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Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines

Amendment to Annex 5 of WHO Technical Report Series, No. 978

NOTE:

This draft document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein which will then be considered by the WHO Expert Committee on Biological Standardization (ECBS). The distribution of this document is intended to provide information on a proposed amendment to the WHO Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines to a broad audience and to ensure the transparency of the consultation process.

The text in its present form does not necessarily represent the agreed formulation of the ECBS. Written comments proposing modifications to this text MUST be received by 24 September 2021 using the Comment Form available separately and should be addressed to the Department of Health Products Policy and Standards, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

Comments may also be submitted electronically to the Responsible Officer: Dr Dianliang Lei at: leid@who.int.

The outcome of the deliberations of the ECBS will be published in the WHO Technical Report Series. The final agreed formulation of the document will be edited to be in conformity with the second edition of the *WHO style guide* (KMS/WHP/13.1).

boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines

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Introduction

The WHO Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines were adopted in 2010 *(1)*. Appendix 2 of these Recommendations addresses the testing of new virus master and working seed lots in non-human primates. Specifically, the document sets out the ways in which such lots should be tested for viscerotropism, immunogenicity and neurotropism, both in terms of clinical evidence and histological lesions, based on comparison against a reference virus approved by the NRA. Following reported discrepancies in the clinical scoring of monkeys during the assessment of working seed lots, WHO received a request from one manufacturer to align the neurotropism assessment outlined in the 2010 Recommendations with that used for the neurovirulence testing of oral poliomyelitis vaccine seed lots *(2)*. In this approach, clinical signs are recorded but do not form part of the assessment or pass/fail criteria.

At its seventy-first meeting in August 2020, the WHO Expert Committee on Biological Standardization expressed its agreement with the request made to WHO *(3)* and recommended that a drafting group be established to consult with as many yellow fever vaccine manufacturers and other stakeholders as possible on a proposed amendment to Appendix 2 of the 2010 Recommendations. At its seventy-third meeting in December 2020, the Committee was updated on the progress that had been made *(4)*. The currently specified approach had now been associated with several technical challenges including: (a) a paucity of data on the performance of the test; (b) the difficulties inherent in conducting a collaborative study involving non-human primates; (c) the lack of an international reference standard and consequent use of different reference materials; (d) reported discrepancies between clinical and histopathological assessments; (e) inconsistencies between staff in the scoring of clinical and histopathological observations; and (f) the sourcing of animals from different locations.

Work on amending Appendix 2 of the 2010 WHO Recommendations commenced in early 2021. On 18–19 March 2021, a virtual WHO working group meeting was held to discuss a proposed draft of the amended text. Overall, there was a consensus among manufacturers and NRAs that clinical evaluation provides important information and should be retained as part of the neurotropism test. However, there was also agreement that the test is somewhat subjective and that analysis can be difficult. It was recognized that there was potential for improvement in both test execution and analysis to increase harmonization between organizations. Based on these working group discussions, the appendix was revised by the WHO drafting group. Following public consultation and further revision, the amendment presented below was reviewed by the Committee at its meeting in October 2021.

No attempt was made at this time to review the 2010 WHO Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines in their entirety and only the issues outlined above have been addressed.

Amendment

Replace Appendix 2 with the following text:

Appendix 2

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

Each virus master and working seed lot should be tested for viscerotropism, immunogenicity and neurotropism in a group of 10 test monkeys. Animals that are in the test vaccine and reference groups should be blinded to the operators throughout the experiment. For the neurotropism test, the test monkeys inoculated with the virus seed lot should be compared with a similar group of 10 monkeys injected with a reference virus. 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. New manufacturers using a new seed should use a homologous preparation known to produce a satisfactory product as a reference material. The reference virus should be approved by the NRA.

A WHO reference virus, 168-73, is available from the National Institute for Biological Standards and Control, Potters Bar, England. This virus is of the same lineage as the WHO primary seed 213-77 (see Appendix 1, Figure 1), but available published data show that it behaves differently to vaccines of at least one other lineage in the monkey test, being much less neurovirulent and producing a higher viraemia. It is likely, though unproven, that 168-73 will be a satisfactory reference for seeds of the 213-77 lineage. While 168-73 is not suitable as a comparator for vaccines of other lineages, its inclusion as a common material would make it possible to compare different tests, and one lineage with another, for information.

The monkeys should be *Macaca mulatta* (i.e. rhesus monkeys) or *Macaca fascicularis* (i.e. cynomolgus monkeys) and should have been demonstrated to be non-immune to yellow fever virus and other flaviviruses using a relevant test (such as the haemagglutination inhibition test, ELISA or seroneutralization assay) immediately prior to injection of the seed virus. Randomized double blind controlled tests should be performed using healthy macaques of both sexes (from 2 to 4 kg body weight and 1 to 2 years old). The monkeys should not have been previously subjected to any experimentation. The test dose should be injected into one frontal lobe of each monkey, under anaesthetic, and the monkeys should be observed for a minimum of 30 days.

