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Recommendations to Assure the Quality, Safety and Efficacy of Live Attenuated Yellow Fever Vaccines

**Proposed replacement of: TRS 872, Annex 2 and Amendment to TRS 872,
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Recommendations published by the WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If a NRA so desires, these Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. The parts of each section printed in small type are comments for additional guidance intended for manufacturers and NRAs, which may benefit from those details.

Table of contents

Introduction.....	3
General Considerations.....	4
Part A. Manufacturing recommendations	7
A.1 Definitions	8
A.2 Certification of the substrain of 17D virus for use in vaccine production	9
A.3 General manufacturing recommendations	9
A.4 Control of source materials.....	9
A.5 Control of vaccine production	12
A.6 Filling and containers	15
A.7 Control tests on final lot	15
A.8 Records.....	17
A.9 Retained samples	18
A.10 Labelling	18
A.11 Distribution and shipping.....	18
A.12 Stability, storage and expiry date	18
Part B. Nonclinical evaluation of live attenuated yellow fever vaccines.....	Error! Bookmark not defined. 9
B.1 Characterization of a new candidate yellow fever vaccine	20
B.2 Immunogenicity and other pharmacodynamic studies	20
B.3 Toxicity assessment	20
Part C. Clinical evaluation of live attenuated yellow fever vaccines	Error! Bookmark not defined.
C.1 General considerations	21
C.2 Safety and immunogenicity studies	21
C.3 Post-marketing studies and surveillance	24
Part D. Recommendations for national regulatory authorities	24
D.1 General	24
D.2 Release and certification	
Authors and Acknowledgements	25
References	28
Appendix 1	
Genealogy of 17D yellow fever vaccine strains	33
Appendix 2	

Tests in non-human primates of new virus master and working seeds 41

Appendix 3

Example, for guidance, of a summary protocol for the testing of new master and working seeds in the monkey safety test as described in Appendix 2 45

Appendix 4

Example, for guidance, of cell-culture techniques for the potency evaluation of yellow fever vaccine53

Appendix 5

Model summary protocol for manufacturing and control of live attenuated yellow fever vaccines54

Appendix 6

Model certificate for the release of live attenuated yellow fever vaccine by national regulatory authorities..... 70

Introduction

Requirements for yellow fever vaccine (Requirements for Biological Substances No. 3) were first formulated by a WHO Study Group in 1958 (1). The Requirements embodied recommendations made by the first WHO Expert Committee on Yellow Fever Vaccine (2), 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 (3), and such approval has been given only to vaccine prepared using seed derived from the 17D strain of yellow fever virus. Yellow fever continues to be the only disease for which a certificate of vaccination is required for entry into some countries and the update of the International Health Regulations (4) increased attention to the need for such certificates. The Requirements have been used also by national regulatory authorities (NRAs) 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 (ECBS) 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 (5). In 1975 the twenty-seventh meeting of the ECBS formulated revised Requirements for yellow fever vaccine (6). Much experience was gained with the preparation of yellow fever vaccine since 1975, and a further revision of the Requirements was approved by ECBS in 1995 (7).

A collaborative study to assess the suitability of a candidate International Standard (IS) for yellow fever vaccine indicated that the use of a standard for measuring potency which has been assigned an arbitrary unitage in International Units (IU) would markedly improve the agreement in the results between laboratories (8, 9). The first IS for yellow fever vaccine with an assigned potency of $10^{4.5}$ IU per ampoule was established in 2003 (10). A proposal to amend the requirements for yellow fever vaccine so that the potency of such vaccines be

expressed in IU per dose and that the dose recommended for use in humans shall not be less than $3.0 \log_{10}$ IU, with no upper limit on the quantity of virus in a dose was approved by ECBS in 2008 (11,12). The availability of an IS for yellow fever vaccine with an assigned potency in IU so that assay in mice and expression of virus titres in LD₅₀ is not required, also impacts on other sections of the requirements established in 1995 (11).

In 2008, ECBS recommended that the requirements for yellow fever vaccines be reviewed as it is over 10 years since they were published and sections on nonclinical and clinical evaluation for new candidate yellow fever vaccines are also required. To facilitate this process, WHO convened a meeting of experts, regulatory professionals and other stakeholders in Geneva, in May 2009 to discuss the scientific basis for the present revision of the requirements and to develop revised recommendations for yellow fever vaccines (13).

The scope of the present Recommendations encompasses live attenuated yellow fever vaccines derived from strain 17D, including 17D-204 and 17DD substrains.

This document should be read in conjunction with the relevant WHO guidelines including those on nonclinical (14) and clinical evaluation (15) of vaccines.

General considerations

The yellow fever virus is small (50 nm) and consists of a nucleocapsid with core protein (13kDa) containing single-stranded, positive-sense RNA surrounded by a lipoprotein envelope (16). The lipoprotein envelope contains two proteins, a small membrane protein (8kDa) and an envelope glycoprotein (53kDa), which is the major target of neutralizing antibodies and has type- and group-specific antigenic determinants. Wild-type yellow fever viruses have genomes of similar length but vary depending on the size of the 3' non-coding region (17,18). Based on sequence analysis, wild-type yellow fever virus strains have been classified into at least seven genotypes: five in Africa and two in South America. The genotypic variation is not accompanied by significant antigenic differences across strains and there is a single serotype (19).

The genome of the yellow fever virus strain from which all 17D vaccines are derived has been completely sequenced and has been found to contain 10,862 nucleotides, which encode three structural and seven non-structural proteins (20). There are two substrains in use today for the manufacture of 17D vaccine, namely 17D-204 and 17DD. 17D-213 is a derivative of 17D-204 that has gained a glycosylation site in the E protein but differs significantly in phenotype from 17D-204. It is sometimes considered to be a substrain of 17D and sometimes referred to as 17D-213. Genomic sequencing has been reported for many of the yellow fever vaccine viruses and their seeds currently used by different manufacturers. These studies show that there are very few nucleotide and amino acid differences between the vaccine strains. The yellow fever vaccine strains that have been and are being used for vaccine manufacture and their history are summarized in Appendix 1.

Yellow fever is a viral haemorrhagic fever that is endemic in 32 countries in Africa and 13 countries in Central and South America (21).

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 (22). There are two epidemiological patterns of yellow fever virus 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. In the Americas, the yellow fever virus circulates by means of an endemic, forest cycle that results in up to several hundred reports of infection primarily in non-immune forest workers per year, with occasional reports of isolated cases of urban yellow fever. 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 (23).

The case-fatality rate of yellow fever can reach as high as 20% to 80% in severely ill patients who are hospitalized (24). Case-fatality rates are highest among young children and the elderly. There are no antiviral drugs for any flavivirus infection including yellow fever so the availability of vaccines is important for both resident populations and travellers.

When 17D vaccine was first used in the late 1930s/early 1940s, some problems were observed which were 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 in 1945. As of 2009, more than 500 million doses of 17D vaccine had been administered (25) so there is a large amount of information available regarding vaccine safety. This vaccine has 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 (EPI) recommended that all countries at risk of yellow fever should incorporate the vaccine in their routine immunization programmes. In Africa, 22 countries have introduced yellow fever vaccine in routine childhood immunization. Routine vaccination coverage in countries at risk in Africa has increased from 16% in 2000 (8 countries) to 43% in 2008. In the Americas, coverage rose from 64% to 91% (21). In this regard it is of note that the limited data on vaccination of individuals with immunosuppression associated with HIV infection suggest that seroconversion is reduced without an increase in adverse events following immunization (AEFI) (26).

Serious adverse reactions that have been reported associated with 17D yellow fever vaccine administration and are of particular note include the following:

- a. Hypersensitivity reactions, including anaphylaxis, are believed to be associated with egg protein due to the vaccine being grown in embryonated chicken eggs. However, gelatine used by some manufacturers may be implicated in some hypersensitivity reactions.
- b. Yellow Fever Vaccine-Associated Neurologic Disease (YEL-AND) is a term recently introduced to define neurologic AEFIs that have occurred in temporal association with YF vaccination since 2000 (27). Encephalitis following 17D vaccination in vaccinees of any age was first described in the 1940s (28). The incidence rate was dramatically reduced to background levels after introduction of the seed lot system for manufacture of 17D

vaccines. However, in the 1950s, there were several individual case reports describing a self-limited encephalitis in infants and very young children that occurred in temporal association with 17D vaccines manufactured in accordance with the seed lot system (see section A.4.2.1). With one exception, these children recovered fully with no sequelae. However, these reports led to the recommendation by WHO that infants 6 months of age and below should not be vaccinated (19). Adoption of this recommendation and unknown factors led to the virtual elimination of post-vaccinal encephalitis by the mid-1960s. However, since 2000, there have been rare case reports of a variety of neurologic AEFIs in 17D vaccinees of all ages, particularly in the elderly (27). Rates of YEL-AND vary in different studies undertaken in different populations, but were observed to range from 0.19 to 0.8 per 100,000 doses in studies in Europe and the US (27,29). Both 17D-204 and 17DD substrain vaccines have been associated with YEL-AND.

c. A total of 51 cases of Yellow Fever Vaccine-Associated Viscerotropic Disease (YEL-AVD) had been identified up to May 2009 (25). The estimated reporting rate is between 0.004 and 0.4 per 100,000 doses, with a case fatality rate up to 64%. All the reported cases occurred after the primary dose (25). The published “index” case is from Brazil in 1975 (30). Currently the mechanism(s) responsible for the clinical picture of YEL-AVD, which can vary from “multi-organ system failure” without much evidence of hepatitis to a fulminant hepatitis resembling the disease Yellow Fever, is unknown (31-34). Available data suggest that YEL-AVD is related to individual, genetically-determined, and currently unknown host factors rather than to the vaccine virus itself. Molecular and animal studies performed to date provide no evidence that 17D vaccine virus mutations have contributed to YEL-AVD (35,36).

