



**World Health  
Organization**

**WHO PROTOCOL:  
NEUROVIRULENCE TEST OF TYPES 1, 2 OR 3  
LIVE POLIOMYELITIS VACCINES (ORAL)  
IN MONKEYS**

*Draft*

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## 1 **Part 1. Description of the test**

2 Monkeys used for neurovirulence tests should satisfy the relevant recommendations in Part E,  
3 section E.4.1.1 of the Recommendations to Assure the Quality, Safety and Efficacy of Live  
4 Attenuated Poliomyelitis Vaccine (oral), and weigh not less than 1.5 kg. The pathogenicity of  
5 the filtered bulk suspension for *Macaca* or *Cercopithecus* monkeys should be tested in  
6 comparison with that of a reference virus preparation for neurovirulence testing (see  
7 Recommendations to Assure the Quality, Safety and Efficacy of Live Attenuated  
8 Poliomyelitis Vaccine (oral) Part A, section A.1.3) by inoculation into the lumbar region of  
9 the central nervous system. A pre-injection serum sample obtained from each monkey should  
10 be shown not to contain any neutralizing antibody in a dilution of 1:4 when tested against no  
11 more than 1000 CCID<sub>50</sub> of each of the three types of poliovirus.

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13 If only the manufacturer performs the neurovirulence test, the histological sections should be  
14 made available to the national regulatory authority for evaluation.

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### 16 **1.1 Number of monkeys**

17 It is recommended that a vaccine and the appropriate homotypic reference virus should,  
18 whenever possible, be tested concurrently in a single group of monkeys. Equal numbers of  
19 animals should be inoculated with the reference virus and the vaccine being tested. Monkeys  
20 should be allocated to vaccine or reference virus and to particular cages using a  
21 randomization procedure.

22

23 The number of monkeys inoculated should be such that at least 11 positive monkeys are  
24 included in the evaluation of the vaccine and at least 11 positive monkeys are included for the  
25 reference preparation for virus types 1 and 2. (A “positive” monkey is one in which neuronal  
26 lesions characteristic of poliovirus are seen in the central nervous system.) For virus type 3,  
27 there should be at least 18 positive monkeys for the reference preparation and a further 18  
28 positive monkeys for the vaccine. More than one vaccine lot may be tested with the same  
29 homotypic reference. The monkeys should, when possible, be from the same quarantine  
30 group and should be allocated randomly to the preparations. If it is not possible to use  
31 monkeys from the same quarantine group for both the homotypic reference and the test  
32 vaccine, monkeys from two quarantine groups should undergo tests with each of the  
33 preparations (with as close to equal numbers as possible from each quarantine group). If a test

1 is done on two working days, equal numbers of monkeys should be inoculated with the  
2 vaccine and the homotypic reference on each working day.

3

4 In order to obtain 11 and 18 positive monkeys, it is usual  
5 to inoculate 12 and 20 monkeys, respectively.

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7 The monkeys are sedated with ketamine hydrochloride or  
8 any other substance that has been shown to be suitable.

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## 10 **1.2 Virus content of vaccines and reference preparations inoculated**

11 The virus contents of the vaccine and the homotypic reference preparation should be adjusted  
12 to be as similar as possible and should be between 5.5 and 6.5 log<sub>10</sub>/0.1ml, based on the virus  
13 concentration determined as described in Part A, section A.4.4.4 of the Recommendations to  
14 Assure the Quality, Safety and Efficacy of Live Attenuated Poliomyelitis Vaccine (oral)<sup>1</sup>. A  
15 target titer of 6.0 log<sub>10</sub>/0.1ml should be prepared. Monkeys should be inoculated with only  
16 one concentration of virus. A back titration of the inoculum should be done after the  
17 inoculation step is completed. The MNVT is valid if the back titration result is within +/-0.5  
18 of 6.0 log<sub>10</sub>/0.1ml, .i.e. if it is between 5.5 and 6.5 log<sub>10</sub>/0.1ml.

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## 20 **1.3 Observation of monkeys**

21 All monkeys should be observed for 17—22 days for symptoms suggestive of poliomyelitis  
22 or other virus infection. Monkeys that survive the first 24 hours but die before the 11th day  
23 after inoculation should be autopsied to determine whether poliomyelitis was the cause of  
24 death. Those that have died from causes other than poliomyelitis should be excluded from the  
25 evaluation.

26

27 Animals that become moribund or are severely paralysed  
28 should be humanely killed and autopsied.

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30 All monkeys that survive the observation period should be euthanized and processed for  
31 analysis.

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<sup>1</sup> Recommendations to Assure the Quality, Safety and Efficacy of Live Attenuated Poliomyelitis Vaccine (oral) revised 2012

1 For the test to be valid, no more than 20% of the animals in each group should show signs of  
2 a concurrent infection during the observation period.