The test dose should consist of 0.25 mL 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 titres of the test virus seed lot and the reference virus should be as close as possible.

Historically, the test dose has consisted of 0.25 mL containing the equivalent of not less than 5000 and not more than 50 000 mouse LD_{50}, as shown by titration in cell culture.

1. Viscerotropism test

The criterion of viscerotropism (indicated by the amount of circulating virus) should 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 should be inoculated at dilutions of 1:10, 1:100 and 1:1000 into at least four cell culture vessels per dilution. In no case should 0.03 mL of serum contain more than 500 (2.7 $\log_{10}$) IU and in no more than one case should 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) should be fulfilled as follows: at least 90% of the test monkeys should be shown to have become immune within 30 days following injection of the test dose, as determined by examining their sera in the yellow fever virus neutralization test described below. In some countries it has been shown that, at low dilutions, some sera contain nonspecific inhibitors that interfere with this test. The NRA may therefore require sera to be treated to remove such substances.

Dilutions of 1:10, 1:40 and 1:160 of serum from each test monkey should 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 should 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 four separate cell culture vessels. The vessels should be handled in accordance with one of the cell culture techniques described in Appendix 4. In addition, 10 vessels should be similarly inoculated with virus as above, along with 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 containing virus and non-immune serum should be compared with the mean number of plaques in the vessels containing 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 should fail to reduce the mean number of plaques by 50% as compared with the vessels containing non-immune serum.

3. Neurotropism test

The monkeys in the test group should be compared with 10 monkeys injected with the reference virus with respect to both clinical evidence of encephalitis and the severity of histological lesions of the nervous system *(5, 6)*.

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

3.1 Clinical evaluation

The monkeys should be examined daily for 30 days by personnel familiar with the clinical signs of encephalitis in primates. All such signs should be recorded individually on a daily basis. Evaluation may include observation from a distance using closed circuit television to gather information. The use of implantable telemetry devices (for example to produce electroencephalograms or to monitor temperature and motor activity) may also be considered.

If necessary, the monkeys may be removed from their cages and examined for signs of motor weakness or spasticity, as described elsewhere *(6)*.

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

0: no general signs or signs of CNS involvement;
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.

Any animal unexpectedly found to be moribund, cachectic or unable to obtain food or water must be euthanized. A monkey that dies receives the score “4” from the day of death until day 30.

The clinical score for each monkey is the average of its daily scores; the clinical score for a group is the arithmetic mean of the individual scores. The timing of the development of clinical signs and their disappearance, as well as their severity, provides evidence of the identity of the test vaccine virus and the reference virus. When the test and reference materials are identical, as required, they will produce identical clinical signs, including in terms of the timescale of their appearance and resolution. It is acknowledged that the clinical evaluation may be imprecise. However, the kinetics and clinical score of monkeys injected with the virus being tested should not significantly differ from those of the monkeys injected with the reference virus.

3.2 Histopathological evaluation

The cervical and lumbar enlargements of the spinal cord and specific structures at five levels of the brain should be examined *(6)* (see Appendix 3). The cervical and lumbar enlargements should each be divided equally into six blocks. The blocks should be dehydrated and embedded in paraffin wax; 15 µm sections should be cut and stained with gallocyanin. Alternatively, 5 µm sections will be suitable for hematoxylin and eosin (H&E) staining or Nissl staining (gallocyanin, cresyl violet), as well as for immunohistochemistry techniques. One section, consisting of two hemisections, should be cut from each block.

Tissue blocks 3–4 mm thick should 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 midlevel of the inferior olives.

These blocks should be dehydrated and embedded in paraffin wax and 15 µm sections cut and stained with gallocyanin. Alternatively, 5 µm sections will be suitable for H&E staining or Nissl staining (gallocyanin, cresyl violet), as well as for immunohistochemistry techniques. A single section, consisting of two hemisections, should be cut from each block.

Sections should be examined microscopically and numerical scores assigned to each hemisection of the cervical and lumbar 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 no 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 *(6)*. These are called “discriminator areas”, whereas structures that are more susceptible to yellow fever virus replication are called “target areas”. Though both rhesus and 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 enlargements are target areas whereas in rhesus monkeys they are discriminator areas. The footnotes to the worksheets provided in Appendix 3 indicate in more detail the discriminator and target areas for the two species. The worksheets also list other anatomical structures that will be present in the brain sections but that are not included in the evaluation of a test sample because they are rarely affected (spared areas).

