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**WORLD HEALTH ORGANIZATION
TECHNICAL REPORT SERIES**

No. 136

**EXPERT COMMITTEE
ON YELLOW FEVER VACCINE**

First Report

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WORLD HEALTH ORGANIZATION

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GENEVA

1957

EXPERT COMMITTEE ON YELLOW FEVER VACCINE

First Session

Geneva, 8-13 April 1957

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This report was originally issued in mimeographed form as document WHO/YFV/14, 15 April 1957.

PRINTED IN SWITZERLAND

EXPERT COMMITTEE ON YELLOW FEVER VACCINE

First Report *

A session of an Expert Committee on Yellow Fever Vaccine was held in Geneva from 8 to 13 April 1957.

The session was opened by Dr M. G. Candau, Director-General of the World Health Organization. In his opening address Dr Candau stated that vaccination against yellow fever has been the concern of WHO from the time the Organization came into being, as this vaccination is officially sanctioned by international health legislation. He pointed out that this meeting had to consider yellow fever vaccine from a technical point of view, its production and control, and gave a short summary of the problems which called for the most immediate attention.

Dr P. Lépine was elected Chairman, Dr A. Gast-Galvis Vice-Chairman, and Dr J. Laigret and Dr F. N. Macnamara Rapporteurs.

The provisional agenda was discussed and adopted.

1. REVISED MINIMUM REQUIREMENTS FOR YELLOW FEVER VACCINE

The Committee noted that the UNRRA Standards for the Manufacture and Control of Yellow Fever Vaccine had been in operation since 1945 and that, whereas these standards had fulfilled a very useful purpose, they now required revision for the following reasons :

(1) they included details of laboratory procedure for the manufacture of the vaccine and thereby hindered manufacturers from developing other methods of vaccine production ;

* The Executive Board, at its twentieth session, adopted the following resolution :
The Executive Board

1. NOTES the first report of the Expert Committee on Yellow-Fever Vaccine ;
2. THANKS the members of the Committee for their work ;
3. AUTHORIZES publication of the report.

(Resolution EB20.R12, *Off. Rec. Wld Hlth Org.*, 1957, 80, 4)

- (2) they made no provision for the use of yellow fever vaccine administered by cutaneous scarification ;
- (3) there was a need for more detailed descriptions of certain tests for potency and safety in order to obtain uniformity among workers ;
- (4) unsatisfactory vaccine might by the chance variation of the incidence of encephalitis in test animals pass the safety tests on a percentage of occasions that was considered unjustifiable.

It was agreed that recommendations on minimum requirements for yellow fever vaccine should be governed by the following principles :

- (1) The minimum requirements, while fulfilling their purpose, should impose as few restrictions as possible on manufacturers of vaccine wishing to improve and develop their products.
- (2) The minimum requirements for a yellow fever vaccine for use in international travel should be described separately from the minimum requirements for the safety of the vaccine in regard to the individual vaccinated.
- (3) The recommendations should be, as far as possible, a pattern on which other recommendations for other vaccines might be based.

In recommending that the minimum requirements for yellow fever vaccine described in Annex 1 (page 10) should be substituted for the UNRRA Standards, the Committee realized that it was impossible to recommend requirements for types of vaccine hitherto not thought of, especially with regard to safety tests. It therefore expressed the hope that, in the event of a new type of vaccine being developed which was proved to be satisfactory, consideration would be given as soon as possible to amending the minimum requirements to make provision for such a vaccine.

The Committee noted that Part III of the recommendations was no more than a guide to those manufacturing vaccine and to those whose duty it might be to approve a vaccine for acceptance for international quarantine procedure.

2. MAINTENANCE OF THE 17D STRAIN

The ideal properties of seed-lot virus differ from those of the vaccine in that the former, although not itself in high titre, should be capable of multiplication to a high titre in the animal or tissue from which the final vaccine is derived, and in that it should be stable under conditions of long storage ; whereas the vaccine virus should be highly concentrated and easily prepared for administration.

The Committee realized that a secondary seed lot which was desiccated might contain an appreciable quantity of non-viable virus which might interfere with the growth of the virus in the host tissue used for vaccine production. Nevertheless, it also pointed out that difficulties in preserving the secondary seed virus in a frozen state might easily arise, unless good and adequate refrigeration (below -30°C) was well maintained.