In 2007, a cluster of five YEL-AVD cases was reported after a mass yellow fever vaccination campaign in Peru with four fatal cases which were confirmed virologically and clinically among 42,000 vaccinees who received the same lot. This was the first (and so far only) occasion that a cluster of YEL-AVD cases has been observed in association with a particular lot of vaccine and it remains unexplained. No quality issues were identified in the manufacture of the vaccine and the characterization of the working seed and batch records were satisfactory. There were no reported problems from nine batches prepared from the same final bulk as the lot associated with YEL-AVD. The virus isolated from one of the individuals was sequenced and found to be vaccine virus with no evidence that it had mutated (37,38). An expert panel convened to investigate the reports found no features of the vaccine lot that would explain the cluster of cases (37), even though deaths were due to extensive replication of vaccine virus in multiple organs. There was no difference identified in quality between this lot and other lots of vaccine so it has been interpreted that there must be cofactors that led to these cases of YEL-AVD.

The rarity of YEL-AVD cases and limited clinical samples makes it difficult to substantiate hypotheses regarding the underlying pathological mechanisms. One potential hypothesis proposes a disconnection between the signalling of innate immune response and the timely activation of the adaptive immune response. Thus, future work that may lead to a more detailed understanding of the immune response induced by the vaccine may help to explain

YEL-AVD pathology. Thus far, risk factors that may be associated with the development of YEL-AVD include age (60 years and above) and a history of thymus disease or ablation.

Between 2007 and 2009 three cases of encephalitis in newborns (aged 10 days to 5 weeks) were reported in which infection in the infants appeared to have resulted from transmission of yellow fever vaccine virus from their recently vaccinated mothers through breastfeeding (39, 40). The onset of symptoms in those infants ranged from 8 to 25 days after maternal vaccination. One of the three cases was confirmed to be vaccine-associated by detection of vaccine virus RNA in the cerebrospinal fluid (CSF) of the infant (39). Maternal breast milk was not tested for evidence of vaccine virus in any of the three cases. Direct blood-to-blood transmission, through a break in the maternal areola and the mucosa of the infant's mouth, was thought to be the possible mode of infection. However, no examination for possible breast lesions was made in any of the cases. These reports are in accordance with the known risk of encephalitis after vaccination of infants less than 6 months of age. Based on these case reports, the potential risk of transmission of yellow fever vaccine virus from vaccinated mothers to breastfeeding infants was recently reviewed by the WHO's Global Advisory Committee on Vaccine Safety which concluded that further research is needed to quantify the potential risk, including the possibility of transmission through breast milk. Such studies might include testing breast milk from vaccinated mothers for presence of vaccine virus and testing their infants for evidence of sero-conversion to the vaccine virus. The committee also noted that the risk of potential transmission might vary, depending on whether mothers are primary vaccinees or have been previously vaccinated, and on the age of the infant when exposed (41).

The first IgM-confirmed transmission of yellow fever vaccine virus through transfusion of blood donated by recently vaccinated military personnel in the US was described in 2009 (42). Serological evidence of infection was confirmed in 3 of 5 transfusion recipients, however, no adverse events or clinical illness attributable to the infection were reported. This documented finding supports the current widely existing recommendations (previously based on a theoretical risk of vaccine virus transmission) for yellow fever vaccine recipients to defer from donating blood products for a period (generally 2 weeks) after vaccination.

It is important to ensure that new master or working seed are confirmed to exhibit levels of neurotropism and viscerotropism that are comparable with those documented for available 17D vaccines. Due to lack of suitable animal models for viscerotropic disease, much weight is currently placed on monkey neurovirulence studies which have a long history. The relevant safety test, performed on monkeys, has therefore been retained in these revised Recommendations.

There have been investigations into alternative animal models. A hamster model has been developed which shows viscerotropic disease (43). However, most wild-type strains, which need to be adapted to hamsters, and viruses from YEL-AVD cases do not show viscerotropic disease in this model. Another study reported results of a mouse model for studying viscerotropic disease caused by yellow fever virus infection, which may have some potential as a small animal model for yellow fever virus (44). The applicability of

these models will have to be established before they can be considered for use in the qualification of virus seeds (See part B).

The thermostability test (See section A.7.4) is undertaken to demonstrate consistency of production and not as a predictive value of real time stability (45). At the end of the incubation period, the geometric mean infectious titre in the incubated final containers shall not have decreased by more than $1.0 \log_{10}$ IU but there is no requirement for the minimum specification to be met.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 *International name and proper name*

The international name should be "Live attenuated yellow fever vaccine". The proper name should be the equivalent of the international name in the language of the country of origin. The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 *Descriptive definition*

Yellow fever vaccine shall consist of a freeze-dried preparation of viable, attenuated yellow fever virus (*Flavivirus hominis*, 17D strain). The preparation shall satisfy all the recommendations formulated below.

A.1.3 *International standards*

An International Standard for yellow fever vaccine is available from the National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK. This material is for use in the calibration of working reference materials for yellow fever vaccine which are included in each potency test so that the potency of vaccines is expressed in IU/dose.

NIBSC 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. This preparation may also be used in antibody assays of clinical trial sera. A non-immune control serum is also available. These preparations are monkey sera.

WHO reference virus 168-73 is available from NIBSC (see Appendix 2).

A.1.4 *Terminology*

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

Adventitious agents

Contaminating microorganisms including bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses that have been unintentionally introduced.

Final bulk

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

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 container of final bulk in one working session and lyophilized under standardized conditions in a common chamber.

International unit (IU)

An international unit (IU) is a unit of potency of measurement for the yellow fever vaccine, based on the determination of the infectivity of a virus preparation resulting in plaque formation in a suitable tissue culture monolayer in parallel with an accepted working standard calibrated in IU against the International Standard for yellow fever 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.

Specific pathogen free (SPF)

Animals that have been shown by the use of appropriate tests to be free from specified pathogenic microorganisms, and also refers to eggs derived from SPF birds (46,47).

Virus master seed lot

A quantity of virus suspension that has been processed at the same time to assure a uniform composition and having been characterized to the extent necessary to support developing the virus working seed lot. The characterized virus master seed lot is used for the preparation of virus working seed lots.

Virus working seed lot

A quantity of virus of uniform composition, fully characterized, only one passage from a virus master seed lot. The virus working seed lot is used for inoculating embryonated chicken eggs in the preparation of vaccine.

WHO primary seed virus (213-77)

A quantity of virus suspension of uniform composition, produced for WHO by the Robert Koch Institute and available to manufacturers for use in the preparation of a virus master seed lot.

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

Currently used substrains 17D-204 and 17DD have well-documented passage history (See Appendix 1), and safety records from non-clinical and clinical studies. Any new candidate 17D virus to be used as a master seed for production would require supporting data to qualify it for use. Virus seed lots that have been certified previously can be used. A yellow fever virus primary seed (213-77) is available from WHO upon request (previously known

as "WHO master seed") (48). Parts B and C of this document provide recommendations for evaluating new candidate 17D vaccine viruses.

Only seed lots derived from viruses that are approved by the national regulatory authority shall be used in the production of yellow fever vaccines.

A.3 General manufacturing recommendations

The general manufacturing recommendations for manufacturing establishments contained in the *Good manufacturing practices for pharmaceutical products: main principles* (49) and the *Good manufacturing practices for biological products* (50) shall apply to establishments manufacturing yellow fever vaccine. Staff directly involved with the production and testing of yellow fever vaccine shall be shown to be immune to yellow fever.

A.4 Control of source materials

A.4. 1 Eggs used for seed virus growth and vaccine production

Virus for the preparation of virus master and working seed lots and all vaccine production shall be grown in embryonated chicken eggs from a closed, SPF flock, which are monitored by methods approved by the national regulatory authority or the national animal health authority.

All chickens are bled when an SPF flock is established, and thereafter a percentage of the birds are bled at specified time intervals to detect exposure of the flock to microbes with the potential to cause quality failure in assessments for adventitious agents. In some countries, SPF flocks are monitored on a weekly basis for quality control. The sera are screened for antibodies to the relevant pathogens. The pathogens may also be detected in the flocks by culture or other detection methods including PCR. Any chicken in an SPF flock that dies should be investigated to determine the cause of death.

Microbes of interest in flock husbandry may vary by geographic region but include as a minimum: avian adenoviruses, avian encephalomyelitis virus, avian infectious bronchitis viruses, avian infectious laryngotracheitis virus, avian leukosis viruses (ALV), avian nephritis virus, avian orthoreoviruses, avian reticuloendotheliosis virus, chicken anemia virus, egg drop syndrome virus, fowl pox virus, infectious bursal disease viruses, influenza A viruses, Marek's disease virus, Newcastle disease virus, *Mycobacterium avium*, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella species*, and *Haemophilus paragallinarum*.

The flock must not have been vaccinated with live Newcastle disease virus vaccine. In addition, flocks should not be receiving any chemotherapeutic agents (e.g. antimicrobial agents and coccidiostats). It is also recommended that eggs be obtained from young hens.

A.4. 2 Yellow fever virus

The substrain of 17D vaccine virus used in the production of vaccine should be certified as described in section A.2.

A.4.2.1 Virus seed lot system

The production of vaccine should be based on the virus master seed lot and virus working seed lot system.

Virus seed lots should be stored in a dedicated temperature-monitored freezer at a temperature that ensures stability, namely less than -60°C. In some laboratories, the virus master and working seed lots are stored in more than one location.

The virus master and working seed lots shall not contain any human protein or added serum or antibiotics.

The virus master and working seed lots shall be free from ALV, mycoplasmas or other adventitious agents as shown by suitable tests (A.4.2.2.3 and A.4.2.2.4).

The inoculum for infecting eggs used in the production of vaccine shall be from a virus 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.4.2.2 Tests on virus master and working seeds

A.4.2.2.1. Identity

Each virus master and working seed lot should be identified as yellow fever virus by immunological assay or by molecular methods and comparison to an appropriate published 17D vaccine virus. An identity test shall be performed on at least one container from each virus master and working seed lot.

