. All mice shall be observed for 21 days, and all deaths shall be recorded. Only deaths considered to be specifically caused by yellow fever virus infection shall be taken into account. Mice paralysed on day 21 shall be counted as alive. The LD₅₀ for vaccine mixed with immune serum shall be more than ten times the LD₅₀ for vaccine mixed with non-immune serum.

A.6.1.2 Test in cell cultures (plaque reduction test)

The technique described in section A.4.1.3. *Immunogenicity test*, (pp. 37–38) shall be used, with dilutions of vaccine with immune and non-immune serum. If a 50% reduction in plaque number at the 1:10 dilution is not observed for the vaccine mixed with immune serum compared with vaccine mixed with non-immune serum, the vaccine shall be rejected.

A.6.2 Potency test

Three final containers shall be selected at random from each filling lot and shall be individually tested on the same day against a reference preparation of yellow fever vaccine approved by the national control

authority. The containers shall be tested by assay in cell cultures or mice by a technique demonstrated to be sensitive.

Before assay but after reconstitution of the vaccine in the volume and diluent recommended by the manufacturer for preparation for human administration, the vaccine shall stand at a temperature between 20°C and 30°C for 20 minutes before further dilution. This material shall be considered as undiluted vaccine.

The titre of the vaccine shall be not less than 1000 mouse LD₅₀, or its equivalent in PFU, in the dose recommended by the manufacturer for use in humans. Each laboratory shall establish to the satisfaction of the national control authority the relationship between mouse LD₅₀ and PFU.

Each laboratory using a cell culture assay shall have the approval of the national control authority for this assay. Appropriate techniques for the potency test are given in Appendix 3.

A.6.3 *Thermal stability test*

Three final containers from the freeze-dried final lot shall be incubated at 37°C for 2 weeks. These containers shall be titrated in parallel with three containers that have been stored at or below the recommended storage temperature. Results should be expressed in infectious units or their equivalent in PFU. A reference reagent for yellow fever virus approved by the national control authority shall be included in each assay. At the end of the incubation period, the geometric mean infectious titre in the incubated final containers shall not have decreased by more than 1.0 log₁₀ unit and shall be at least equal to the required minimum number of infectious units per human dose.

Procedures for carrying out the above test and for the interpretation of results, including the specification of confidence limits, should be approved by the national control authority. A suitable test for determination of virus content (i.e. titre) is described in Appendix 3.

A.6.4 *Tests for bacteria and fungi*

The requirements concerning tests for bacteria and fungi as described in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (21) shall apply to yellow fever vaccine.

A.6.5 *Tests for other agents*

In some countries the national control authority requires that the vaccine should be shown by suitable tests to be free from ALV or other agents. If

these tests have been performed on the final bulk they may be omitted for the final lot.

A.6.6 General safety tests

Each final lot shall be tested for the absence of abnormal toxicity in mice and guinea-pigs by appropriate tests approved by the national control authority.

A.6.7 Residual moisture

The residual moisture in a representative sample of each freeze-dried final lot shall be determined by a method approved by the national control authority. The upper limit of the moisture content shall be approved by the national control authority on the basis of stability tests.

Generally, moisture levels of 3% or less are considered satisfactory.

A.6.8 Residual ovalbumin

The content of residual ovalbumin shall be limited to a level approved by the national control authority.

A.6.9 Test for endotoxin

Each final lot shall be tested for endotoxin. The test and limits shall be approved by the national control authority.

A.6.10 Inspection of final containers

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

A.7 Records

The requirements in section 8 of Good Manufacturing Practices for Biological Products (18, pages 27–28) shall apply.

A.8 Samples

The requirements in section 9.5 of Good Manufacturing Practices for Biological Products (18, page 29) shall apply.