The Committee considered that until more evidence was forthcoming that the genetic quality of the virus might be in any way affected by conditions of storage or by desiccation, although such conditions might reduce the titre of the preparation concerned, no requirements should be made concerning desiccation of the secondary seed lots. The Committee, however, emphasized that if any change was observed to occur in a secondary seed lot whether this had been desiccated or not, the whole seed lot should be discarded.

In view, however, of the long periods for which the primary seed lots were likely to remain stored, the Committee recommended that all primary seed lots should be stored desiccated.

The Committee noted that whereas 17D virus could be obtained from organizations holding type cultures, such organizations could not be expected to provide quantities of seed virus which had undergone the full safety tests.

The Committee considered that any laboratory making yellow fever vaccine should be capable of conducting safety tests on its own seed lots, but it recognized that considerable advantages would accrue if there existed a central laboratory which could provide seed-lot virus in quantity. Such a laboratory could ensure that the seed-lot virus was of the best available type, and subjected to safety tests of greater accuracy than might otherwise be the case. Research into various aspects of yellow fever vaccine production might there more easily be centralized.

The Committee agreed that the establishment of a laboratory of this nature deserved full consideration.

3. YELLOW FEVER VACCINE ADMINISTERED BY CUTANEOUS SCARIFICATION

A vaccine administered by cutaneous scarification has been used very widely and with notable success in French African territories, so that at the present day practically no cases of yellow fever are observed there. Owing, however, to the presence of yellow fever among the wild animals, the virus could not be eradicated, and repeated vaccinations of the population

might be necessary for an indefinite period. Therefore, the value of an inexpensive and effective vaccine should be emphasized. By growing the virus in mouse brain, using either the Dakar strain or the 17D strain of yellow fever virus, an inexpensive vaccine can be prepared. In view of the slight differences existing between the antigenic powers of the two strains, and taking into consideration the various complications which the strains may produce, the Committee considered that the choice of the virus strain to be employed should be left to the local authorities, having due regard to the local prevalence of yellow fever and the resulting morbidity.

The Committee agreed that mass vaccination with vaccines produced from either of the two strains was preferable to no vaccination, and pointed out that a degree of herd immunity resulting from a vaccine which produced an immunity rate in vaccinated persons of only 80% would nevertheless be of considerable advantage in preventing the spread of yellow fever.

The Committee recommended that the titre of 17D vaccine given by scarification should be as high as possible, in order to produce as high an immunity rate as possible after vaccination.

The Committee agreed that, as far as was possible and reasonable, vaccine administered by scarification should pass the same safety tests as vaccine given by subcutaneous injection.

It was emphasized that, although vaccination by scarification might be less expensive than vaccination by subcutaneous injection, and may be performed by auxiliary personnel, it should be supervised by medically qualified staff.

The Committee recognized that, although encephalitic complications were known to follow vaccination both with vaccinia and with yellow fever virus vaccines, there was insufficient evidence to show whether the two different pathological processes were synergistic or antagonistic. Therefore, the Committee considered that the use of a combined yellow fever and smallpox vaccine should not be condemned without further evidence, bearing in mind the potential advantages of such a vaccination procedure.

4. THE MOUSE PROTECTION TEST FOR YELLOW FEVER

It is important that the results of a yellow fever protection test done in one laboratory should be capable of evaluation in terms of the results obtained by other methods and in other laboratories.

There are many uses of protection tests, some requiring more sensitive methods, but it was felt that the methods to be used for survey purposes

should be sufficiently stringent for positive results to be accepted with considerable certainty.

The Committee considered that a reference protection test was desirable and recommended the procedures described in Annex 2 (page 18). It was the opinion of the Committee that this test was suitable for survey purposes on primate sera and at the same time was desirable as a reference procedure in that its results are less likely to be influenced by extraneous factors than those obtained by other methods.

The Committee recommended that WHO should be asked to study the establishment of an international reference preparation of a yellow fever immune simian serum. The Committee further considered that a reference human serum or pool of sera non-immune to yellow fever should be made available. The Committee recommended that if possible both these reference sera should be non-immune to other Group-B arthropod-borne viruses.

The Committee further recommended that initially a strain of virus such as described in Annex 2 (page 18) should be made available to laboratories wishing to employ the standardized protection test.