A.4.2.2.2. Genotype characterization

For any new virus master and working seed, it is recommended that the first three, consecutive consistency vaccine lots be analyzed for consensus sequence changes from the seed virus (total genome sequence). The sequence results should be used to demonstrate the consistency of the production process.

Routine sequence analysis of final bulk vaccine is not recommended.

A.4.2.2.3 Tests for bacteria, fungi and mycoplasmas

Each virus master and working seed lot should be tested for bacterial, fungal, and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 (52) and 5.3 (53) of the *General requirements for the sterility of biological substances*, or by a method approved by the national regulatory authority.

Nucleic Acid Amplification Techniques (NAT) alone or in combination with cell culture, with an appropriate detection method, might be used as an alternative to one or both of the compendial mycoplasma detection methods after suitable validation and agreement from national regulatory authority (54).

A.4.2.2.4. Tests for adventitious agents

Each virus master and working seed lot should be tested for ALV and other adventitious agents relevant to the passage history of the seed virus. In addition, each virus working seed lot should be tested in both cells and eggs for the other adventitious agents.

Neutralization of yellow fever virus is necessary for many tests because the virus is cytopathogenic. Where antisera are used to neutralize yellow fever virus, the antigen used to generate the antisera should be produced in cell cultures (other than those derived from chickens) and free from extraneous agents. After neutralization of the yellow fever virus by hyper-immune antibody preparation, the virus pool should be inoculated on cell cultures of human cells, simian cells, chicken cells. Following inoculation, the cell cultures should be observed microscopically for cytopathic changes. At the end of the observation period, the cells should be tested for haemadsorbing viruses. The cell cultures, the method of incubation and the period of observation shall be approved by the national regulatory authority. A specific monoclonal antibody may be used instead of a hyper-immune polyclonal serum.

Each virus master or working seed lot should also be tested in animals that may include guinea-pigs, adult mice, suckling mice and embryonated chicken eggs, as appropriate. For test details refer to the *WHO Requirements for measles vaccines (Live)* (55, section 4.2.1.1).

New molecular methods with broad detection capabilities are being developed for detection of adventitious agents. These methods include degenerate NAT for whole virus families with analysis of the amplicons by hybridization, sequencing or mass spectrometry; NAT with random primers followed by analysis of the amplicons on large oligonucleotide micro-arrays of conserved viral sequencing or digital subtraction of expressed sequences; and high throughput sequencing. These methods might be used in the future to supplement existing methods or as alternative methods to both *in vivo* and *in vitro* tests after appropriate validation and approval of the national regulatory authority (54).

Each virus master and working seed lot should be tested for and shown to be free from *Mycobacterium avium* by an appropriate test approved by the national regulatory authority.

Nucleic acid amplification techniques might be used as an alternative to mycobacteria microbiological culture method and/or to the *in vivo* guinea-pigs test for the detection of mycobacteria after suitable validation and approval of the national regulatory authority (54).

Additional testing for ALV and adventitious agents may be performed on control eggs for the virus working seed lot (e.g. fowl pox, salmonella, mycobacteria).

A.4.2.2.5. Tests in non-human primates

Each virus master and working seed lot should be tested for neurotropism, viscerotropism and immunogenicity in non-human primates as described in Appendix 2.

A.4.2.2.6. Virus titration for infectivity

Each virus master and working seed lot should be assayed for yellow fever virus infectivity in a sensitive assay in cell cultures as described in Appendix 4.

A.5 Control of vaccine production

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

A.5.1 Tests on uninoculated control eggs

If monitoring of the flocks supplying embryonated chicken eggs is not under the direct responsibility of the vaccine manufacturer, an SPF Certificate and Quality Control Certificate (with test results) should be available from the supplier. The following tests shall be performed:

A sample of 2% of, but in any case not less than 20 and not more than 80 uninoculated embryonated eggs from the batch used for vaccine production shall be incubated under the same conditions as the inoculated embryonated eggs. At the time of virus harvest, the uninoculated embryonated eggs shall be processed in the same manner as the inoculated embryonated eggs, and the extract from the control embryos shall be shown to be free from haemagglutinating agents and ALV and other adventitious agents by methods approved by the national regulatory authority.

A.5.2 Single harvests

After inoculation and incubation at a controlled temperature and humidity, only living and normal chicken embryos shall be harvested. The age of embryos at the time of harvest shall be calculated from the initial introduction of the eggs into the incubator and shall be no more than 12 days. The number of rejected eggs may be estimated to monitor the consistency of the production.

After homogenization and centrifugation, the embryonic extract shall be kept at -60°C or below until further processing.

All intermediates should be maintained under conditions shown by the manufacturer to retain the desired biological activity. Storage periods should be approved by the national regulatory authority.

A.5.3 Tests on single harvests

A.5.3.1 Sampling

Samples required for the testing of single harvests should be taken immediately on harvesting prior to further processing. If the tests are not performed immediately, the samples taken for tests on single harvests should be kept at a temperature of -60°C or below and subjected to no more than one freeze-thaw cycle.

A.5.3.2 Identity

Each single harvest or group of single harvests from a daily production should be identified as yellow fever virus by immunological assay on cell culture using specific antibodies or by molecular methods approved by the national regulatory authority.

A.5.3.3 Tests for bacteria, fungi and mycoplasmas

Each single harvest or group of single harvests from a daily production should be tested for bacterial, fungal, and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 (52) and 5.3 (53) of the *General requirements for the sterility of biological substances*, or by a method approved by the national regulatory authority.

Nucleic acid amplification techniques alone or in combination with cell culture, with an appropriate detection method, might be used as an alternative to one or both of the compendial mycoplasma detection methods after suitable validation and agreement of the national regulatory authority (54).

A.5.3.4 Tests for adventitious agents

Each single harvest or group of single harvests from a daily production should be tested for and shown to be free from *Mycobacterium avium* by an appropriate test approved by the national regulatory authority.

Nucleic acid amplification techniques might be used as an alternative to mycobacteria microbiological culture method and/or to the in vivo guinea-pigs test for the detection of mycobacteria after suitable validation and approval of the national regulatory authority (54).

A.5.3.5 Virus titration

The live yellow fever virus content of each single harvest or group of single harvests from a daily production shall be determined by titration in cell culture against a reference preparation and the titre should be expressed in IU/ml (see Appendix 4).

A.5.4 Final bulk

The final bulk shall be prepared from one or several single harvests. The addition of any stabilizing agents shall be approved by the national regulatory authority. The following tests shall be performed, 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 and subjected to no more than one freeze-thaw cycle.

A.5.4.1 Sterility tests for bacteria and fungi

Each final bulk should be tested for bacterial and fungal sterility as specified in Part A, sections 5.2 of the *General requirements for the sterility of biological substances (52)*, or by the methods approved by the national regulatory authority.

A.5.4.2 Stabilizers

If a stabilizing agent is added, its concentration shall be measured. The method used and permitted levels shall be approved by the national regulatory authority.

A.5.4.3 Virus titration (if performed)

The live yellow fever virus content of each final bulk shall be determined by titration in cell culture against a reference preparation and the titre should be expressed in IU/ml (see Appendix 4).

A.6 Filling and containers

The general requirements concerning filling and containers given in *Good Manufacturing Practices for Biological Products (50)* 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. The vaccine shall be freeze-dried.

Single- and multiple-dose containers may be used.
Failure to achieve adequate drying will result in a product that is susceptible to rapid deterioration even at 0°C . 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 regulatory authority with adequate data to prove the stability of the vaccine under appropriate conditions of storage and shipping (See section A.12).

A.7 Control tests on final lot

Samples should be taken from each final vaccine lot to be tested and fulfil requirements of this section. All the tests and specifications including methods used and the permissible limits for the different parameters listed under this section, unless otherwise specified, should be approved by the national regulatory authority.

A.7.1 Inspection of final containers

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

A7.1.1 Appearance

The appearance of the freeze-dried vaccine and the reconstituted vaccine should be described with respect to its form and colour. If reconstitution with the product diluent does not allow for the detection of particulates, an alternative diluent may be used.

If the glass used for the final containers does not permit inspection of the contents e.g. with tinted glass, visual inspection should be performed on the reconstituted vaccine and the observations shall comply with the specifications approved by the national regulatory authority.

A.7.2 Identity

An identity test shall be performed on at least one container from each final 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 or a monoclonal antibody known to be free from neutralizing agents that react with other flaviviruses shall be used.

A sensitive test in cell cultures (plaque reduction test) shall be used for the identity test. Dilutions of vaccine are mixed with immune and non-immune serum. A suitable test is described in Appendix 2 (see 2.Immunogenicity test). 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.

Molecular tests may also be used after suitable validation and approval of the national regulatory authority.

A.7.3 Potency

Three final containers shall be selected at random from each final lot and shall be individually tested on the same day against a reference preparation of yellow fever vaccine calibrated in IU approved by the national regulatory authority. The containers shall be assayed in cell cultures demonstrated to be of adequate sensitivity and approved by the national regulatory authority (see Appendix 4).

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 be held at a temperature between 20°C and 30°C for 20 minutes before further dilution. This material shall be considered as undiluted vaccine.

The dose recommended for use in humans shall not be less than 3.0 log₁₀ IU. The release specification shall be approved by the national regulatory authority.

An internal upper limit may be established by each manufacturer to monitor the consistency of production. E.g. based on mean titre in IU/dose + 3 standard deviations. The upper limit should be approved by the national regulatory authority.

Existing release specifications should not be changed unless justified by clinical data and approved by the national regulatory authority.

Major changes to existing vaccines e.g. during production or in formulation and which may have a potential impact on the efficacy of the vaccine, should be justified by clinical data and approved by the national regulatory authority.

Specifications for new manufacturers (including manufacturers with production transfer) should be set by clinical trial, and expressed in IU.

