ordinate for the 50 per cent haemolytic complement dose where \( Y/(1−Y) = 1.0 \). Calculate the activity in haemolytic units (CH50/ml) from the expression:

\[
C_d = \frac{C_n \times 5}{a − b} \times 100
\]

\( a \) = mean complement activity (CH50/ml) of complement control,
\( b \) = complement activity (CH50/ml) of tested sample.

The test is not valid unless:
- the anticomplementary activities found for ACA negative control and ACA positive control are within the limits stated in the leaflet accompanying the reference preparation,
- the complement activity of the complement control (\( a \)) is in the range 80 to 120 CH50/ml.

Table 2.6.17.-2

<table>
<thead>
<tr>
<th>Tube Number</th>
<th>Volume of diluted complement in millilitres (for example 1:250)</th>
<th>Volume of gelatin barbital buffer solution in millilitres</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>7</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>8</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>11</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>12</td>
<td>1.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Three tubes at 100 per cent haemolysis — 1.3 ml of water.

Test for anticomplementary activity

Prepare a complement dilution having 100 CH50/ml by diluting titrated guinea-pig complement with gelatin barbital buffer solution. If necessary, adjust the immunoglobulin to be examined to pH 7. Prepare incubation mixtures as follows for an immunoglobulin containing 50 mg/ml:

Table 2.6.17.-3

<table>
<thead>
<tr>
<th>Immunoglobulin to be examined</th>
<th>Complement control (in duplicate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin (50 mg/ml)</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Gelatin barbital buffer</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>Complement</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

Carry out the test on the immunoglobulin to be examined and prepare ACA negative and positive controls using human immunoglobulin BRP, as indicated in the leaflet accompanying the reference preparation. Higher or lower volumes of sample and of gelatin barbital buffer solution are added if the immunoglobulin concentration varies from 50 mg/ml; for example, 0.47 ml of gelatin barbital buffer solution is added to 0.33 ml of immunoglobulin containing 30 mg/ml to give 0.8 ml. Close the tubes and incubate at 37 °C for 60 min. Add 0.2 ml of each incubation mixture to 9.8 ml of gelatin barbital buffer solution to dilute the complement. Perform complement titrations as described above on each tube to determine the remaining complement activity (Table 2.6.17.-2). Calculate the anticomplementary activity of the preparation to be examined relative to the complement control considered as 100 per cent, from the expression:

\[
\frac{a − b}{a} \times 100
\]

For each test, use not fewer than ten monkeys that are seronegative for the virus to be tested. For each monkey, inject not more than 0.5 ml of the material to be examined into the thalamic region of each hemisphere, unless otherwise prescribed. The total amount of virus inoculated in each monkey must be no less than the amount contained in the recommended single human dose of the vaccine. As a check against the introduction of wild neurovirulent virus, keep a group of not fewer than four control monkeys as cage-mates or in the immediate vicinity of the inoculated monkeys. Observe the inoculated monkeys for 17 to 21 days for symptoms of paralysis and other evidence of neurological involvement; observe the control monkeys for the same period plus 10 days. Animals that die within 48 h of injection are considered to have died from non-specific causes and may be replaced. The test is not valid if: more than 20 per cent of the inoculated monkeys die from non-specific causes; serum samples taken from the control monkeys at the time of inoculation of the test animals and 10 days after the latter are killed show evidence of infection by wild virus of the type to be tested or by measles virus. At the end of the observation period, carry out autopsy and histopathological examinations of appropriate areas of the brain for evidence of central nervous system involvement. The material complies with the test if there is no unexpected clinical or histopathological evidence of involvement of the central nervous system attributable to the inoculated virus.

2.6.18. TEST FOR NEUROVIRULENCE OF LIVE VIRUS VACCINES

Monkeys used in the neurovirulence test comply with the requirements given in the monograph on Poliomyelitis vaccine oral (0215) and weigh not less than 1.5 kg. The pathogenicity for Macaca or Cercopithecus monkeys is tested in comparison with that of a reference virus preparation for neurovirulence testing by inoculation into the lumbar region of the central nervous system after sedation with a suitable substance, for example, ketamine hydrochloride. A sample of serum taken before
the injection shall be shown not to contain neutralising antibody at a dilution of 1:4 when tested against not more than 1000 CCID<sub>50</sub> of each of the three types of poliovirus.

**Number of monkeys.** The vaccine and the appropriate homotypic reference virus are tested concurrently in the same group of monkeys. Equal numbers of animals are inoculated with the vaccines to be examined and the reference preparation. The animals are allocated randomly to treatment groups and cages and their identity is coded so that the treatment received by each animal is concealed from the observers and the evaluators of the sections. The number of monkeys inoculated in such a way that in the evaluation of both the vaccine and the reference preparation not fewer than eleven positive monkeys are included for type 1 and type 2 virus and not fewer than eighteen positive monkeys for type 3 virus (positive monkeys are those that show specific neuronal lesions of poliovirus in the central nervous system).