The Committee recognized that the mouse protection test was more specific than other serological tests for the presence of yellow fever antibodies. Nevertheless, it considered that on rare occasions sera of persons who had been infected with a related virus might, especially shortly after infection, neutralize sufficient yellow fever virus to become "positive" in a yellow fever protection test. The Committee recommended that for survey purposes approximately 100 mouse LD₅₀ should be used as a test dose (see Annex 2, page 20).

Infection with a related virus of a person already immune to yellow fever can result in a marked rise in the yellow fever antibody titre of the serum. Diagnosis of yellow fever by demonstrating in the serum a rise of yellow fever antibody titre should not be made without other supporting evidence. If, however, the acute phase serum were clearly negative and the second or convalescent sample clearly positive, the diagnosis of yellow fever would be justifiable. The Committee emphasized that in every case suspected of being yellow fever, every endeavour should be made to obtain a sample of acute phase serum as early in the course of the disease as possible.

The Committee recommended that further research should be undertaken upon antibodies cross-reacting with yellow fever virus and upon the development of methods whereby such cross-reactions could be distinguished or eliminated.

5. ENCEPHALITIS FOLLOWING YELLOW FEVER VACCINATION

Encephalitis following yellow fever vaccination has been observed in the Americas, Europe and Africa. Today its occurrence is negligible in South American countries where 17D vaccine is given by subcutaneous injection to persons of all ages and of both sexes. Nevertheless, in Europe and Africa a small, yet significant, number of cases continue to occur, and cases have been reported following vaccination both with the 17D strain of virus and the strain developed by mouse-brain passage.

There can be no reasonable doubt that cases of encephalitis may be consequent upon vaccination with both the mouse brain and 17D strains. The Committee felt that it could not accept the explanation that the encephalitic episodes were in all cases due to the chance occurrence of encephalitis from another cause. It was noted that the occurrence of five encephalitis cases among the 1800 infants vaccinated with 17D vaccine in France in 1952-1953 had a probability of occurring by chance of less than 1.28×10^{-7} .

The Committee noted that cases of encephalitis tended to occur in groups, sometimes in space, sometimes in time. The grouping of the cases did not appear in most instances to be related to specific vaccine lots, but to other, unknown causes. The Committee therefore emphasized the need for research to ascertain the factors that made one person more likely to develop encephalitis than another. The Committee noted that a most important factor was the age of the person vaccinated, and observed that the majority of cases of encephalitis occurred in children less than one year old. The Committee therefore recommended that research should be conducted to find practical methods of protecting infants against yellow fever other than by vaccination, particularly in those regions where encephalitis had been observed to follow vaccination. Of major importance was the need for a method to protect, although perhaps only temporarily, an infant proceeding on international travel. The Committee stressed that in the meantime the dose of vaccine administered to infants should be no less than that administered to adults.

6. RECOMMENDATIONS

The Committee wished to emphasize the importance of mass vaccination in the control of yellow fever, both for the protection of individuals and for the prevention of spread of the disease in international travel. The

following recommendations were made in the hope that they will increase the use and effectiveness of yellow fever vaccination.

The Committee recommended that :

1. The minimum requirements for yellow fever vaccine described in Annex 1 be substituted for the UNRRA Standards for the Manufacture and Control of Yellow Fever Vaccine.

2. The appropriate bodies of WHO be requested :

(a) to study the recommendations on minimum requirements and to make recommendations for their amendment if considered necessary ;

(b) to make available a reference yellow fever immune serum as specified in Annex 2 ;

(c) to make available a reference serum non-immune to yellow fever virus as specified in Annex 2.

3. WHO be requested to study the possibility of designating a laboratory which would undertake research into various aspects of yellow fever and its vaccine and would supply vaccine seed lots to manufacturers.

4. A standardized yellow fever virus neutralization test be adopted for the purpose of comparing the results obtained in different laboratories.

5. Research be conducted upon, *inter alia* :

(a) the need to standardize the age of monkeys used in safety tests for yellow fever vaccine ;

(b) the use of monkeys other than *Macaca mulatta* (*Macacus rhesus*) for the safety tests ;

(c) the effect of desiccation and of varying conditions of storage on the characteristics and genetic properties of modified yellow fever virus used for vaccination ;

(d) the growth curves and the genetic homogeneity of the 17D strain and of the French neurotropic strain to determine means for obtaining preparations of maximum active and minimum inactive virus content ;

(e) practical methods of protecting infants against yellow fever other than by vaccination ;

(f) factors which make one person more likely than another to develop encephalitis after yellow fever vaccination ;

(g) cases of encephalitis following vaccination with regard to late sequelae ;

(h) the incidence and characteristics of antibodies cross-reacting with yellow fever virus and the development of methods whereby such cross-reactions could be detected or eliminated.