A.9 Labelling

The requirements in section 7 of Good Manufacturing Practices for Biological Products (18, pages 26–27) shall apply, with the addition of the following:

The label on the carton or the leaflet accompanying the container shall:

- state that the vaccine fulfils Part A of these Requirements;
- state the nature of the preparation, specifying the strain of yellow fever virus in the vaccine, the minimum number of infectious units per human dose, and the origin of the substrate used to prepare the vaccine (i.e. whether the flock was specific-pathogen free (SPF)).
- state the nature and quantity of any residual antibiotic present in the vaccine;
- indicate that the vaccine contains proteins derived from eggs;
- indicate that contact of the vaccine with disinfectants is to be avoided;
- indicate that the dose shall be the same for persons of all ages;
- indicate the volume and nature of the diluent to be added to reconstitute the vaccine, and specify that only the diluent supplied by the manufacturer should be used;
- state that the vaccine is not recommended for administration to children less than 6 months of age;
- state that the reconstituted vaccine shall be kept at 2–8 °C and in the dark and shall be used or discarded within 1 hour after the container is opened.

A.10 Distribution and shipping

The requirements in Good Manufacturing Practices for Biological Products (18, section 8) shall apply.

A.11 Storage and expiry date

The requirements in Good Manufacturing Practices for Biological Products (18) shall apply.

A.11.1 Storage conditions

Before being distributed by the manufacturing establishment, or before being issued from a depot for the maintenance of vaccine reserves, all vaccines shall be kept at all times at a temperature below –20 °C.

If possible, storage should be at a temperature below –25 °C.

The manufacturer shall recommend conditions of storage and shipping that will ensure the vaccine conforms to the requirements of potency until the expiry date stated on the label.

A.11.2 Stability of vaccine and expiry date

The stability of the vaccine shall be established at the recommended storage temperature and calculated from the date of a given satisfactory potency test. The expiry date shall be fixed with the approval of the national control authority and shall be no less than 2 years after the potency test.

Accelerated degradation studies at 37°C may provide useful additional information.

Part B. National control requirements

B.1 General

The general requirements for control laboratories contained in the Guidelines for National Authorities on Quality Assurance for Biological Products (22) shall apply.

The national control authority shall give directions to manufacturers concerning the yellow fever virus strains to be used in vaccine production and concerning the recommended human dose.

The national control authority should take into consideration information available on strains before deciding on those permitted for vaccine production.

In addition, the national control authority shall provide or approve a reference preparation of live yellow fever virus (see section A.1.3) for tests for virus concentration (see sections A.4.4.5 and A.6.2) and shall specify the virus content required to achieve adequate immunization of humans at the recommended dose.

B.2 Release and certification

A vaccine lot shall be released only if it fulfils the national requirements and/or Part A of the present Requirements. A protocol based on the model given in Appendix 1, signed by the responsible official of the manufacturing establishment, shall be prepared and where appropriate submitted to the national control authority in support of a request for release of vaccine for use. For subsequent routine lot release, a less comprehensive document may suffice, a model for which is found in Annex 4 of this volume of the WHO Technical Report Series.

At the request of the manufacturing establishment, the national control authority shall provide a certificate that states whether the vaccine meets all national requirements and/or Part A of the present Requirements. The certificate shall be based on the model given in Appendix 2.

The purpose of the certificate is to facilitate the exchange of live yellow fever virus vaccine among countries.

Authors

The draft of these revised Requirements was prepared by Dr V. Grachev, Scientist, Biologicals; Dr D. Magrath, Chief, Biologicals (1987–1994); and Dr E. Griffiths, Chief, Biologicals, World Health Organization, Geneva, Switzerland.