A.7.4 Thermal stability

The thermostability test is to demonstrate consistency of production. Additional guidance on evaluation of vaccine stability is provided in the *WHO guidelines on stability evaluation of vaccines (45)*.

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. A reference preparation calibrated in IU approved by the national regulatory 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 IU.

A.7.5 Sterility tests for bacteria and fungi

Each final lot should be tested for bacterial and fungal sterility as specified in Part A, sections 5.2 of the *General requirements for the sterility of biological substances (52)*, or by the methods approved by the national regulatory authority.

A.7.6 General safety test

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

This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the national regulatory authority.

A.7.7 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 regulatory authority. The upper limit of the moisture content shall be approved by the national regulatory authority on the basis of stability tests.

A.7.8 Residual ovalbumin

The content of residual ovalbumin should be determined and be within limits approved by the national regulatory authority.

A.7.9 Endotoxin content

The vaccine in the final container should be tested for endotoxin by a *Limulus amoebocyte lysate* test. The endotoxin content should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and approved by the national regulatory authority.

A.7.10 Residual antibiotics (if applicable)

If any antibiotics is added in the vaccine production, the content of the residual antibiotics should be determined and be within limits approved by the national regulatory authority.

A.8 Records

The requirements given in Section 8 of *Good Manufacturing Practices for Biological Products (50)* shall apply.

A.9 Retained samples

The requirements given in Section 9.5 of *Good Manufacturing Practices for Biological Products (50)* shall apply.

A.10 Labelling

The requirements given in Section 7 of *Good Manufacturing Practices for Biological Products (50)* 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 Recommendations;
- state the nature of the preparation, specify the substrain of yellow fever virus in the vaccine, the minimum number of infectious units per human dose, and that SPF eggs were used
- 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 contraindicated in children aged under 6 months and is not recommended for those aged 6-8 months (19), except in specific circumstances and it should be in accordance with available official recommendations;
- state that the reconstituted vaccine should be used without delay, or if not used immediately, should be stored between 2°C and 8°C, protected from direct light and used within 6 hours (56).

A.11 Distribution and shipping

The requirements given in Section 8 of *Good Manufacturing Practices for Biological Products (50)* shall apply. Further guidance is provided in the *WHO Model guidance for the storage and transport of time and temperature-sensitive pharmaceutical products (57)*.

A.12 Stability, storage and expiry date

A.12.1 Stability testing

Adequate stability studies form an essential part of vaccine development. Current guidance on evaluation of vaccine stability is provided in the *WHO guidelines on stability evaluation of vaccines (45)*. Stability testing should be performed at different stages of production, namely on single harvests or pool of single harvests, final bulk, final lot. In addition, such studies should be undertaken on reconstituted vaccine. Stability-indicating parameters should be defined or selected appropriately according to the stage of production. It is advisable to assign a shelf-life to all in-process materials during vaccine production, in particular intermediates such as single harvests and final bulk.

The stability of the vaccine in its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the national regulatory authorities on at least three consecutive lots of final product. Accelerated thermal stability tests may be undertaken to give additional information on the overall stability of a vaccine.

The formulation of vaccine should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with national regulatory authorities. Following licensure, ongoing monitoring of vaccine stability is recommended to support shelf-life specifications and to refine the stability profile (45). Data should be provided to the national regulatory authority as per local regulatory requirements.

A.12.2 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 approved by the national regulatory authority.

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. These shall be approved by the national regulatory authority. The vaccine should have been shown to meet the release specifications for a period equal to that between the date of release and the expiry date.

A.12.3 Expiry date

The expiry date should be defined on the basis of shelf-life and supported by the stability studies with the approval of the national regulatory authority.

A.12.4 Expiry of reconstituted vaccine

For single dose containers, the reconstituted vaccine should be used immediately. For multi-dose containers, the container should be kept in the dark at 2-8°C and the expiry time

for use of an opened container should be defined by stability studies, approved by the national regulatory authority, but not more than 6 hours (56).

Part B. Nonclinical evaluation of live attenuated yellow fever vaccines

The nonclinical evaluation of candidate live attenuated yellow fever vaccines derived from substrains of the 17D strain should be based on *WHO guidelines on nonclinical evaluation of vaccines (14)*.

Any new candidate 17D strain that is not already in use by a manufacturer should be characterized with respect to immunogenicity and safety and compared to at least one strain in current use for the manufacture of a licensed vaccine. In the case of manufacturing changes for an existing vaccine, re-characterization of the vaccine strain may be required.

The following specific issues should be considered.

B.1 Characterization of a new candidate yellow fever vaccine

Any new candidate virus requires supporting data that would qualify it for use. The new candidate virus should be identified by historical records that include information on the origin of the virus, its method of attenuation, whether the virus has been biologically or genetically cloned prior to generation of the master seed, genetic sequence information and the passage level.

To assess genotypic and phenotypic stability, virus from each production passage level should be characterized by laboratory and animal tests in comparison with a currently acceptable vaccine. These tests may include full genome sequencing, growth in permissive and semi-permissive cell cultures, plaque size estimation by plaque assays, and mosquito infectivity and dissemination.

Seed viruses used in the manufacture of vaccine intended for clinical trials should be tested as described in Appendix 2 to demonstrate that the seed virus is suitable for use in vaccine production.

B.2 Immunogenicity and other pharmacodynamic studies

The non-clinical studies should indicate that the new candidate yellow fever vaccine induces neutralizing antibodies in mice and non-human primates to yellow fever virus. A currently licensed yellow fever vaccine should be included as a control in such studies.

B.3 Toxicity assessment

In the early development of a new candidate yellow fever vaccine and prior to the initiation of clinical trials in humans, toxicity assessment including systemic toxicity and local tolerance should be considered in relevant species in accordance with the WHO guidelines (14). The toxicology assessment should include an evaluation of neurotropism and viscerotropism. If the vaccine candidate is to be licensed to include women of child bearing potential, at an appropriate point in development, a reproductive toxicity study will

need to be conducted, in accordance with the WHO guidelines (14), and would require administration of the vaccine to pregnant animals once in the early phase of implantation/organogenesis, as this is the phase which is most at risk.

These studies must demonstrate that the new candidate yellow fever vaccine is safe and suitable for use in humans.

Appropriate safety characterization studies should be conducted, which will include an evaluation of neurotropism and viscerotropism, according to the accepted protocol, which suggests monkey as the relevant species and the use of the 17D vaccine as a comparator (see section A.4.2.2.5 and Appendix 2).

Part C. Clinical evaluation of live attenuated yellow fever vaccines

Clinical trials should adhere to the principles described in the *WHO guidelines for good clinical practice (GCP) for trials on pharmaceutical products* (58) and to the *WHO guidelines on clinical evaluation of vaccines: regulatory expectations* (15). All clinical trials should be approved by the relevant national regulatory authorities.

Some of the issues that are specific to the clinical evaluation of yellow fever vaccines derived from the 17D strain are discussed in the following sections. These sections should be read in conjunction with the general guidance mentioned above. It is also recommended that manufacturers should consult with relevant national regulatory authorities regarding the overall clinical development program.

The section considers the provision of clinical data required 1) when a new candidate live attenuated yellow fever vaccine derived from the 17D virus is developed and, 2) when there have been major changes to the manufacturing process of an established vaccine, including preparation of new virus master seed lot of an established strain. Clinical evaluation of vaccine manufactured using a new working seed lot is not required provided that the passage level is not more than one from the master seed lot, the working seed has been characterized and consistency of the manufacturing process has been demonstrated.

C.1 General considerations

Due to the success of 17D vaccines over the past 70 years studies of vaccine efficacy are not feasible. Therefore, clinical studies should assess the safety and immunogenicity of a candidate yellow fever vaccine in comparison with at least one licensed vaccine. The assessment of immunogenicity should be based on the elicitation of neutralizing antibodies, which are thought to be the basis of protection (59) although the actual mechanism of protection is not known (60,61,62).

The relative risk of YEL-AVD and YEL-AND for a new candidate yellow fever vaccine versus approved vaccines cannot be estimated from pre-approval studies but should be addressed as part of post-marketing surveillance.

C.2 Safety and immunogenicity studies

C.2.1 Assessment of the immune response

The demonstration of an immune response to vaccination should be based on the measurement of neutralizing antibody titres pre and post-vaccination. Neutralizing antibody may be determined either by the plaque reduction neutralization test (PRNT) or using the \log_{10} neutralization index (LNI). Geometric mean titers (GMTs), seroconversion rates and reverse cumulative distributions (RCD) should be provided. Seroconversion may be defined as either a fourfold increase in neutralizing antibody or the induction of measurable neutralizing antibody in a previously seronegative individual. It is desirable to consider these two phenomena separately in the comparison between a novel 17D vaccine and a licensed one used as control.

The flavivirus haemagglutination inhibition (HAI) test may be used to determine whether or not individuals enrolled into vaccine studies are flavivirus naïve (see below). It is not suitable for assessing responses to vaccination.

C.2.2 Immunogenicity studies

New candidate yellow fever vaccines (i.e. manufactured using a newly derived 17D strain) should be compared with at least one well-established and licensed 17D yellow fever vaccine. It is preferable that the comparative vaccine(s) selected should have been in widespread use for some years so that some data on effectiveness are available as well as a reliable description of the safety profile.

If the candidate vaccine has been produced by an existing manufacturer from a new virus master seed lot the comparison should be against a lot derived from the existing virus master seed.

C.2.3 Population

Safety and immunogenicity studies should be undertaken initially in healthy adults aged 18-60 years, preferably in need of vaccination against yellow fever. Subjects may be resident in non-endemic or endemic areas and should have no history of yellow fever or vaccination against yellow fever.