6. WHO be requested to promote a regular exchange of reports and information between laboratories specializing in yellow fever research.

Annex 1

PROPOSED RECOMMENDATIONS ON MINIMUM REQUIREMENTS FOR YELLOW FEVER VACCINE

Definitions used for the purpose of this document

Embryo pulp shall mean the embryos and/or their juices after harvesting and milling with or without centrifugation but without the addition of diluent.

Vaccine shall mean the product ready for distribution from place of manufacture.

Prepared vaccine shall mean the vaccine ready for administration.

Immune. This adjective shall be applied to a person or an animal whose serum shall give a positive result in a yellow fever mouse protection test.^a

Non-immune. This adjective shall be applied to a person or an animal whose serum shall give a negative result in a yellow fever mouse protection test.^a

Mouse LD₅₀. That quantity of virus which has a 0.5 probability of producing fatal specific encephalitis in a mouse of a susceptible species 3-6 weeks of age after intracerebral inoculation as described in the potency test.

Vaccine lot. This refers to material which is processed together as a unit and which has a uniform composition.

Seed-lot system. The system whereby a quantity of virus is processed together as a "lot" with a uniform composition from which material is drawn for subsequent passage. The *primary seed lot* is that from which material is drawn for inoculating all *secondary seed lots*. *Secondary seed lots* are one passage removed from the primary seed lot. Material is drawn from *secondary seed lots* for inoculating cultures for the production of vaccine; thus no vaccine is more than two passages removed from the virus of the *primary seed lot*.

^a See Annex 2, section 7, page 21.

Date of manufacture. This shall be the date on which the *vaccine lot* first passes a satisfactory potency test. It may be at a variable period after the preparation of the product, depending upon the manufacturing conditions and demands on vaccine.

Date of issue. This shall be the date on which distribution of a *vaccine lot* is commenced.

Date of distribution. This shall be the date on which a particular portion of a lot is distributed.

Expiry date. The date after which distributed vaccine may not be used.

I. Minimum Requirements for Vaccine for Use in International Travel

A. Yellow fever vaccine for subcutaneous injection

1. The *vaccine* shall be immunogenic and of a live strain of modified yellow fever virus of a type which has in well-conducted trials involving 100 or more *non-immune* persons after subcutaneous injection failed to render *immune* less than 5% of those vaccinated.

2. The *vaccine virus* shall be of a type not transmissible from man to man by natural ways of infection, including the bite of an insect vector.

3. The *vaccine* shall be of a type which protects against yellow fever for a period lasting from not more than nine days after vaccination until at least six years after vaccination.

4. The *vaccine* shall not contain any extraneous pathogenic agent which the person vaccinated may transmit to humans or animals (see Part II).

5. The *prepared vaccine* shall contain not less than 1000 *mouse LD₅₀* of modified yellow fever virus per dose.

6. Each *vaccine lot* shall be tested for potency not more than six months before the *date of issue*, and subsequently if distribution of the lot is not completed within the *expiry date*, at intervals equal to the period between the *date of distribution* and the *expiry date*.

The potency test shall be conducted as follows :

(a) Three containers are taken at random from a *vaccine lot*.

(b) The *vaccine* as *prepared* for administration is called the undiluted vaccine. This shall stand at a temperature between 20-30°C for 20 minutes before dilution. Dilutions 1 : 10, 1 : 100, 1 : 1000, 1 : 10 000 and 1 : 100 000 are made up from the content of each container using

0.75% bovine albumin, fraction V, in phosphate-buffered isotonic sodium chloride (pH 7.4) as diluent.

(c) Injection of the mice is commenced immediately after the dilutions have been made. Three series of mice of a highly susceptible strain, 3-6 weeks of age, are injected intracerebrally under ether anaesthesia with the vaccine dilutions. The dose is 0.03 ml using not less than six mice for each dilution.

(d) The mice are observed for 21 days. Deaths assumed to be caused by typical yellow fever virus infection are recorded. (Mice paralysed on the twenty-first day after injection shall be counted as alive in making the calculations.)