Three separate scores should be calculated for each monkey: (a) discriminator areas only; (b) target areas only; and (c) discriminator plus target areas. These three scores should be calculated as shown in the sample worksheets provided in Appendix 3.

Overall mean scores should 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 of these overall mean scores should be considered when determining virus seed lot acceptability. For the histological criterion of the neurotropism test to be satisfied, both of the overall mean scores for the test monkeys should not be significantly greater (at the 5% significance level) than the overall mean scores for the monkeys injected with the reference virus.

Both the clinical and histological criteria of the neurotropism test should be satisfied in order for the virus seed lot to meet the requirements for use in production.

It is acknowledged that clinical observations without telemetry may be more subjective than histological scoring. However, any failure to meet the statistical criteria should result in failure of the batch. Any exception made to this rule should be rare and would only be acceptable after a thorough investigation of the conducting of the tests, including a review of historical in-house data. Clinical observation should be included in the review and the record of the ultimate decision even if the findings do not meet the statistical criteria for a pass. However, any decision to ignore the statistical evaluation of clinical signs should be a rare and exceptional event involving close discussion with the NRA.

Authors and acknowledgements

The first draft of this amendment of Appendix 2 of the WHO Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines was prepared by a WHO drafting group comprising Dr P. Minor, St Albans, the United Kingdom; Dr J. Martin, National Institute for Biological Standards and Control, the United Kingdom; Professor A. D.T. Barrett, University of Texas Medical Branch Sealy Center for Vaccine Development, the United States of America (USA); Dr G. Cirefice, European Directorate for the Quality of Medicines & HealthCare, France; Dr E. Grabski, Paul-Ehrlich-Institut, Germany; Mrs F. Garnier, Agence nationale de sécurité du médicament et des produits de santé, France; Mrs V. Pithon, Agence nationale de sécurité du médicament et des produits de santé, France; and Dr D. Lei, World Health Organization, Switzerland, taking into consideration the discussions

and consensus reached during a WHO working group meeting to amend the WHO Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines, held virtually via Zoom video conferencing, 18–19 March 2021 and attended by: Professor A.D.T. Barrett, University of Texas Medical Branch Sealy Center for Vaccine Development, the USA; Dr R.M. Bretas, Agência Nacional de Vigilância Sanitária, Brazil; Dr G. Cirefice, European Directorate for the Quality of Medicines & HealthCare , France; Dr M. Diagne, Direction des Laboratoires, Senegal; Dr A. Dieng, Direction de la Pharmacie et du Médicament, Senegal; Dr E. Grabski, Paul-Ehrlich-Institut, Germany; Mrs F. Garnier, Agence nationale de sécurité du médicament et des produits de santé, France; Dr Y. Li, National Institutes for Food and Drug Control, China; Dr J. Martin, National Institute for Biological Standards and Control, the United Kingdom; Dr P. Minor, St Albans, the United Kingdom; Mrs V. Pithon, Agence nationale de sécurité du médicament et des produits de santé, France; Dr M.F. Reis e Silva Thees, Agência Nacional de Vigilância Sanitária, Brazil; Dr J. Wang, National Institutes for Food and Drug Control, China; Dr Y. Wang, National Institutes for Food and Drug Control, China; Dr M. Xu, National Institutes for Food and Drug Control, China; Dr A. Trapkova, Federal Service for Surveillance in Healthcare, Russian Federation; Dr D. Yakunin, Federal Service for Surveillance in Healthcare, Russian Federation; Dr F. Cano, Agence nationale de sécurité du médicament et des produits de santé, France; and Dr G. Cooper, National Institute for Biological Standards and Control, the United Kingdom. The following participants attended the meeting as representatives of vaccine manufacturers: M. da Luz Fernandes Leal; M. da Silva Freire; R.C. Guimarães; A. Hommaand; and R. Marchevsky (*Bio-Manguinhos/Fiocruz*, Brazil); A. Malkin; and A. Sinyugina (*Chumakov Federal Scientific Center for Research & Development of Immune-and-Biological Products of Russian Academy of Sciences*, Russian Federation); Y. Chen; H. Wang; N. Li; X. Zhu; and C. Jia (*Beijing Institute of Biological Products Co., Ltd*, China); C. Logvinoff; C. Allain; and E. Coppens (*Sanofi Pasteur*, France); and A.M. Diatta; and A.A. Sall (*Institut Pasteur de Dakar*, Senegal); The WHO Secretariat for the working group comprised: Dr I. Knezevic; and Dr D. Lei, Technical Specifications and Standards Unit, Access to Medicines and Health Products Division, World Health Organization, Switzerland; and Dr M. Janssen, Prequalification unit, Regulation and Prequalification, World Health Organization, Switzerland.

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