Studies in children should be undertaken only after adult studies have demonstrated that the safety profile is acceptable. In accordance with national and regional recommendations it is likely that inclusion of children aged 9 months or more would be possible and desirable in endemic countries. However, some national regulatory authorities have agreed that studies in children are not always required provided that the studies in adults are satisfactory and taking into account the overall experience with the use of 17D vaccines in children.

The study exclusion criteria should reflect the current contraindications to administration of live attenuated yellow fever vaccines (e.g. pregnancy, known allergy to vaccine components and immunosuppression).

C.2.4 Endpoints and analyses

The protocol should state the primary objective(s) of the study. The neutralizing antibody response to the candidate vaccine should be demonstrated to be non-inferior versus an appropriate licensed yellow fever vaccine based primarily on GMTs and/or seroconversion rates. The primary endpoint should be selected according to the study population and the anticipated immune response. For example, very high seroconversion rates are expected in healthy adults, which has implications for the selection of the non-inferiority margin and therefore the sample size calculation. Further details on demonstrating non-inferiority are described in the *WHO guidelines on clinical evaluation of vaccines: regulatory expectations (15)*.

The primary analysis should be conducted in subjects who are flavivirus naïve. If the HAI results are obtained only after vaccination (rather than being used to screen subjects for study eligibility before enrolment) the results for neutralizing antibody against yellow fever should be analyzed overall and separately for those who were flavivirus-naïve or non-naïve to assess any effect of pre-existing antibody to a heterologous flavivirus (e.g. dengue or West Nile viruses) on the response to yellow fever vaccine.

Other immunological parameters should be compared in planned secondary analyses (e.g. percentages reaching predefined titres).

C.2.5 Dose ranging studies

Dose ranging studies may be undertaken for new vaccines based on a 17D virus seed to determine the minimum dose of virus (in IU) required to provide adequate immune responses. These data could also be used to support the derivation of the minimum viral titre that should be present in the vaccine at the end of shelf-life. The assessment of safety of a 17D yellow fever vaccine during clinical studies should be in accordance with the *WHO guidelines on clinical evaluation of vaccines: regulatory expectations (15)*.

C.2.6 Concomitant administration with other vaccines

An evaluation of the effects of co-administration of a yellow fever vaccine with other vaccines should be considered taking into account which vaccines are most likely to be given concomitantly in different age groups and populations.

If a yellow fever vaccine is to be used in an EPI programme simultaneously with other vaccines, it is particularly important that the effects of co-administration should be evaluated. For example, some studies in children have shown that co-administration with measles-mumps-rubella (MMR) combined vaccines has resulted in lowered serological responses to yellow fever vaccines (63).

Immune responses to all other antigens co-administered with the yellow fever vaccine should be measured at least in subsets. While the study will usually be powered only to demonstrate non-inferiority with respect to neutralizing antibody against yellow fever the protocols should at least include planned secondary analyses of antigen-specific responses. If these analyses indicate that immune responses are lower on co-administration with a new yellow fever vaccine compared to the licensed vaccine(s) national regulatory authorities will need to consider the potential clinical consequences on a case by case basis.

C2.7 Viraemia

Assessment of viraemia is not routinely required for a 17D derived vaccine because it is usual that recipients of yellow fever vaccines have a transient viraemia.

A low level viraemia is known to occur after 17D vaccination. Titers of virus in blood have traditionally been determined by counting plaques in tissue culture monolayers that have been infected with serial dilutions of serum samples. More recently reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR have been used instead of the plaque assay. Quantitative RT-PCR assays should include generation of a standard curve using quantitative RT-PCR of 17D vaccine virus so that the results can be expressed as PFU equivalents or genomic equivalents. Currently there is no international standard available for quantitative RT-PCR assays.

C.2.8 Pre-licensure safety data

The general approach to the assessment of safety of a new 17D yellow fever vaccine during clinical studies should be in accordance with the *WHO guidelines on clinical evaluation of vaccines: regulatory expectations* (15). Planned safety studies should be supported by a clear scientific rationale. However, given the long history of the use of 17D vaccines, the national regulatory authority may decide that sufficient data can be obtained from the immunogenicity studies in relatively small numbers. Where a new 17D seed, which has not been used previously, is investigated, larger scale studies may be needed.

An appropriate pharmacovigilance plan should be developed and approved by the national regulatory authority prior to licensure.

C.3 Post-marketing studies and surveillance

Enhanced safety surveillance (particularly for detection of YEL-AND and YEL-AVD) should be undertaken during the initial post-approval years in collaboration with national regulatory authorities. The total duration of enhanced surveillance should be regularly reviewed by the national regulatory authority. Case definitions for YEL-AVD are being developed by the Brighton Collaboration and should be used when finalized (64).

If particular issues arise during pre-licensure studies or during post-licensure safety surveillance then it may be necessary to conduct specific post-licensure safety studies.

Part D. Recommendations for national regulatory authorities

D.1 General

The general recommendations for control laboratories given in the *Guidelines for national authorities on quality assurance for biological products* (51) should apply. These guidelines specify that no new biological substance should be released until consistency of manufacturing and quality as demonstrated by a consistent release of batches has been established. The detailed production and control procedures and any significant changes in them that may affect quality, safety and efficacy of yellow fever vaccine should be

discussed with and approved by the national regulatory authority. For control purposes, the national regulatory authority should obtain the International Standard for potency testing and, where necessary, establish national working reference preparation(s) calibrated against the International Standard.

D.2 Release and certification

A vaccine lot should be released only if it fulfils the national requirements and/or Part A of the present Recommendations. A protocol based on the model given in Appendix 5, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the national regulatory authority in support of a request for release of vaccine for use. A statement signed by the appropriate official of the national regulatory authority should be provided if requested by a manufacturing establishment and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these Recommendations. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory potency test as well as assigned expiry date on the basis of shelf life should be stated. A copy of the official national release document should be attached. The certificate should be based on the model given in Appendix 6. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

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Appendix 1

Genealogy of 17D yellow fever vaccine strains

Two live attenuated yellow fever vaccines were developed in the 1930s: the French neurotropic vaccine (FNV) prepared from wild-type strain French viscerotropic virus passaged in mouse brain, and the “17D” vaccine, prepared from wild-type strain Asibi 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 (1).

The 17D vaccine was developed by Theiler and Smith in 1937 and has been shown to protect against all seven known genotypes of wild-type yellow fever virus.

There are two substrains of the 17D vaccine that are used in vaccine production today (Figure 1), namely 17D-204 and 17DD. Some vaccines are also prepared from a distinct substrain of 17D-204 (17D-213) using seed viruses 112/95 and 213/77.

The 17D-204 vaccine substrain is utilized in all countries, except Brazil, where the 17DD vaccine substrain is used. The 17D-204 vaccine was developed from the original attenuated 17D by continued chick embryo passage (without neuronal tissue) from passage 176 to passage 204. Subsequently, the virus was passaged in embryonated chicken eggs and most currently manufactured vaccines are manufactured at passage levels between 235 and 240. The 17DD vaccine was derived by passage in whole chick embryonic tissue with the neuronal tissue removed from passage 176-195; however subsequent passages were undertaken independently in Brazil such that its development differed from 17D-204. This vaccine was passaged in embryonated chicken eggs and all currently manufactured vaccines are at passage levels 287. During the 1970s and 1980s it became apparent that some vaccines had been prepared in eggs contaminated with avian leucosis virus (ALV) and so a number of manufacturers prepared ALV-free seeds of 17D virus in order to remove the endogenous retrovirus. The Robert Koch Institute in Germany, on behalf of WHO, established a new seed lot from 17D-204 substrain at passage 237, termed 213-77, which was certified free of ALV contamination, and is used at passage 239-240 in embryonated chicken eggs (2, 3). 213-77 is considered by some, but not all, scientists, to be a substrain of 17D due to acquisition of an envelope protein glycosylation site compared to 17D-204 substrain and is sometimes referred to as 17D-213 (2).

Over the years there have been many manufacturers of yellow fever vaccines (see Figure 1). The 17D-204 substrain vaccine has been manufactured in France, Senegal, South Africa, United States, the Netherlands, United Kingdom, Germany and India, and 17DD substrain vaccine in Brazil and Colombia. The 17D-213 substrain vaccine has been manufactured in Nigeria and Russia, plus Berna Biotech (now Crucell) in Switzerland has developed a vaccine derived from seed virus 112/95 but not yet marketed by the latter. At the present time there are only six producers: Sanofi Pasteur in France and United States (17D-204), Institut Pasteur, Dakar, Senegal (17D-204), Federal State Unitary Enterprise of Chumakov Institute of Poliomyelitis and Viral Encephalitides (17D-213), Beijing Tiantan Biological

Products Co., Ltd. (17D-204), and Bio-Manguinhos, FioCruz, Rio de Janeiro, Brazil (17DD). Currently, four of the manufacturers are prequalified by the WHO to provide yellow fever vaccine for use in developing countries (http://www.who.int/immunization_standards/vaccine_quality/yellow_fever/en/index.html/). The others produce yellow fever vaccine for domestic use.

The 17D-204 vaccine virus genome has 10,862 nucleotides in length and encodes a 3,411 amino acid polyprotein, which is flanked by a 5' non-coding region of 118 nucleotides and a 3' non-coding region of 511 nucleotides (4,5). The 5' terminus has a type 1 cap followed by two conserved nucleotides (AG) and the 3' terminus lacks a poly A tract (4). The polyprotein encodes 10 proteins: the structural proteins-capsid (C), membrane (M) and envelope (E) proteins are encoded by the N-terminal one-third of the polyprotein; and the nonstructural (NS) proteins; NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are encoded by the C-terminal two-thirds of the polyprotein. The major immunogen is the E protein, which encodes epitopes inducing neutralizing antibodies that are primarily responsible for the protective immune response. Monoclonal antibodies have identified a number of overlapping epitopes on the E protein (6,7). Physically these epitopes are either yellow fever strain-, yellow fever type-, complex-specific or flavivirus genus common, while biologically some of these epitopes are associated with haemagglutination inhibition (HI), which may or may not be associated with neutralization (6 -17). Overall, few epitopes are involved in neutralization and very few elicit high titer neutralization (6, 12, 16). Monoclonal antibodies have been generated against yellow fever wild-type and vaccine strains. Wild-type specific (6, 8, 10, 11, 14, 15, 17) and vaccine-specific epitopes (i.e. recognize 17D and FNV viruses only) (7, 8, 10, 11, 14, 15, 17), 17D-204 and 17DD substrain specific epitopes (8, 9, 13, 14) have all been identified on the E protein. To date, few epitopes have been mapped to specific amino acids on the E protein: two yellow fever type-specific epitopes have been mapped to amino acids 71/72 and 153/155, a wild-type epitope to amino acid 173 and a 17D-204 substrain specific epitope to amino acids 305 and 325 (18-20). Human cytotoxic T cell epitopes are found on the E structural protein and the NS1, NS2B and NS3 nonstructural proteins (21-22).