(e) After the period of observation the 50% mortality end-point for the three series is calculated according to the method of Reed & Muench.⁹

(f) The potency of the vaccine is expressed in *mouse LD₅₀* per human dose and is determined by calculating the geometrical mean value of the three end-points obtained by testing three containers from the same *vaccine lot*.

(g) If any two of the three end-points calculated differ by $2.5 \log_{10}$ or more, seven more containers shall be tested. If any of the ten have titres such as to contain less than 1000 *mouse LD₅₀* per human dose, the lot shall be condemned. If the lot is satisfactory, the potency of the vaccine shall be expressed as the geometrical mean of the *mouse LD₅₀* per human dose.

7. Between the *date of issue* and the *date of distribution* the *vaccine* must be kept constantly at a temperature below -5°C .

8. The period between the *date of distribution* and the *expiry date* shall be governed by the conditions of storage (see below 11 (f)), but must not be more than one year.

9. The conditions of storage, including those during shipping, must be such that at the *expiry date* the *prepared vaccine* shall still conform with minimum requirement 5.

10. A label printed on or attached to each container shall show :

- (a) the words "yellow fever vaccine";
- (b) the lot number and *expiry date*;
- (c) the name of the manufacturer, suitably abbreviated if necessary.

11. Instructions accompanying each container shall show :

- (a) the words "living yellow fever vaccine for subcutaneous injection";

- (b) a statement : " This yellow fever vaccine for subcutaneous injection has passed the requirements for use in international travel specified in Part I of Annex 1 of WHO Technical Report No. 136 " ;
- (c) the volume and kind of diluent to be added, and instructions for administration, including requirements of the temperature at which the *prepared vaccine* must be kept and the period within which it must be administered ;
- (d) the volume of the dose, and the words " The dose shall be the same for persons of all ages " ;
- (e) the number of *mouse LD₅₀* per dose ;
- (f) a statement of the conditions of storage and shipping requirements ;
- (g) the name and address of the manufacturer.

B. Yellow fever vaccine for cutaneous scarification

If the vaccine is to be given by cutaneous scarification the minimum requirements of a yellow fever vaccine given by subcutaneous injection shall be met, except that the following substitutions shall be made :

- (1) In minimum requirement 1, for " subcutaneous injection " read " cutaneous scarification " .
- (2) In minimum requirement 5, for " 1000 *mouse LD₅₀* of modified yellow fever virus per dose " read " 500 000 *mouse LD₅₀* per ml " .
- (3) In minimum requirement 6, under (b), read " The *vaccine* as *prepared* for administration is called the undiluted vaccine. If necessary the *vaccine* may be *prepared* by using diluent (see below) provided it has been shown in well conducted experiments that the excipient used is not deleterious to the vaccine virus. Dilutions 1 : 10, 1 : 100, 1 : 1000, 1 : 10 000, 1 : 100 000, 1 : 1 000 000 and 1 : 10 000 000 are made up . . . " .
- (4) In minimum requirement 6, under (c), commencing the second sentence, read " Three series of mice of a susceptible strain, 3-6 weeks of age, are injected intracerebrally under ether anaesthesia with vaccine diluted at 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} . The dose is . . . " .
- (5) In minimum requirement 6, under (g), read " If any two of the three end-points calculated differ by $2.5 \log_{10}$ or more, seven more containers shall be tested. If any of the ten have titres such as contain less than 500 000 *mouse LD₅₀* per ml, the lot shall be condemned. If the lot is satisfactory, the potency of the vaccine shall be expressed as the geometrical mean of the *mouse LD₅₀* per ml " .
- (6) In minimum requirement 11, under (a) and (b), for " subcutaneous injection " read " cutaneous scarification " .

- (7) In minimum requirement 11 (d), delete " the volume of the dose, and ".
- (8) In minimum requirement 11 (e), for " per dose " read " per ml ".

II. Minimum Safety Requirements

A. Yellow fever vaccine for subcutaneous injection

1. The *vaccine* and *seed lots* shall not contain any human protein or added serum.

2. The vaccine virus shall be grown in chick-embryos. Developing eggs shall be obtained from chickens free of agents pathogenic to man. Only living typical chick-embryos from eggs injected with virus shall be harvested. The age of the harvested embryo shall be computed from the initial introduction of the egg into the incubator, and shall be not more than 12 days. The inclusion of the heads of the embryos in preparing the pulp is optional.