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References

1. The resurgence of deadly yellow fever. *Expanded Programme on Immunization update*. Geneva, World Health Organization, March 1992 (unpublished document available on request from Expanded Programme on Immunization, World Health Organization, 1211 Geneva 27, Switzerland).
2. Rice CU et al. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evaluation. *Science*, 1985, **229**:727–733.
3. Bres PLJ. A century of progress in combating yellow fever. *Bulletin of the World Health Organization*, 1986, **64**:775–786.
4. Rey M et al. Aspects épidémiologiques et cliniques des encéphalites consécutives à la vaccination anti-amarille (d'après 248 cas observés dans quatre services hospitaliers de Dakar à la suite de la campagne 1965). [Epidemiological and clinical aspects of encephalitis following yellow fever vaccination (report of 248 cases from four hospital services in Dakar after the 1965 campaign).] *Bulletin de la Société médicale d'Afrique noire de langue française*, 1966, **11**:560–574.
5. *Requirements for biological substances 3. Requirements for Yellow Fever Vaccine 4. Requirements for Cholera Vaccine. Report of a Study Group*. Geneva, World Health Organization, 1959 (WHO Technical Report Series, No. 179).
6. *Expert Committee on Yellow Fever Vaccine. First Report*. Geneva, World Health Organization, 1957 (WHO Technical Report Series, No. 136).
7. *International health regulations (1969)*, 3rd annotated ed. Geneva, World Health Organization, 1983 (Article 66.4).
8. *WHO Expert Committee on Biological Standardization. Twenty-second report*. Geneva, World Health Organization, 1970: 21 (WHO Technical Report Series, No. 444).
9. Requirements for Yellow Fever Vaccine (Requirements for Biological Substances No. 3, revised 1975). In: *WHO Expert Committee on Biological*

Standardization. Twenty-seventh report. Geneva, World Health Organization, 1976, Annex 1 (WHO Technical Report Series, No. 594).

10. **Meegan JM.** *Yellow fever vaccine.* Geneva, World Health Organization, 1991 (unpublished document WHO/EPI/GEN/91.6, available on request from Expanded Programme on Immunization, World Health Organization, 1211 Geneva 27, Switzerland).
11. **Drouet A.** Méningo-encéphalite après vaccination anti-amarile par la souche 17D: deux observations. [Meningo-encephalitis following yellow fever vaccination using strain 17D: two cases.] *Revue de médecine interne*, 1993, 14(4):257–259.
12. **Galazka AM et al.** *The immunological basis for immunization of yellow fever.* Geneva, World Health Organization, 1993 (unpublished document WHO/EPI/GEN/93.18; available on request from Expanded Programme on Immunization, World Health Organization, 1211 Geneva 27, Switzerland).
13. **Monath TP et al.** Ontogeny of yellow fever 17D vaccine. RNA oligonucleotide fingerprint and monoclonal antibody analysis of vaccines produced worldwide. *Journal of general virology*, 1983, 64:627–637.
14. **Waters TD et al.** Yellow fever vaccination, avian leukosis virus and cancer risk in man. *Science*, 1972, 177:76–77.
15. Requirements for Yellow Fever Vaccine (Requirements for Biological Substances No. 3, revised 1975, addendum 1987). In: *WHO Expert Committee on Biological Standardization. Thirty-eighth Report.* Geneva, World Health Organization, 1988, Annex 9 (WHO Technical Report Series, No. 771).
16. Production and testing of the WHO yellow fever virus primary seed lot 213–77 and reference batch 168–73. In: *WHO Expert Committee on Biological Standardization. Thirty-sixth report.* Geneva, World Health Organization, 1987, Annex 6 (WHO Technical Report Series, No. 745).
17. Good manufacturing practices for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Thirty-second Report.* Geneva, World Health Organization, 1992, Annex 1 (WHO Technical Report Series, No. 823).
18. Good manufacturing practices for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report.* Geneva, World Health Organization, 1992, Annex 1 (WHO Technical Report Series, No. 822).
19. **Fox JP, Penna HA.** Behaviour of 17D yellow fever virus in rhesus monkeys: relation to substrain, dose and neural or extraneural inoculation. *American journal of hygiene*, 1943, 38:152–172.
20. **Levenbook IS, Pelleu LJ, Elisberg BL.** The monkey safety test for neurovirulence of yellow fever vaccines: the utility of quantitative clinical evaluation and histological examination. *Journal of biological standardization*, 1987, 15:305–313.
21. General Requirements for the Sterility of Biological Substances (Requirements for Biological Substances No. 6, revised 1973). In: *WHO Expert Committee on Biological Standardization. Twenty-fifth Report.*

Geneva, World Health Organization, 1973, Annex 4 (WHO Technical Report Series, No. 530).

22. Guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second Report*. Geneva, World Health Organization, 1992, Annex 2 (WHO Technical Report Series, No. 822).