3

#### 4 **1.4 Number of sections examined**

5 The lumbar cord, the cervical cord, the lower and upper medulla oblongata, the  
6 mesencephalon, the thalamus, and the motor cortex of each monkey, as a minimum, should  
7 be subjected to histological examination.

8

9 Sections should be cut at a thickness of 15µm and stained with gallocyanin.

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11 If adequately justified, sections may be cut at a thickness of 8-  
12 15 µm, and Nissl staining may be used as an alternative to  
13 gallocyanin.

14

15 The minimum number of sections examined should be as follows:

16

17 — 12 sections representative of the whole of the lumbar enlargement

18 — 10 sections representative of the whole of the cervical enlargement

19 — 2 sections from the medulla oblongata

20 — 1 section from the pons and cerebellum

21 — 1 section from the midbrain

22 — 1 section each from the left and the right of the thalamus and cerebral cortex.

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#### 24 **1.5 Scoring of virus activity**

25 In the evaluation of virus activity in the hemisections of the spinal cord and brain stem, a  
26 method of scoring the severity of the lesions should be used. Since the type of damage,  
27 whether cellular infiltration or destruction of neurons, is important, the lesions should be  
28 scored as follows:

29

30 *Score*

31 1 Cellular infiltration only (this is not sufficient for the monkey to be considered as  
32 positive)

33 2 Cellular infiltration with minimal neuronal damage

34 3 Cellular infiltration with extensive neuronal damage

1 4 Massive neuronal damage with or without cellular infiltration

2

3 The scores obtained should be recorded on a standard form (see Part 3).

4

5 A monkey with neuronal lesions in the sections but which shows no needle tract should be  
6 regarded as positive.

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8 A monkey showing a needle tract in the sections but no neuronal lesions should not be  
9 regarded as positive.

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11 A section that shows damage due to trauma but no specific virus lesion is not included in the  
12 score.

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14 Severity scores are based on hemisection readings of the lumbar (L), cervical (C), and brain  
15 (B) histological sections. The lesion score (LS) for each positive monkey is calculated as  
16 follows:

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$$19 \quad LS = \left\{ \left[ \frac{\sum \text{ of L scores} }{\text{No. of hemisections}} \right] + \left[ \frac{\sum \text{ of C scores} }{\text{No. of hemisections}} \right] + \left[ \frac{\sum \text{ of B scores} }{\text{No. of hemisections}} \right] \right\} \div 3$$

20

21 A mean lesion score is calculated for each group of positive monkeys.

22

### 23 **1.6 Evaluation of neurovirulence test**

24 The comparison of the virus activity in the vaccine and the reference preparation should be  
25 based on the activity in the lumbar enlargement of the cord and the degree of spread of  
26 activity from this region to the cervical enlargement and the brain.

27

28 The acceptance or rejection of the vaccine should be based on the total score of all the test  
29 animals. Individual animals showing unusually high activity, either in the lumbar region or as  
30 the result of spread from this region, should also be taken into consideration in the final  
31 evaluation.

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1 The filtered bulk suspension passes the test if the required number of animals is positive and  
 2 if none of the clinical and histopathological examinations shows a significant difference in  
 3 pathogenicity between the vaccine virus and the reference material.

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5 Criteria for the acceptance of vaccines after neurovirulence testing are given in Part 2.

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7

8 **Part 2 Criteria for the acceptance of vaccines after neurovirulence testing**

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10 It is recommended that each laboratory should perform a minimum of four neurovirulence  
 11 tests (referred to here as “qualifying” tests) on each reference vaccine (Types 1, 2, and 3) to  
 12 provide sufficient data on the activity of such reference vaccines for the development of  
 13 criteria for the acceptability of test vaccines. On practical grounds, each of these tests should  
 14 include a homotypic lot of production vaccine tested concurrently with the reference so that  
 15 the results of the tests may be used in assessing vaccines in addition to providing information  
 16 on the reference. The minimum number of animals in each of these tests is as specified in  
 17 section 1.1 for each poliovirus type. The overall mean Lesion Score ( $M$ ) for the replicate tests  
 18 on each reference virus is calculated together with the pooled estimate ( $s^2$ ) of the within-test  
 19 variance and the within-test deviation ( $s$ ).

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21 Criteria for the validity of the results of a test of a reference preparation can be determined by  
 22 each laboratory only on the basis of the data accumulated after the four qualifying tests. No  
 23 generally applicable criteria can therefore be given. For laboratories with limited experience  
 24 with neurovirulence testing, the following empirical method of establishing acceptable limits  
 25 for the mean Lesion Score for the reference ( $\bar{X}_{ref}$ ) may be helpful:

26

27 *Lower Limit      Upper Limit*

28

29 For Types 1 and 2  $M - s$   $M + s$

30

31 For Type 3  $M - \frac{s}{2}$   $M + s$

32

33

1 A neurovirulence test in which the mean Lesion Score for the reference ( $\bar{X}_{ref}$ ) is not  
 2 consistent with previous experience (within the acceptable limits referred to above) should  
 3 not be used for assessing a test vaccine.