More than one batch of vaccine may be tested with the same homotypic reference. Monkeys from the same quarantine group are used wherever possible, otherwise monkeys from two groups are used and equal numbers from each group are treated with the vaccine and the reference preparation. If the test is carried out on two working days, an equal number of monkeys from each group are inoculated on each day with the vaccine and the homotypic reference preparation.

**Virus content.** The virus contents of the vaccine and the homotypic reference preparation are adjusted so as to be as near as possible equal and between 10<sup>5.5</sup> and 10<sup>5.5</sup> CCID<sub>50</sub>/0.1 ml.

**Observation.** All monkeys are observed for 17 to 22 days for signs of poliomyelitis or other virus infection. Monkeys that survive the first 24 h but die before the 11th day after inoculation are autopsied to determine whether poliomyelitis was the cause of death. Animals that die from causes other than poliomyelitis are excluded from the evaluation. Animals that become moribund or are severely paralysed are killed and autopsied. All animals that survive until the end of the observation period are autopsied. The test is not valid if more than 20 per cent of the animals show intercurrent infection during the observation period.

**Number of sections examined.** The lumbar cord, the cervical cord, the lower and upper medulla oblongata, the midbrain, the thalamus and the motor cortex of each monkey, as a minimum, are subjected to histological examination. Sections are cut with a thickness of 15 μm and stained with gallocyanin. The minimum number of sections examined is as follows:

- (a) 12 sections representative of the whole of the lumbar enlargement,
- (b) 10 sections representative of the whole of the cervical enlargement,
- (c) 2 sections from the medulla oblongata,
- (d) 1 section from the pons and cerebellum,
- (e) 1 section from the midbrain,
- (f) 1 section from the left and the right of the thalamus,
- (g) 1 section from the left and the right motor cerebral cortex.

**Scoring of virus activity.** For the evaluation of virus activity in the hemisections of the spinal cord and brain-stem, a score system for the severity of lesions is used, differentiating cellular infiltration and destruction of neurons as follows:

1. Cellular infiltration only (the monkey is not counted as positive),
2. Cellular infiltration with minimal neuronal damage,
3. Cellular infiltration with extensive neuronal damage,
4. Massive neuronal damage with or without cellular infiltration.

The scores are recorded on a standard form<sup>(3)</sup>. A monkey with neuronal lesions in the sections but that shows no needle tract is counted as positive. A monkey showing a needle tract in the sections, but no neuronal lesions is not regarded as positive. A section that shows damage from trauma but no specific virus lesions is not included in the score.

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

\[
LS = \frac{1}{3} \left( \frac{1}{S_{L,\text{hemisect}}} \cdot S_{L,\text{hemisect}} + \frac{1}{S_{C,\text{hemisect}}} \cdot S_{C,\text{hemisect}} + \frac{1}{S_{B,\text{hemisect}}} \cdot S_{B,\text{hemisect}} \right)
\]

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

**Evaluation.** The comparison of the virus activity in the vaccine and the reference preparation is based on the activity in the lumbar enlargement of the cord and the degree of spread of activity from this region to the cervical enlargement and the brain. Acceptance or rejection is based on the total score of all the test animals. Individual animals showing evidence of unusually high activity, either in the lumbar region or as the result of spread from this region, are also taken into consideration in the final evaluation. The monovalent bulk passes the test if the required number of animals is positive and if none of the clinical and histopathological examinations shows a significant difference in pathogenicity between the vaccine virus and the reference material. Criteria for acceptance are given below.

**Criteria.** A suitable number of neurovirulence qualifying tests (for example, four tests) is carried out on each reference vaccine (types 1, 2 and 3) to provide data on the activity of such vaccines that will serve as the basis of the criteria for vaccines to be tested. The overall mean lesion score (M) for the replicate tests on each reference virus is calculated together with the pooled estimate of the within-test variance (<s>2</s>) and the within-test deviation (s). Validity criteria for the results of a test on a reference preparation are established on the basis of the cumulative data from the qualifying tests. No generally applicable criteria can be given; for laboratories with limited experience, the following empirical method for setting acceptable limits for the mean lesion score for the reference preparation (X<sub>ref</sub>) may be helpful (see Table 2.6.19.1):

<table>
<thead>
<tr>
<th>Type</th>
<th>Lower limit</th>
<th>Upper limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Types 1 and 2</td>
<td>M - s</td>
<td>M + s</td>
</tr>
<tr>
<td>Type 3</td>
<td>M - s/2</td>
<td>M + s</td>
</tr>
</tbody>
</table>

If the mean lesion score for the vaccine to be tested is X<sub>test</sub> and C<sub>1</sub>, C<sub>2</sub> and C<sub>3</sub> are constants determined as described below, then:

- the vaccine is not acceptable if:
  \[ X_{\text{test}} - X_{\text{ref}} > C_1 \]
- the vaccine may be retested once if:
  \[ C_1 < X_{\text{test}} - X_{\text{ref}} < C_2 \]