Appendix 1

Example, for guidance, of a summary protocol for the production and testing of yellow fever vaccine¹

Master virus seed lot

Source of 17D substrain	_____
Master virus seed lot no.	_____
Name and address of manufacturer	_____
Passage level	_____
Date of inoculation of embryos	_____
Temperature of incubation	_____
Date of harvest	_____
Age of embryos (at harvest)	_____
Number of containers	_____
Conditions of storage	_____

Production control (A.4)

Monkey safety test (A.4.1.3)

Species	_____
Number inoculated	_____
Master virus seed lot no.	_____
Reference virus lot no.	_____
Date of serology tests before inoculation	_____
Dilution used for the inoculation	_____
Volume and route of inoculation	_____
Date of inoculation	_____
Number of mouse LD ₅₀ or PFU inoculated	_____
Date of end of the test	_____

¹ Based on Requirements for yellow fever vaccine (Requirements for Biological Substances No. 3, revised 1995). In: *WHO Expert Committee on Biological Standardization. Forty-sixth Report*. Geneva, World Health Organization, 1997, Annex 2 (WHO Technical Report Series, No. 872).

Viscerotropism test (master virus seed lot)

Specify if mice or cells are used for virus titration.

Monkey no.	Titre of circulating virus on:			Maximum titre of circulating virus
	Day 2	Day 4	Day 6	
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

Result (pass or fail)

Immunogenicity test (master virus seed lot)

Specify if mice or cells are used for virus titration.

Monkey no.	Seroneutralization titre:	
	Day 0	Day 30
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

Result (pass or fail) _____

Neurotropism test (master virus seed lot)

Summary clinical results

Date of inoculation: _____

Master virus seed lot no.:		Reference virus lot no.:	
Monkey no.	Clinical score	Monkey no.	Clinical score
1		11	
2		12	
3		13	
4		14	
5		15	
6		16	
7		17	
8		18	
9		19	
10		20	
Group mean		Group mean	

Result (pass or fail) _____

Histological worksheet

The worksheets below are provided as an example of how the histological score would be calculated for a cynomolgus monkey with lesions graded as shown.

Species: cynomolgus

Pathology no:

Monkey no:

Corpus striatum & thalamus	Block I:		Block II:		Total
	L	R	L	R	
N. caudatus ^a	1	0	2	2	(5/4 =) 1.25
Globus pallidus ^{a,b}	0	1	2	0	(3/4 =) 0.75
Putamen ^{a,b}	2	0	1	1	(4/4 =) 1.00
N. ant./med. thalami ^{a,b}	1	1	0	1	(3/4 =) 0.75
N. lat. thalami ^{a,b}	1	2	1	1	(5/4 =) 1.25
Hypothalamus	0	1	0	0	(1/4 =) 0.25

^a Discriminator area for rhesus.

^b Discriminator area for cynomolgus.

Mesencephalon (Block III)	L	R	Total
Colliculi superior	0	0	0
Corpus geniculatum med.	0	0	0
N. oculomotorius	0	0	0
N. ruber	0	0	0
Substantia nigra ^c	2	2	(4/2 =) 2.00

^c Target area for rhesus and cynomolgus.

Pons (Block IV)	L	R	Total
N. abducens	0	0	0
N. vestibularis	0	0	0
N. trigeminus	0	0	0
N. facialis	0	1	(1/2 =) 0.5
Formatio reticularis	1	0	(1/2 =) 0.5
Oliva superior	0	0	0

Medulla oblongata (Block V)	L	R	Total
N. hypoglossus	0	0	0
N. glossopharyngeus	0	0	0
N. vestibularis	0	0	0
N. trigeminus	0	0	0
N. ambiguus	0	0	0
Formatio reticularis	0	0	0
Oliva inferior	0	0	0

Cerebellum (Blocks IV and V)	L	R	Total
N. dentatus	0	0	0
Other nuclei	0	0	0

Spinal cord	I		II		III		IV		V		VI		Total		
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L&R
Cervical enlargement ^{a, d}	2	3	2	3	2	2	2	2	1	2	2	2	11	14	(25/12 =) 2.08
Lumbar enlargement ^{a, d}	2	2	1	2	1	2	1	2	0	1	0	2	5	11	(16/12 =) 1.33

^a Discriminator area for rhesus.