The genomes of 17DD (23, 24), 17D-204 (4,5) and 17D-213 (23, 24) vaccine viruses and parent wild-type Asibi virus have been sequenced (25). Unfortunately, the original 17D virus is not available. The three substrains differ slightly in sequence, thus justifying their classification as substrains (24), but they share 20 amino acid substitutions and four nucleotide changes in the 3' non-coding region. The capsid gene and 5' non-coding region of wild-type Asibi and 17D vaccine viruses were identical in sequence (Table 1). At the present time the molecular basis of attenuation of 17D vaccine is not known. Mouse models indicate that multiple mutations might be responsible for the attenuated phenotype.

Genomic sequences have been published for various 17D vaccines, some by manufacturers and some by academic laboratories, these include vaccines prepared in Brazil (23,24), China (*unpublished Genbank accession # FJ654700*), France (5, 26), Senegal (27), South Africa (28), United States (29), and American Type Culture Collection (ATCC) (4). The original published sequence of 17D-204 vaccine (4) is based on the virus obtained from ATCC.

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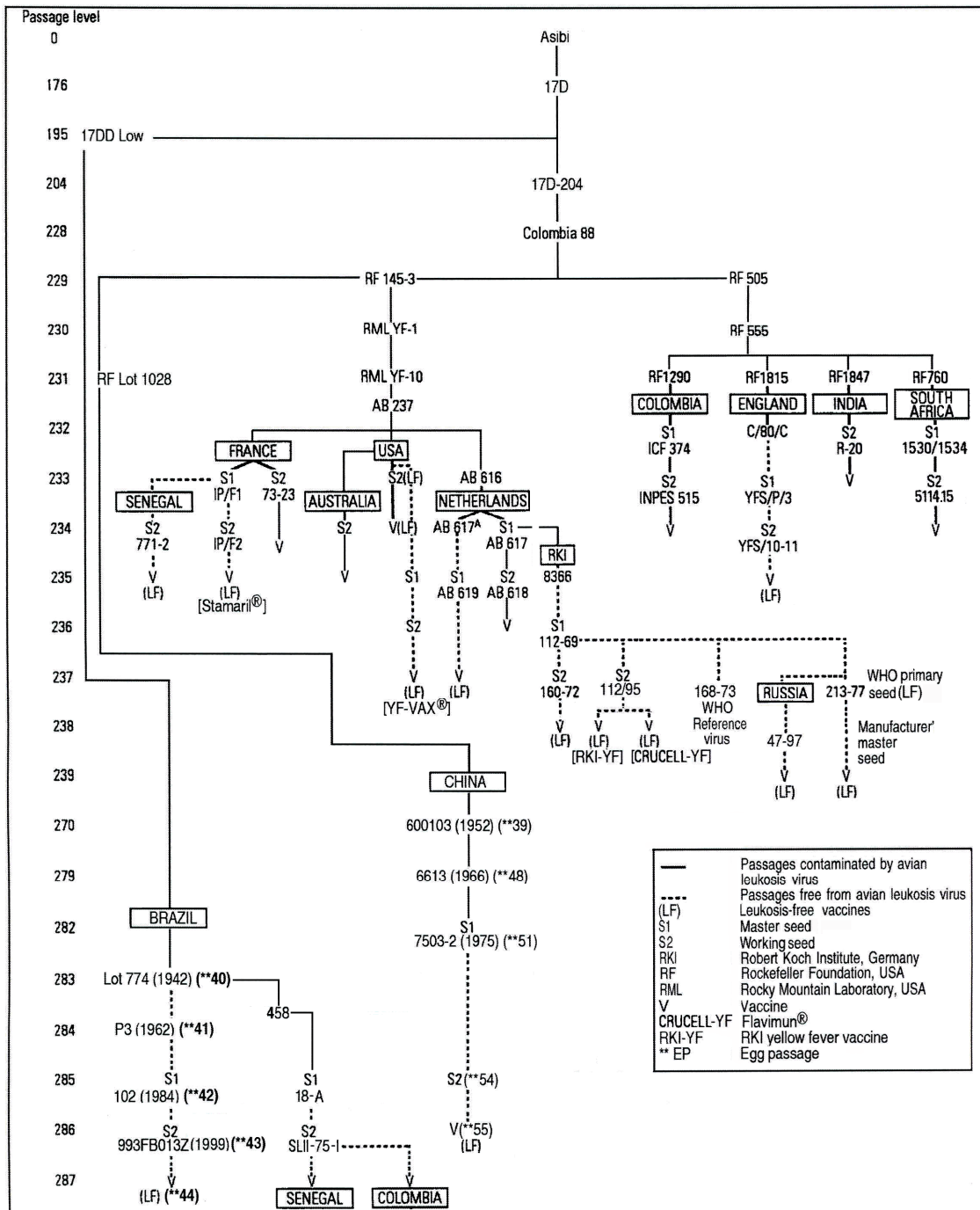
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Table 1. Amino acid differences and nucleotide differences in the 3' non-coding region between wild-type Asibi virus and attenuated 17D vaccines

Nucleotide	Gene	Amino acid*	Asibi	17D-204, 17D-213 and 17DD vaccine viruses
854	M	36	Leu	Phe
1127	E	52	Gly	Arg
1482		170	Ala	Val
1491		173	Thr	Ile
1572		200	Lys	Thr
1870		299	Met	Ile
1887		305	Ser	Phe
2112		380	Thr	Arg
2193		407	Ala	Val
3371	NS1	307	Ile	Val
3860	NS2A	118	Met	Val
4007		167	Thr	Ala
4022		172	Thr	Ala
4056		183	Ser	Phe
4505	NS2B	109	Ile	Leu
6023	NS3	485	Asp	Asn
6876	NS4A	146	Val	Ala
7171	NS4B	95	Ile	Met
10142	NS5	836	Glu	Lys
10338		900	Pro	Leu
10367	(3' NCR)	-	U	C
10418		-	U	C
10800		-	G	A
10847		-	A	C

* The 20 amino acids and 4 nucleotide changes in the 3' non-coding region identified in this table are conserved in any vaccine virus derived from the 17D strain.

Figure 1: History and genealogy of 17D vaccines and reference viruses: status as of October 2010



Note 1: This diagram only provides information on a historical overview of the use of strains derived from 17D yellow fever vaccine strain (as of October 2010). It does not indicate any WHO "qualification" or "approval" of the strains or vaccines in the context of this document.

Note 2: The First International Standard for yellow fever vaccine (Code 99/616) was derived from a bulk vaccine derived from seed S2 YFS/10-11 (England).

Note 3: The 17D-204 vaccines from Australia, Colombia, Germany, India, the Netherlands, and South Africa, plus the 17DD vaccines from Colombia and Senegal are not manufactured today.

Appendix 2

Tests in non-human primates of new virus master and working seeds

Each virus master and working seed lot shall be tested for viscerotropism, immunogenicity and neurotropism in a group of 10 test monkeys. Animals that are in the test vaccine and the reference groups should be blinded to the operators throughout the experiment. 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.

A WHO reference virus, 168-73, is available from NIBSC. This virus is of the same lineage as the WHO primary seed 213-77 (see appendix 1, figure 1), and unpublished data indicate that it is less neurovirulent in monkeys than strains of at least one other lineage known to produce an acceptable vaccine. Existing manufacturers should use a homologous reference; for example where their existing working seed is to be replaced by another derived from the same master seed, the existing seed can be used as the reference material provided it has been shown to produce a vaccine with satisfactory properties. It is recommended that sufficient stocks of such a reference are kept for all future anticipated replacements of the working seeds.

It is likely, but unproven, that 168-73 will be a satisfactory reference for seeds of the 213-77 lineage.

A new manufacturer with a new seed should use a homologous preparation known to produce a satisfactory product as reference. The inclusion of 168-73 as a common material would make it possible to compare different tests and one lineage with another for information.

The reference virus shall be approved by the national regulatory 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 virus by the haemagglutination inhibition test immediately prior to injection of the seed virus. They shall be healthy and shall not have been previously subjected to any experimentation. The test dose shall be injected into one frontal lobe of each monkey under anesthetic, and the monkeys shall be observed for a minimum of 30 days.

The test dose shall consist of 0.25ml containing not less than 5000 ($3.7 \log_{10}$) IU and not more than 50 000 ($4.7 \log_{10}$) IU as shown by titration in cell culture. In addition, the virus titer of the test virus seed lot and the reference virus shall be as close as possible.

Historically, the test dose shall consist of 0.25 ml containing the equivalent of not less than 5000 and not more than 50 000 median mouse lethal dose (mouse LD₅₀), as shown by a titration in cell culture.

1. 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 per dilution. In no case 0.03 ml of serum shall contain more than 500 ($2.7 \log_{10}$) IU and in no more than one case shall 0.03 ml of serum contain more than 100 ($2.0 \log_{10}$) IU.

2. 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 regulatory 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 4. 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 0.2 ml 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 4. 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.