---

<sup>(3)</sup> A suitable form is shown in the Requirements for Poliomyelitis Vaccine (Oral) (Requirements for Biological Substances No. 7, World Health Organization).
If the vaccine is retested, the means of the lesion scores for the vaccine to be tested and the reference vaccine are recalculated. The vaccine is not acceptable if:

$$X_{\text{test} 1} + X_{\text{test} 2} - 2 \times X_{\text{ref} 1 + \text{ref} 2} > C_3$$

The constants $C_1$, $C_2$ and $C_3$ are calculated from the expressions:

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

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

$$C_3 = 1.6 \sqrt{\frac{2s^2}{N_1}}$$

$N_1$ = number of positive monkeys per vaccine test,

$N_2$ = number of positive monkeys in the two tests,

2.3 = normal deviate at the 1 per cent level,

2.6 = normal deviate at the 0.5 per cent level,

1.6 = normal deviate at the 5 per cent level.

A neurovirulence test in which the mean lesion score for the reference ($X_{\text{ref}}$) is not compatible with previous experience is not used for assessing a test vaccine. If the test is valid, the mean lesion score for the vaccine to be tested ($X_{\text{test}}$) is calculated and compared with that of the homotypic reference vaccine.

01/2005:20620

2.6.20. ANTI-A AND ANTI-B HAEMAGGLUTININS (INDIRECT METHOD)

Prepare in duplicate serial dilutions of the preparation to be examined in a 9 g/1 solution of sodium chloride R. To each dilution of one series add an equal volume of a 5 per cent V/V suspension of group A, red blood cells previously washed three times with the sodium chloride solution. To each dilution of the other series add an equal volume of a 5 per cent V/V suspension of group B red blood cells previously washed three times with the sodium chloride solution. Incubate the suspensions at 37 °C for 30 min then wash the cells three times with the sodium chloride solution. Leave the cells in contact with a polyvalent anti-human globulin reagent for 30 min. Without centrifuging, examine each suspension for agglutination under a microscope.

01/2005:20621 corrected

2.6.21. NUCLEIC ACID AMPLIFICATION TECHNIQUES

1. INTRODUCTION

Nucleic acid amplification techniques are based on two different approaches:

1. amplification of a target nucleic acid sequence using, for example, polymerase chain reaction (PCR), ligase chain reaction (LCR), or isothermal ribonucleic acid (RNA) amplification,

2. amplification of a hybridisation signal using, for example, for deoxyribonucleic acid (DNA), the branched DNA (bDNA) method. In this case signal amplification is achieved without subjecting the nucleic acid to repetitive cycles of amplification.

In this general chapter, the PCR method is described as the reference technique. Alternative methods may be used, if they comply with the quality requirements described below.

2. SCOPE

This section establishes the requirements for sample preparation, in vitro amplification of DNA sequences and detection of the specific PCR product. With the aid of PCR, defined DNA sequences can be detected. RNA sequences can also be detected following reverse transcription of the RNA to complementary DNA (cDNA) and subsequent amplification.

3. PRINCIPLE OF THE METHOD

PCR is a procedure that allows specific in vitro amplification of segments of DNA or of RNA after reverse transcription into cDNA.

Following denaturation of double-stranded DNA into single-stranded DNA, two synthetic oligonucleotide primers of opposite polarity, anneal to their respective complementary sequences in the DNA to be amplified. The short double-stranded regions which form as a result of specific base pairing between the primers and the complementary DNA sequence, border the DNA segment to be amplified and serve as starting positions for in vitro DNA synthesis by means of a heat-stable DNA polymerase.

Amplification of the DNA occurs in cycles consisting of:

- heat denaturation of the nucleic acid (target sequence) into two single strands;
- specific annealing of the primers to the target sequence under suitable reaction conditions;
- extension of the primers, which are bound to both single strands, by DNA polymerase at a suitable temperature (DNA synthesis).

Repeated cycles of heat denaturation, primer annealing and DNA synthesis results in an exponential amplification of the DNA segment limited by the primers.

The specific PCR product known as an amplicon can be detected by a variety of methods of appropriate specificity and sensitivity.

4. TEST MATERIAL

Because of the high sensitivity of PCR, the samples must be optimally protected against external contamination with target sequences. Sampling, storage and transport of the test material are performed under conditions that minimise degradation of the target sequence. In the case of RNA target sequences, special precautions are necessary since RNA is highly sensitive to degradation by ribonucleases. Care must be taken since some added reagents, such as anticoagulants or preservatives, may interfere with the test procedure.

5. TEST METHOD

5.1. Prevention of contamination

The risk of contamination requires a strict segregation of the areas depending on the material handled and the technology used. Points to consider include movement of personnel, gowns, material flow and air supply and decontamination procedures. The system should be sub-divided into compartments such as:

- master-mix area (area where exclusively template-free material is handled, e.g. primers, buffers, etc.),