^d Target area for cynomolgus.

Calculations:

Discriminator areas (globus pallidus, putamen, n. ant./med. thalami, n. lat. thalami):

$$\text{Lesion score} = \frac{(0.75 + 1.00 + 0.75 + 1.25)}{4} = 0.94$$

Target areas (s. nigra, cervical enlargement, lumbar enlargement):

$$\text{Lesion score} = \frac{(2.00 + 2.08 + 1.33)}{3} = 1.80$$

Discriminator plus target areas:

$$\text{Lesion score} = \frac{0.94 + 1.80}{2} = 1.37$$

Summary histopathology results

Date of inoculation: _____ Species: _____

Master virus seed lot no.:			Reference virus lot no.:		
Monkey no.	Discriminator area score	Discriminator plus target area score	Monkey no.	Discriminator area score	Discriminator plus target area score
1			11		
2			12		
3			13		
4			14		
5			15		
6			16		
7			17		
8			18		
9			19		
10			20		
Group mean			Group mean		

Result (pass or fail) _____

Identity test (A.4.1.4)

Comparison of virus titre in presence of normal serum with that in presence of immune serum

Method _____

Date _____

Results _____

Sterility tests (A.4.1.5)

Number of containers tested	_____		
	<i>bacteria</i>	<i>fungi</i>	<i>mycoplasmas</i>
Date of inoculation	_____	_____	_____
Media used	_____	_____	_____
Observation period	_____	_____	_____
Results	_____	_____	_____

Tests for avian and other viruses

Method	_____
Date	_____
Results	_____

Working virus seed lot

Working virus seed lot no.	_____
Name and address of manufacturer	_____
Passage level	_____
Date of inoculation of embryos	_____
Temperature of incubation	_____
Date of harvest	_____
Age of embryos (at harvest)	_____
Number of containers	_____
Conditions of storage	_____

Monkey safety test (A.4.1.3)

Species	_____
Number inoculated	_____
Working virus seed lot no.	_____
Reference virus lot no.	_____
Date of serology tests before inoculation	_____
Dilution used for inoculation	_____
Volume and route of inoculation	_____
Date of inoculation	_____
Number of mouse LD ₅₀ or PFU inoculated	_____
Date of end of the test	_____

Viscerotropism test (working virus seed lot)

Specify if mice or cells are used for virus titration. See p. 52 for an example of the viscerotropism test worksheet.

Result (pass or fail) _____

Immunogenicity test (working virus seed lot)

Specify if mice or cells are used for virus titration. See p. 53 for an example of the immunogenicity test worksheet.

Result (pass or fail) _____

Neurotropism test (working virus seed lot)

Please see pp. 54–58 for examples of the clinical and histological worksheets

Result (pass or fail) _____

Identity test (A.4.1.4)

Comparison of virus titre in presence of normal serum with that in presence of immune serum

Method _____

Date _____

Results _____

Sterility tests (A.4.1.5)

No. of containers tested _____

	<i>bacteria</i>	<i>fungi</i>	<i>mycoplasmas</i>
Date of inoculation	_____	_____	_____
Media used	_____	_____	_____
Observation period	_____	_____	_____
Results	_____	_____	_____

Tests for avian and other viruses

Method _____

Date _____

Results _____

Control of single harvests (A.4.3)

Name and address of manufacturer _____

Laboratory reference no. _____

Information on source materials*Virus used to inoculate embryos*

Derived from master seed virus
lot no. _____

Working virus seed lot, reference
no. and source _____

Passage level of working virus
seed lot _____

Source of eggs

Is the flock under direct control
of manufacturer? _____

Is the flock monitored for
compliance with these
Requirements? _____

Results of tests on flock or control tissues

Tests for salmonella _____

Tests for fowl pox virus _____

Tests for avian mycobacteria _____

Tests for other microbial agents
(give details) _____

Information on manufacture

Date of inoculation of embryos _____

Temperature of incubation _____

Date of harvest _____

Age of embryos (at harvest) _____

Results of sterility tests _____

Control of final bulk (A.4.4)*Sterility tests (A.4.4.1)*

	<i>bacteria</i>	<i>fungi</i>	<i>mycoplasmas</i>
Date of inoculation	_____	_____	_____
Media used	_____	_____	_____
Observation period	_____	_____	_____
Results	_____	_____	_____