3. The *vaccine* shall be prepared by the *seed-lot system*. *Primary* and *secondary seed lots* shall be prepared.

4. No *vaccine* shall be issued which is more than one passage removed from a *seed lot* which has passed all tests prescribed below.

5. Safety tests shall be conducted on *primary* and *secondary seed lots* and all *vaccine lots*. *Seed lots* shall pass safety tests (a) and (b), and each *vaccine lot* shall pass safety test (b).

(a) *Monkey safety test*

The monkeys shall be of the species *Macaca mulatta* (*Macacus rhesus*), and shall have been proven to be *non-immune* just prior to injecting the seed virus. They shall be healthy and shall not have been previously inoculated. Not less than ten monkeys shall be used for each test. The test dose shall consist of 0.25 ml containing not less than 5000 *mouse LD₅₀*, as shown by a titration conducted by the method described in the potency test. The test dose shall be injected into the frontal lobe of each monkey. The monkeys shall be observed for a minimum period of 30 days.

(1) The degree of viscerotropism as indicated by the amount of circulating virus shall be determined as follows :

Blood serum obtained from each of the test monkeys on the second, fourth and sixth days after inoculation shall be injected undiluted and in at least two tenfold dilutions in aliquots of 0.03 ml intracerebrally into groups of at least six mice of the same quality as

used in the potency test. Circulating virus shall be demonstrated in at least one sample of serum from at least nine of the monkeys, but in no case shall 0.03 ml of serum contain more than 100 *mouse* LD_{50} . From one of these positive samples the virus shall be identified as that of yellow fever by a neutralization test using specific yellow fever immune serum.

(2) Not more than one monkey shall fail to become *immune* within 30 days subsequent to injection of the test dose.

(3) The degree of neurotropism as indicated by the incidence of clinical manifestations of encephalitis and death shall be observed. Not more than two of the monkeys under test shall develop encephalitis manifested by paralysis or inability to stand, with or without subsequent death of the animal.

(b) *Sterility of the vaccine and seed lots*

(1) *Bacteriological safety*

The sterility, except for the presence of live yellow fever virus, of the product must be maintained at all times while processing. The *vaccine* and *seed lots* shall be sterile as indicated by tests on the contents of not less than three containers selected at random when the total filled is 100 or less, plus one additional container for each additional 50 containers filled. Not less than the equivalent of five human doses from each container, or the entire content if less than five human doses are present, shall be tested. Duplicate cultures shall be made with incubation at 37°C and 22°C using dextrose broth, both aerobic and anaerobic tubes, and Brewer's or Linden's thioglycollate media. Chocolate agar slants shall also be used to detect contaminants. The cultures shall be observed for 10 days. If contamination appears in any of the tubes planted, the test may be repeated with the same number of containers and a lot shall be discarded if the same type of organism appears in more than one test, but no lot shall be passed until the final test shows no growth throughout.

(2) *Guinea-pig safety test for extraneous pathogens*

The equivalent of 8-10 human doses shall be injected intraperitoneally into each of two or more normal guinea-pigs weighing 300-500 grams. The animals shall remain healthy for 21 days. If both of the animals show reactions the entire *vaccine lot* or *seed lot* shall be regarded as unsatisfactory. If one animal shows reaction the test shall be repeated using three test animals. If in the repeated test one of the three animals shows reactions the product is unsatisfactory.

6. Each dose of *prepared vaccine* shall contain not more than 0.25 mg protein nitrogen.

7. The *vaccine* shall be in sealed containers.

8. Instructions accompanying the vaccine shall state that it must be used less than three hours after the container is opened.

9. When a vaccine and its mode of production conform to the requirements in Part II A, the following statement shall appear in the accompanying instructions: "This yellow fever vaccine for subcutaneous injection has passed the requirements for safety specified in Part II of Annex 1 of WHO Technical Report No. 136."

B. Yellow fever vaccine for cutaneous scarification

The minimum requirements of a yellow fever vaccine given by subcutaneous injection shall be met, except that the following substitutions shall be made:

(1) For minimum requirement 2, substitute "The virus may be grown in tissues from or in animals, including the chick-embryo, but the colonies or stocks from which the animals are derived must be free of pathogens capable of transmission to man by cutaneous scarification."