4  
 5 If the test is valid, the mean Lesion Score for the test vaccine ( $\bar{X}_{test}$ ) is compared with that of  
 6 the homotypic reference vaccine ( $\bar{X}_{ref}$ ) as follows ( $C_1$ ,  $C_2$  and  $C_3$  are constants defined  
 7 below).

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 9 The vaccine is not acceptable if:

$$\bar{X}_{test} - \bar{X}_{ref} > C_1$$

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 13 The vaccine may be retested once at the discretion of the national regulatory authority if:

$$C_1 < \bar{X}_{test} - \bar{X}_{ref} < C_2$$

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 16  
 17 If the vaccine is retested, the means of the Lesion scores for the test and reference vaccines  
 18 are recalculated, combining the data from both tests, and the vaccine is rejected if:

$$\bar{X}_{test1+test2} - \bar{X}_{ref1+ref2} > C_3$$

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 22 The constants  $C_1$ ,  $C_2$  and  $C_3$  are calculated as follows:

$$C_1 = 2.3 \sqrt{\frac{2s^2}{N_1}}$$

$$C_2 = 2.6 \sqrt{\frac{2s^2}{N_1}}$$

$$C_3 = 1.6 \sqrt{\frac{2s^2}{N_2}}$$

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 33 Where  $N_1$  = number of positive monkeys per group per vaccine test,

34  $N_2$  = number of positive monkeys per group for the two tests combined,

- 1           2.3 = normal deviate at the 1% level,
- 2           2.6 = normal deviate at the 0.5% level,
- 3           1.6 = normal deviate at the 5% level.

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In some countries, the national regulatory authority may permit an experienced manufacturer to accumulate data on the qualifying tests of the Types 1 and 2 international preparations for neurovirulence as serial batches of vaccine are tested and released, rather than wait until the data are available from the four qualifying tests before the release of any future vaccine.

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It is assumed that the values of the constants  $C_1$ ,  $C_2$ , and  $C_3$  will be calculated by each laboratory for each reference vaccine. As experience with the reference accumulates, it is recommended that laboratories should recalculate the values of  $s^2$  and  $M$  using data from their last 10 tests only, and update their values of  $C_1$ ,  $C_2$ , and  $C_3$  accordingly.

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Estimates of the probability that test vaccines with a true Lesion Score double that of the reference vaccine will be rejected, are given in Table 1 for different coefficients of variation. The number of animals needed for the test have been chosen so that, for a single test, the analysis recommended above will result in the rejection of approximately 1% of test vaccines that are identical to the homotypic reference, on the assumption that, in each laboratory, the within-test variation is similar to that observed in the qualifying tests with that reference.

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**Table 1**

**Estimated probability that test vaccines with a true Lesion Score double that of the reference vaccine will be rejected for different coefficients of variation.**

Total number of positive animals per test	Coefficient of Variation <sup>a</sup>					
	0.4	0.6	0.8	1.0	1.2	1.4
24 <sup>b</sup>	99%	96%	77%	55%	39%	28%
40	99%	99%	95%	80%	62%	47%

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<sup>a</sup> The coefficient of variation is defined as the within-test standard deviation divided by the mean Lesion Score. Boxes show acceptable coefficients of variation.

<sup>b</sup> Divided equally between tests on Type 1 and 2 vaccines.

<sup>c</sup> Corresponds to tests on Type 1 and 2 vaccines.

<sup>d</sup> Corresponds to tests on Type 3 vaccines.

In tests on vaccines which satisfy the above criteria of acceptability, individual animals may occasionally develop extremely high Lesion Scores. Such findings should be taken into consideration in evaluating the acceptability of vaccines, but precise criteria for use in making a decision are difficult to define.

Sample calculations are shown in Tables 2 and 3.