Protein content (A.4.4.3)

Method _____
Date _____
Result _____

Stabilizers and preservatives (A.4.4.4)

Name of stabilizer _____
Quantity or percentage _____
Name of preservative _____
Quantity or percentage _____
Date _____

Virus titration (A.4.4.5)

Method _____
Date _____
Result _____

Control tests on final lot (A.6)

Identity test (A.6.1)

Comparison of virus titre in presence of normal serum with
that in presence of immune serum

Method _____
Date _____
Result _____

Potency test (A.6.2)

Date of test _____
Reference batch no. _____

Relationship between titre as determined by inoculation in mice,
expressed in LD₅₀ per human dose, and/or the titre as determined in
cell culture, expressed in plaque forming units (PFU), per human
dose is: _____

	Vaccine samples			Reference samples
	1	2	3	
Virus concentration in each container	_____	_____	_____	_____
Mean virus titre per human dose, with 95% fiducial limits	_____			_____
Thermal stability test (A.6.3)				
Method	_____			
Date	_____			
Results	_____			
Sterility tests (A.6.4)				
	<i>bacteria</i>		<i>fungi</i>	
Date of inoculation	_____		_____	
Media used	_____		_____	
Observation period	_____		_____	
Results	_____		_____	
Tests for other agents (if performed) (A.6.5)				
Method	_____			
Date	_____			
Results	_____			
General safety test (A.6.6)				
<i>Tests in mice</i>				
Date of inoculation	_____			
No. of mice tested	_____			
Volume and route of injection	_____			
Observation period	_____			
Results (give details of deaths)	_____			
<i>Tests in guinea-pigs</i>				
Date of inoculation	_____			
No. of guinea-pigs tested	_____			

Volume and route of injection _____
Observation period _____
Results (give details of deaths) _____

Residual moisture (A.6.7)

Method _____
Date _____
Result _____

Residual ovalbumin (A.6.8)

Method _____
Date _____
Result _____

Endotoxin (A.6.9)

Method _____
Date _____
Result _____

Inspection of final containers (A.6.10)

Method _____
Date _____
Result _____

Submission addressed to national control authority

Name (typed) and signature
of head of production laboratory _____

Date _____

Certification by person taking overall responsibility for production
and control of the vaccine:

I certify that lot no. ____ of yellow fever vaccine satisfies national
requirements and/or Part A of the Requirements for Biological

Substances No. 3 (Requirements for Yellow Fever Vaccine, revised 1995).

Signature

Name (typed)

Date

Appendix 2

Model certificate for the release of yellow fever vaccine by national control authorities¹

The following final lots of yellow fever vaccine produced by _____² in _____,³ whose numbers appear on the labels of the final containers, meet all national requirements⁴ and Part A⁵ of Requirements for Biological Substances No. 3 (Requirements for Yellow Fever Vaccine), revised 1995⁶ and comply with Good Manufacturing Practices for Pharmaceutical⁷ and Biological⁸ Products.

Lot no.	Date of last potency test by manufacturer	Expiry date	Lot no.	Date of last potency test by manufacturer	Expiry date
_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____

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

The Director of the National Control Laboratory (or Authority as appropriate):⁹

Name (typed) _____

Signature _____

Date _____

¹ To be completed by the national control authority of the country where the vaccine has been manufactured, and to be provided by the vaccine manufacturer to importers.

² Name of manufacturer.

³ Country.

⁴ 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 control authority.

⁵ With the exception of provisions on distribution and shipping, which the national control authority may not be in a position to assess.

⁶ WHO Technical Report Series, No. 872, 1998, Annex 2.

⁷ WHO Technical Report Series, No. 823, 1992, Annex 1.