(2) In minimum requirement 5, for the first paragraph read "Safety tests shall be conducted on *primary* and *secondary seed lots* and all *vaccine lots*. *Seed lots* shall pass safety tests (a) and (b), and each *vaccine lot* shall pass safety tests (b) (1) and (2). At intervals of not more than six months safety test (b) (3) shall be carried out on at least one *vaccine lot*."

(3) At the end of minimum requirement 5, there shall be inserted the following:

"(3) *Monkey safety test for extraneous pathogens*. Not less than two yellow fever *immune* monkeys which shall be healthy and not have been previously inoculated with any virus other than yellow fever virus shall be employed. The test dose shall consist of 0.25 ml containing not less than 5000 *mouse LD₅₀* and shall be injected into the frontal lobe of each monkey. The monkeys shall be observed for a period of 30 days. The monkeys shall remain well and afebrile (rectal temperature less than 40°C). No organism of any nature shall be isolated from specimens of sera taken from the monkeys on the second, fourth, sixth and eighth days after their injection, when the undiluted sera are injected into susceptible mice (0.03 ml) intracerebrally as described in (a) (1). If one monkey becomes sick the test shall be repeated using three more yellow fever *immune* monkeys, all of which shall remain

well. If this test is not satisfactory the lot shall be discarded, and subsequent lots must be similarly tested until at least two lots have passed the test."

(4) Minimum requirement 6 shall not apply.

(5) In minimum requirement 9, for "Part II A" read "Part II B", and for "subcutaneous injection" read "cutaneous scarification".

III. Recommendations for the Manufacture of Vaccine for Subcutaneous Injection

The following recommendations were in part embodied in the UNRRA Standards² and are in part new.

1. The production method employed should be essentially as described in reports.^{3, 5-8, 10, 11}

2. The primary seed virus should be the 17D strain of yellow fever virus of the 200-300 sub-culture cultivated in chick-embryo tissue culture,¹ or in developing chick-embryo.

3. The *primary* and *secondary seed lots* should be grown in the developing chick-embryos.

4. The *vaccine* and *primary seed lots* should, as soon as possible after harvesting, be dried in the final container from the frozen state under high vacuum. The *vaccine* and *primary seed lots* should contain less than 1.0% of moisture, preferably less than 0.5%, as determined by the phosphorus pentoxide method.

5. On harvesting the chick-embryos the juice of the *embryo pulp* should contain sufficient virus so that after desiccation and rehydration to its original volume, not less than 150 000 *mouse LD₅₀* per ml are present.

6. In the monkey safety test (Part II A, section 5 (a), page 14), onset and duration of the febrile reaction as well as symptoms and pathology should not be such as to indicate a change in the properties of the virus.⁴

7. The ampoules containing the vaccine should be flame-sealed, of suitable size, and prepared from glass which is of the best quality available, especially with regard to resistance against temperature fluctuations and breakage, and to low alkalinity.

8. The greatest care should be exercised when deviating from these recommendations, as any deviation might result in a vaccine which would not pass the minimum requirements of Parts I and II or might be for other reasons unacceptable.

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Annex 2**RECOMMENDED STANDARDIZED PROCEDURES
FOR THE YELLOW FEVER VIRUS NEUTRALIZATION TEST
ON SERA OF PRIMATES (MOUSE PROTECTION TEST)****1. The virus**

The strain recommended is the neuroadapted yellow fever virus commonly referred to as French neurotropic. The passage level shall be high and at least the last 200 consecutive passages shall have been by the intracerebral route in mice. The stock virus shall consist of a suspension of adult mouse brain in undiluted heat-inactivated non-immune human or simian serum, cleared by centrifugation, desiccated by vacuum from the frozen state and subsequently stored at a temperature below -20°C . The rehydrated lyophilized product shall contain not less than 10^6 mouse LD_{50} per 0.03 ml.

2. *Diluent for virus*

0.75% bovine albumin, fraction V, in phosphate-buffered isotonic sodium chloride, pH 7.4, prepared from water purified by ion-exchange resins or by glass distillation.

3. *Non-immune serum*

A serum or pool of sera of human or simian origin proved by protection test to be devoid of yellow fever antibodies and itself controlled in a quantitative protection test, as described here, in comparison with a reference non-immune serum.

4. *Immune serum*

A serum or pool of sera taken not less than six months following inoculation from one or more monkeys immunized by a single subcutaneous injection of French neurotropic or unmodified pantropic yellow fever virus. The neutralizing power of this serum shall have been determined by titration in a protection, test as described here, in comparison with a reference immune serum.