3. 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 (1, 2).

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

3.1 Clinical evaluation

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 (2).

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.

3.2 Histological evaluation

The cervical and lumbar enlargements of the spinal cord and specific structures at five levels of the brain shall be examined (2) (see Appendix 3). The cervical and lumbar enlargements shall each be divided equally into six blocks. The blocks shall be dehydrated and embedded in paraffin wax; 15- μ m sections shall be cut and stained with gallocyanin. One section, consisting of two hemisections, shall be cut from each block.

Tissue blocks 3-4 mm 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- μ m sections shall be cut and stained with gallocyanin. 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 3) 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 (2). 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 3) 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 3. 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.

References

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Appendix 3

Example, for guidance, of a summary protocol for the testing of yellow fever vaccine in the monkey safety test as described in Appendix 2

Species _____

Number of monkeys inoculated _____

Master virus seed lot no. _____

Reference virus lot no. _____

Date of serology tests before inoculation _____

Dilution of yellow fever virus used for the inoculation _____

Volume and route of inoculation _____

Date of inoculation _____

Number of IU inoculated _____

Date of end of the test _____

Viscerotropism test (virus master seed lot)

Specify cell line 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 (virus master seed lot)

Specify cell line 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 (virus master 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:

Virus master 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)

Appendix 4

Example, for guidance, of cell-culture techniques for the potency evaluation of yellow fever vaccine

Vero cells or PS cells (1) may be used. (Note that PS cells are latently infected with swine fever virus and their importation is prohibited in certain countries). A Vero-cell seed and a description of a method for Vero-cell cultivation may be obtained from the World Health Organization.

A reference vaccine calibrated in IU shall be included in all assays and potency expressed as IU/dose.

Monolayers of the cell substrate are prepared in 6-well (35-mm) tissue culture plates. Serial four-fold dilutions of the reconstituted test and reference vaccine are prepared in inoculated in duplicate in the plate wells and incubated at 36°C for 1 hour. After this incubation period, the inoculum is replaced by 3 ml of agarose or 3.3% carboxyl methyl cellulose (CMC) overlay and the plates are further incubated at 36°C for 7 days. The agarose or CMC overlay is removed the cell cultures are stained with, either naphthalene black or crystal violet, washed and air-dried. The virus plaques are counted. In calculating the titre all dilutions should be considered in which the average number of plaques per well is between 1 and 30. The potency in IU/dose is calculated relative to the standard vaccine

For the test to be considered valid:

- the control cells should not show any plaque-forming or other cytopathic effect
- the reference vaccine should be within $10^{0.5}$ (0.5 Log₁₀) IU of its established mean titre.

Since yellow fever virus is light sensitive the vaccine should be protected from direct light during storage and testing.

Further detailed guidance is available in the *Manual of Laboratory Methods for testing vaccines used in the WHO Expanded Programme on Immunization. 1997 WHO/VSQ/97.04 Part II Potency control of live viral vaccines, Chapter 16 Yellow fever vaccine.*

Reference

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.

Appendix 5

Model summary protocol for manufacturing and control of live attenuated yellow fever vaccines

The following protocol is intended for guidance, and indicates the information that should be provided as a minimum by the manufacturer to the national regulatory authority.

Information and tests may be added or deleted as required by the national regulatory authority, if applicable.

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

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that accompanies the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot release certificate from the national regulatory authority of the country in which the vaccine was produced/released stating that the product meets the national requirements as well as Part A recommendations of this document published by WHO.

Summary information on the finished product (final lot)

International name:	_____
Trade name:	_____
Product licence (marketing authorization) number	_____
Country:	_____
Name and address of manufacturer:	_____
Site of manufacture of final lot:	_____
Name and address of licence holder if different:	_____
Virus strain	_____
Origin and short history	_____
Authority that approved virus strain	_____
Date approved	_____
Final lot number:	_____
Final bulk number:	_____
Volume of final bulk:	_____
Final product	_____

Type of container: _____

Number of doses per container: _____

Number of filled containers in this final lot: _____

Date of manufacture of final lot (filling or lyophilizing, if applicable): _____

Date on which last determination of virus concentration was started or date of start of period of validity:: _____

Shelf-life approved (months): _____

Expiry date: _____

Diluent: _____

Storage conditions: _____

Volume of single human dose: _____

Volume of vaccine per container: _____

Number of doses per container: _____

Prescribed virus concentration per single human dose: _____

Antibiotics added: _____

Release date: _____

Production information

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

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

Starting materials

The information requested below is to be presented on each submission. Full details on Master and working seed-lots upon first submission only and whenever a change has been introduced.

Virus Master seed lot

Source of 17D substrain _____

Master virus seed lot number. _____

Name and address of manufacturer _____

Passage level _____

Date of inoculation of embryos _____

Date of harvest _____

Age of embryos (at harvest) _____

Number of containers _____

Conditions of storage _____

Date VMS was established: _____

Date approved by the National Regulatory Authority: _____

Information on source materials

Source of eggs _____

Is the flock under direct control of manufacturer? _____

Is the flock monitored for compliance with these Recommendations? _____

Tests on virus master seed lot production (A.4.2.2)

Identity test (A.4.2.2.1)

Method used _____

Date test on _____

Date test off _____

Results _____

Lot number of reference reagents _____

Genotype characterization A.4.2.2.2.

Method used _____

Date test began and ended _____

Results _____

Lot number of reference reagents _____

Freedom from bacteria, fungi and mycoplasmas (A.4.2.2.3.)

Tests for bacteria and fungi

Method used _____

Number of vials tested _____

Volume of inoculum per vial _____

Volume of medium per vial _____

Observation period (specification) _____

Incubation	Media used	Inoculum	Date test began	Date test ended	Results
20–25 °C	_____	_____	_____	_____	_____
30–36 °C	_____	_____	_____	_____	_____
Negative control	_____	_____	_____	_____	_____

Tests for mycoplasmas

Method used _____

Volume tested _____

Media used _____

Temperature of incubation _____

Observation period (specification) _____

Positive controls (list of species used and results) _____

	Date test began	Date test ended	Results
Sub cultures at 3 rd day	_____	_____	_____
Sub cultures at 7 th day	_____	_____	_____
Sub cultures at 14th day	_____	_____	_____
Sub cultures at 21th day	_____	_____	_____

Indicator cell-culture method (if applicable)

Cell substrate used _____

Inoculum _____

Date of test _____

Passage number _____

Negative control _____

Positive controls _____

Date of staining _____

Results _____

Tests for ALV and other adventitious agents (A.4.2.2.4.)

Method

Volume tested _____

Date test on _____

Date test off _____

Result

Tests for Avian Mycobacteria:

Method

Media used _____

Temperature of incubation _____

Volume tested _____

Date test on _____

Date test off _____

Result _____

Safety test on animals (guinea-pigs, mice and embryonated chicken eggs

Species used _____

Number of animals inoculated _____

Volume injected per animal _____

Inoculation route _____

Date test on _____

Date test off _____

Result _____

Testing in non-human primates (A.4.2.2.5)

See Appendix 2

Virus titration for infectivity (A.4.2.2.6.)

Method _____

Date _____

Result _____

Virus working seed lot

Working virus seed lot number. _____

Name and address of manufacturer _____

Passage level _____

Date of inoculation of embryos _____

Temperature of incubation _____

Date of harvest _____

Age of embryos (at harvest) _____

Date of filling _____

Date of lyophilized (if appropriate) _____

Number of containers _____

Conditions of storage _____

Date virus working seed lot was established _____

Date approved by the National Regulatory Authority _____

Information on source materials

Source of eggs _____

Is the flock under direct control of manufacturer? _____

Is the flock monitored for compliance with these Recommendations? _____

Tests on virus working seed lot production (A.4.2.2)

Identity test (A.4.2.2.1)

Method used _____

Date test on _____

Date test off _____

Results _____

Lot number of reference reagents _____

Genotype characterization A.4.2.2.2.

Method used _____

Date test began and ended _____

Results _____

Lot number of reference reagents _____

Freedom from bacteria, fungi and mycoplasmas (A.4.2.2.3.)

Tests for bacteria and fungi

Method used _____

Number of vials tested _____

Volume of inoculum per vial _____

Volume of medium per vial _____

Observation period (specification) _____

Incubation	Media used	Inoculum	Date test began	Date test ended	Results
20–25 °C	_____	_____	_____	_____	_____
30–36 °C	_____	_____	_____	_____	_____
Negative control	_____	_____	_____	_____	_____

Tests for mycoplasmas

Method used _____

Volume tested _____

Media used _____

Temperature of incubation _____

Observation period (specification) _____

Positive controls (list of species used and results) _____

	Date test began	Date test ended	Results
Sub cultures at 3 rd day	_____	_____	_____
Sub cultures at 7 th day	_____	_____	_____
Sub cultures at 14th day	_____	_____	_____
Sub cultures at 21th day	_____	_____	_____

Indicator cell-culture method (if applicable)

Cell substrate used _____

Inoculum _____

Date of test _____

Passage number _____

Negative control _____
Positive controls _____
Date of staining _____
Results _____

Tests for ALV and other adventitious agents (A.4.2.2.4.)

Method _____
Volume tested _____
Date test on _____
Date test off _____
Result _____

Tests for Avian Mycobacteria:

Method _____
Media used _____
Temperature of incubation _____
Volume tested _____
Date test on _____
Date test off _____
Result _____

Tests for other adventitious agents on cell culture

Human diploid cells / Monkeys kidney cells / Primary chick embryo fibroblast cells

Method used _____
Test on cell culture _____
Type of Cells _____
Cell strain _____
Lot number of antiserum _____
Volume tested _____
Temperature of incubation _____
Date test on _____
Date test off _____
Date of haemadsorbing (if applicable) _____
Result _____

Test for adventitious agents on eggs (Avian Viruses)

Allantoic Cavity

Lot number of antiserum _____
Number of eggs inoculated _____
Volume inoculated per egg _____

Temperature of incubation _____

Inoculation date _____

Date of harvest _____

Date of haemagglutination test _____

Result _____

Yolk sac

Number of eggs inoculated _____

Volume inoculated per egg _____

Temperature of incubation _____

Inoculation date _____

Date of collect embryo observation _____

Result _____

Safety test on animals (guinea-pigs, mice and embryonated chicken eggs)

Species used _____

Number of animals inoculated _____

Volume injected per animal _____

Inoculation route _____

Date test on _____

Date test off _____

Result _____

Testing in non-human primates (A.4.2.2.5)

See Appendix 2

Virus titration for infectivity (A.4.2.2.6.)