⁸ WHO Technical Report Series, No. 822, 1992, Annex 1.

⁹ Or his or her representative.

Appendix 3

Techniques for the potency evaluation of yellow fever vaccine

Cell-culture technique

Vero cell

Vero-cell seed and a description of a method for Vero-cell cultivation may be obtained from the World Health Organization. Monolayers of Vero cells are prepared in 35-mm Petri dishes, and 4 dishes are inoculated with each virus dilution (0.2ml per dish). After incubation for 1 hour at 36°C, the virus dilution is replaced by 3 ml of an overlay medium consisting of Leibovitz medium No. 15 or MEM medium at pH 7.1–7.2, bovine fetal serum (final concentration, 5%), and agarose (final concentration, 0.5%). Five days after inoculation, 0.1 ml of the overlay medium, with the addition of a 1:50 000 dilution of neutral red dye, is added. Plaques are counted on day 6. When plaque-forming titre is calculated, all dilutions should be considered in which the average number of plaques per dish is between 1 and 30.

Alternatively, equal amounts (0.2 ml) of a Vero-cell suspension (approximately 6×10^5 cells/ml) and virus dilution in Leibovitz medium No. 15 or MEM medium are placed in each of the 16-mm flat-bottomed wells in sterile trays suitable for cell culture. The trays are sealed and incubated for 4 hours at 36°C. After incubation, 0.4 ml of overlay medium, consisting of Leibovitz medium No. 15 or MEM medium, bovine fetal serum (3%), and carboxymethylcellulose (low viscosity sodium salt) (1.6%), is added to each well. The trays are resealed and incubated at 36°C for 5 days. On the sixth day, the trays are drained, washed with saline, stained with 1% naphthalene black or any other suitable stain and thoroughly rinsed with tap water. The plaques are then counted. All dilutions in which the average number of plaques per well is between 1 and 30 should be considered in calculating titre.

*PS cell*¹

This assay technique is a modification of that used by De Madrid & Porterfield (1). Equal amounts (0.2 ml) of a PS-cell suspension (approximately 6×10^5 cells/ml) and virus dilution in Leibovitz medium No. 15 (2) are placed in each of the 16-mm flat-bottomed wells

¹ These cells are latently infected with swine fever virus and their importation is prohibited in certain countries.

in sterile trays suitable for cell culture. The trays are sealed and incubated for 4 hours at 36°C. After incubation, 0.4ml of overlay medium, consisting of Leibovitz medium plus bovine fetal serum (3%) and carboxymethylcellulose (low-viscosity sodium salt) (1.6%), is added to each well. The trays are resealed and incubated at 36°C for 5 days. On the sixth day, the trays are drained, washed with saline, stained with 1% naphthalene black and thoroughly rinsed with tap water. The plaques are then counted. All dilutions in which the average number of plaques per well is between 1 and 20 should be considered in calculating titre.

Mouse LD₅₀ technique

The mouse LD₅₀ is the quantity of virus suspension estimated to produce fatal, specific encephalitis in 50% of intracerebrally inoculated mice.

Appropriate serial dilutions of the reconstituted vaccine are made in diluent for yellow fever virus (0.75% solution of bovine albumin (fraction V) in phosphate-buffer which has been demonstrated to be free of yellow fever virus inhibitors).

Mice of a strain highly susceptible to yellow fever virus, aged 4–6 weeks, are injected intracerebrally under anaesthesia with 0.03ml of vaccine dilution. Groups of at least 6 mice are used for each dilution, and the series of dilutions should result in mortality rates after inoculation spanning the range 0–100%. Inoculation of the mice should be performed immediately after the dilutions have been made. All deaths are recorded during a period of 21 days. Mice dying from unrelated causes are removed from both the numerator and denominator of mortality calculations. Mice paralysed on the twenty-first day are counted as alive.

References

1. De Madrid AT, Porterfield JS. A simple micro-culture method for the study of group B arboviruses. *Bulletin of the World Health Organization*, 1969, 40:113–121.
2. Leibovitz A. The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. *American journal of hygiene*, 1963, 78:173–180.