5. *Mice*

Mice used for the test shall be healthy individuals, 4 to 6 weeks old, of a strain highly susceptible to intracerebral inoculation of yellow fever virus. They shall be randomized from the litters and used in groups of not less than six.

6. *Procedure*

(a) *General.* Two or more containers of the virus shall be rehydrated and pooled in an amount of diluent equal to 10 times the original volume of virus suspension in the containers and allowed to stand at room temperature for 15 minutes. Further dilutions are prepared in the same diluent as needed. Sera, and any required dilutions thereof, for the test are measured out in advance into separate containers, and to each of these is added an equal volume of the appropriate dilution of virus. No serum-virus mixture shall be allowed to remain at room temperature more than 15 minutes before incubation. The serum-virus mixtures shall be incubated at 37°C for one hour and then chilled in an ice-water bath. Inoculations shall begin at once and be completed as expeditiously as possible. At least one group of mice shall be injected intracerebrally with each test serum-virus mixture, 0.03 ml per mouse, and at least two groups shall be injected with

each control mixture as described below. The period of observation shall be 10 days.

(b) *Controls.* The volume of serum-virus mixtures for all controls shall be sufficient for the inoculation of at least 12 mice.

For control of the challenge dose, at least five serial tenfold dilutions of virus (expected to cover the range from 100% mortality to 100% survival) are added in equal volume to separate containers of undiluted non-immune serum.

For control of neutralizing potency of sera and specificity of the virus preparation, serial fourfold dilutions of immune serum shall be made in undiluted non-immune serum as expected to cover the range from complete neutralization to no neutralization of the test dose of virus to be described hereafter, and to each of these shall be added an equal volume of the appropriate dilution of virus.

Virus and antibody titres are to be determined by the method of Reed & Muench.

Scheme for test, when expected titre of virus in non-immune serum is $10^{6.5}$

	<i>Virus dilutions to be added</i>					
Undiluted non-immune serum	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
	(12 mice to be injected with each of the 5 mixtures)					
	<i>Dilutions</i>					
Immune serum, expected titre 1 : 64	1 : 1	1 : 4	1 : 16	1 : 64	1 : 256	1 : 1024
	To each container is added an equal volume of a $10^{-4.5}$ dilution of virus (12 mice to be injected with each of the 6 mixtures)					

A sufficient quantity of $10^{-4.5}$ dilution of virus is made to serve the above control purposes and also for addition to the test sera.

(c) *Qualitative test.* Using the results of previous assays of the virus preparation as a guide, a dilution of the virus shall be made which it is estimated will, after mixture with serum and incubation for one hour, contain at the time of inoculation approximately 100 mouse LD_{50} (75-300 LD_{50}) per 0.03 ml. For qualitative tests, this dilution of virus is added in equal volume to each test serum. The same dilution of virus is added to the immune serum and its dilutions for control of neutralizing potency and specificity of virus.

(d) *Quantitative tests* upon sera known or believed to contain antibody may be made by preparing dilutions of these sera as for the control immune serum, and adding to each dilution an equal volume of the test dilution of virus mentioned in (c) above.

7. Interpretation

(a) Deaths occurring before the fourth day and others considered to be non-specific shall not be taken into account.

(b) The validity of the test from the standpoint of potency of virus shall be determined by the titration of the virus in non-immune serum. If the titre is such that the test dose is shown to be 75 to 300 mouse LD₅₀, results may be accepted, whether neutralization occurs or not. In the event that the virus dose is less than 75 mouse LD₅₀, only negative results are to be accepted as valid, and sera which have given neutralization are to be retested. In the event that the virus dose is more than 300 mouse LD₅₀, only positive results are to be accepted as valid.

(c) The specificity of neutralization by test sera and the freedom of the virus preparation from contaminating pathogens shall be gauged from the neutralizing effect of the control immune serum.

(d) The results with test sera are to be evaluated according to the following table :

Number of mice living on the 3rd day	Number of mice living on the 10th day		
	Negative	Inconclusive	Positive
4	0	1-3	4
5	0-1	2-3	4-5
6	0-1	2-4	5-6
7	0-2	3-4	5-7
8	0-2	3-5	6-8
9	0-2	3-6	7-9
10	0-3	4-6	7-10
11	0-3	4-7	8-11
12	0-3	4-8	9-12

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