Method _____

Date _____

Result _____

Control of vaccine production (A.5)

Information on source materials _____

Source of eggs _____

Is the flock under direct control of manufacturer? _____

Is the flock monitored for compliance with these Recommendations? _____

Virus used to inoculate embryos

Derived from master seed virus lot number. _____

Working virus seed lot, reference number and source _____

Passage level of working virus seed lot _____

Information on manufacture

Date of inoculation of embryos _____
Quantity of inoculated embryos _____
Temperature of incubation _____
Date of harvest _____
Age of embryos (at time of harvest) _____
Quantity of harvested embryos _____
Number of rejected eggs (ratio) _____
Number of containers _____
Conditions of storage _____
Expiry date _____

Tests on uninoculated control eggs (A.5.1)

Number of eggs used _____

Test for Haemagglutinating agents

Directly on allantoic fluid: _____

Method _____

Volume tested _____

Date of test _____

Result _____

After a passage in SPF eggs

Method _____

Volume tested _____

Route of inoculation _____

Date test on _____

Date test off _____

Result _____

Test for other adventitious agents on cell culture

Human diploid cells / Monkey kidney cells / Primary chick embryo fibroblast cells

Cell type _____

Volume tested _____

Temperature of incubation _____

Date test on _____

Date test off _____

Result _____

Test for ALV _____

Method _____

Volume tested _____

Date test on _____

Date test off _____

Result _____

Tests on control tissues

Test for Salmonella:

Method: _____

Volume tested _____

Date test on _____

Date test off _____

Tests for Avian Mycobacteria:

Method _____

Media used _____

Temperature of incubation _____

Volume tested _____

Date test on _____

Date test off _____

Result _____

Test for Fowl Pox Virus:

Method: _____

Volume tested: _____

Volume of negative controls _____

Date test on _____

Date test off _____

Result _____

Tests for ALV (if applicable)

Method used _____

Volume tested _____

Temperature _____

Date test on _____

Date test off _____

Result _____

Test for Haemagglutinating agents on embryonated hen eggs (avian viruses)

Allantoic Cavity

Number of eggs inoculated _____

Volume inoculated per egg _____

Temperature of incubation _____

Inoculation date _____

Date of harvest _____

Date of haemagglutination test _____

Result _____

Yolk sac

Number of eggs inoculated _____

Volume inoculated per egg _____

Temperature of incubation _____

Inoculation date _____

Date of collect embryo observation _____

Result _____

Test for other extraneous agents on cell culture

Human diploid cells / Monkey kidney cells / Primary chick embryo fibroblast cells

Cell type _____

Volume tested _____

Temperature of incubation _____

Date test on _____

Date test off _____

Result _____

Tests on single harvests (A.5.3)

Identity test (A.5.3.2)

Date test on _____

Date test off _____

Result _____

Freedom from bacteria, fungi and mycoplasmas (A.5.3.3)

Tests for bacteria and fungi

Method used _____

Number of vials tested _____

Volume of inoculum per vial _____

Volume of medium per vial _____

Observation period (specification) _____

Incubation	Media used	Inoculum	Date test began	Date test ended	Results
20–25 °C	_____	_____	_____	_____	_____
30–36 °C	_____	_____	_____	_____	_____
Negative	_____	_____	_____	_____	_____

control

Tests for mycoplasmas

Method used _____
Volume tested _____
Media used _____
Temperature of incubation _____
Observation period (specification) _____
Positive controls (list of species used and results) _____

	Date test began	Date test ended	Results
Sub cultures at 3 rd day	_____	_____	_____
Sub cultures at 7 th day	_____	_____	_____
Sub cultures at 14th day	_____	_____	_____
Sub cultures at 21th day	_____	_____	_____

Indicator cell-culture method (if applicable)

Cell substrate used _____
Inoculum _____
Date of test _____
Passage number _____
Negative control _____
Positive controls _____
Date of staining _____
Results _____

Tests for Adventitious agents (A5.3.4)

Tests for *Mycobacterium avium*

Method _____
Media used _____
Temperature of incubation _____
Volume tested _____
Date test on _____
Date test off _____
Result _____

Virus titration (A.5.3.5)

Method _____
Date _____
Result _____

Control of final bulk (A.5.4)

Sterility Tests (A. 5.4.1)

Tests for bacteria and fungi

Method used _____
 Number of vials tested _____
 Volume of inoculum per vial _____
 Volume of medium per vial _____
 Observation period (specification) _____

Incubation	Media used	Inoculum	Date test began	Date test ended	Results
20–25 °C	_____	_____	_____	_____	_____
30–36 °C	_____	_____	_____	_____	_____
Negative control	_____	_____	_____	_____	_____

Stabilizers if added (A.5.4.3)

Name of stabilizer _____
 Quantity or percentage _____
 Date _____

Virus titration (A.5.4.4) if performed

Method _____
 Date _____
 Result _____

Filling and containers (A.6)

Lot number _____
 Date of filling _____
 Volume of final bulk filled _____
 Filling volume per container _____
 Number of containers filled (gross) _____
 Date of lyophilization _____
 Number of containers rejected during inspection _____
 Number of containers sampled _____
 Total number of containers (net) _____
 Maximum period of storage approved _____
 Storage temperature and period _____

Control tests on final lot (A7)

Inspection of final containers (A.7.1)

Appearance
Date of test
Results
Before reconstitution
After reconstitution
Diluent used
Lot number of diluent used

Identity test (A.7.2)

Method used _____
Date test on _____
Date test off _____
Results _____
Lot number of reference reagents _____

Potency test (A.7.3)

Date of test _____
Reference batch number _____
Specification _____
Titre of reference batch (IU/0.5) _____
Vaccine Virus concentration (IU/human dose)
Vial 1 _____
Vial 2 _____
Vial 3 _____
Mean virus titre per human dose, with 95%
fiducial limits _____

Thermal stability test (A.7.4)

Date of test _____
Reference batch number _____
Titre of reference batch (IU/0.5ml) _____
Vaccine held at 37° for 14 days _____
Vaccine Virus concentration (IU/human dose)
Vial 1 _____
Vial 2 _____
Vial 3 _____
Mean virus titre per human dose, with 95%
fiducial limits _____
Loss in titre (in log₁₀IU) _____

Sterility Tests (A.7.5)

Tests for bacteria and fungi

Method used _____
 Number of vials tested _____
 Volume of inoculum per vial _____
 Volume of medium per vial _____
 Observation period (specification) _____

Incubation	Media used	Inoculum	Date test began	Date test ended	Results
20–25 °C	_____	_____	_____	_____	_____
30–36 °C	_____	_____	_____	_____	_____
Negative control	_____	_____	_____	_____	_____

General safety test (A.7.6) if performed

Tests in mice _____
 Date of inoculation _____
 No. of mice tested _____
 Volume and route of injection _____
 Observation period _____
 Results (give details of deaths) _____

Tests in guinea-pigs _____
 Date of inoculation _____
 No. of mice tested _____
 Volume and route of injection _____
 Observation period _____
 Results (give details of deaths) _____

Residual moisture (A.7.7)

Method _____
 Specification _____
 Date _____
 Result _____

Residual ovalbumin (A.7.8)

Method _____
 Specification _____
 Date _____
 Result _____

Endotoxin (A.7.9)

Method _____

Specification _____

Date _____

Result _____

Residual antibiotics (if applicable) (A.7.10)

Method _____

Specification _____

Date _____

Result _____

Submission addressed to national regulatory authority

Name of Head of Production (typed) _____

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

I certify that lot no. _____ of yellow fever vaccine , whose number appears on the label of the final container, meets all national requirements and/or satisfies Part A of the Recommendations for Biological Substances No. 3 (Recommendations for live attenuated yellow fever vaccine, revised 2010)

Signature: _____

Name (typed): _____

Date: _____

Appendix 6

Model certificate for the release of live attenuated yellow fever vaccine by national regulatory authorities

LOT RELEASE CERTIFICATE

The following lot(s) of yellow fever vaccine produced by _____⁽¹⁾ in _____⁽²⁾, whose numbers appear on the labels of the final containers, meet all national requirements⁽³⁾ and Part A⁽⁴⁾ of the WHO recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines (_____)⁽⁵⁾, and comply with Good Manufacturing Practices for Pharmaceutical Products: Main Principles⁽⁶⁾ and Good Manufacturing Practices for Biological Products⁽⁷⁾. As a minimum, this certificate is based on examination of the summary protocol of manufacturing and control.

The certificate may include the following information:

- Name and address of manufacturer;
- Site(s) of manufacturing;
- Trade name and/common name of product
- Marketing authorization number
- Lot number(s) (including sub-lot numbers, packaging lot numbers if necessary)
- Type of container
- Number of doses per container
- Number of containers/lot size
- Date of start of period of validity (e.g. manufacturing date) and/or expiry date
- Storage condition
- Signature and function of the authorized person and authorized agent to issue the certificate
- Date of issue of certificate
- Certificate number

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

Name (Typed)

Signature

Date

1 Name of manufacturer

2 Country of origin

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

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

5 WHO Technical Report Series, No. ____, YYYY, Annex __.

6 WHO Technical Report Series, No. 908, 2003, Annex 4.

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