**Table 2 Sample calculations of results of qualifying tests of reference**

Basic data		Type 1	Type 2	Type 3
Overall mean Lesion Score ( $M$ ) (initial four tests)		1.110	0.878	1.043
Within-test pooled variance ( $s^2$ ) of $M$		0.444	0.236	0.686
Within-test pooled standard deviation ( $s$ ) (square root of $s^2$ )		0.666	0.486	0.828
Coefficient of variation (CV) ( $CV = s/M$ )		$\frac{0.666}{1.110} = 0.60$	$\frac{0.486}{0.878} = 0.55$	$\frac{0.828}{1.043} = 0.79$
Upper and lower limits for satisfactory test result. Mean Lesion Score of reference vaccine ( $\bar{X}_{ref}$ )		$M + s = 1.110 + 0.666 = 1.776$ $M - s = 1.110 - 0.666 = 0.444$	$M + s = 0.878 + 0.486 = 1.364$ $M - s = 0.878 - 0.486 = 0.392$	$M + s = 1.043 + 0.828 = 1.871$ $M - \frac{s}{2} = 1.043 - \frac{0.828}{2} = 0.629$
Constants for assessing acceptability of difference between mean Lesion Score of test vaccine ( $\bar{X}_{test}$ ) and mean Lesion score of reference vaccine ( $\bar{X}_{ref}$ )	$C_1 = 2.3 \sqrt{\frac{2s^2}{N_1}}$  $C_2 = 2.6 \sqrt{\frac{2s^2}{N_1}}$  $C_3 = 1.6 \sqrt{\frac{2s^2}{N_2}}$	$2.3 \sqrt{\frac{2 \times 0.444}{12}} = 2.3\sqrt{0.074} = 0.626$  $2.6 \sqrt{\frac{2 \times 0.444}{12}} = 2.6\sqrt{0.074} = 0.707$  $1.6 \sqrt{\frac{2 \times 0.444}{24}} = 1.6\sqrt{0.037} = 0.308$	$2.3 \sqrt{\frac{2 \times 0.236}{12}} = 2.3\sqrt{0.039} = 0.456$  $2.6 \sqrt{\frac{2 \times 0.236}{12}} = 2.6\sqrt{0.039} = 0.516$  $1.6 \sqrt{\frac{2 \times 0.236}{24}} = 1.6\sqrt{0.020} = 0.224$	$2.3 \sqrt{\frac{2 \times 0.686}{20}} = 2.3\sqrt{0.069} = 0.602$  $2.6 \sqrt{\frac{2 \times 0.686}{20}} = 2.6\sqrt{0.069} = 0.681$  $1.6 \sqrt{\frac{2 \times 0.686}{40}} = 1.6\sqrt{0.034} = 0.296$

**Table 3**  
**Examples of tests with Type 1 reference and vaccine**

Example	Mean Lesion Scores		Difference between vaccine and reference ( $\bar{X}_{test} - \bar{X}_{ref}$ )	Conclusion/Action <sup>a</sup>
	Reference ( $\bar{X}_{ref}$ )	Reference ( $\bar{X}_{test}$ )		
One test	0.826	1.188	0.362	Less than $C_1$ : vaccine acceptable
Two tests 1 <sup>st</sup>	0.826	1.493	0.667	More than $C_1$ but less than $C_2$ :
2 <sup>nd</sup>	1.285	1.209	-0.076	Vaccine unacceptable: retest is permissible -
Combined tests	1.056	1.351	0.295	Less than $C_3$ : vaccine acceptable
Two tests 1 <sup>st</sup>	0.826	1.493	0.667	More than $C_1$ but less than $C_2$ :
2 <sup>nd</sup>	1.285	1.405	0.120	Vaccine unacceptable: retest is permissible -
Combined tests	1.056	1.449	0.393	More than $C_3$ : vaccine fails

<sup>a</sup> For values of  $C_1$ ,  $C_2$  and  $C_3$  see Table 2

### **Part 3 Form on which to report the score of virus activity for each histological section from all monkeys included in the neurovirulence test**

Although the test requires that at least 11 monkeys should be positive after inoculation with the vaccine and reference virus for Types 1 and Types 2, provision is made on the following form (pp. 12-13) for recording results for 12 monkeys that may be inoculated and survive the test. A separate form to record the lesions in 20 monkeys will be required for Type 3.

Records for each vaccine and reference preparation must be on separate forms.

On the forms, the method of scoring the lesions used for all sections from all areas is that already indicated in Part 1.5, namely:

1. Cellular infiltration only.
2. Cellular infiltration with minimal neuronal damage.
3. Cellular infiltration with extensive neuronal damage.
4. Massive neuronal damage with or without cellular infiltration.

A model of the certificate of compliance with the international requirements for the neurovirulence testing of monovalent OPV bulks in monkeys is given below.

### **Certificate of compliance with the recommendations for the neurovirulence testing of monovalent OPV bulks in monkeys**

Monovalent bulk no. \_\_\_\_\_

Date of certification \_\_\_\_\_

I certify that the above monovalent bulk complies with the recommendations for tests in monkeys for neurovirulence published in WHO Technical Report Series, No. xxx.

A.4.4.5.2

Signature \_\_\_\_\_

Name (typed) \_\_\_\_\_

Date \_\_\_\_\_



