2.6.21. TEST FOR EXTRANEOUS AGENTS USING EMBRYONATED HENS’ EGGS

GENERAL PROVISIONS

a) In the following tests, chickens and/or chicken material such as eggs and cell cultures shall be derived from chicken flocks free from specified pathogens (SPF) (5.2.2).

b) Cell cultures for the testing of extraneous agents comply with the requirements for the master cell seed of chapter 5.2.4. Cell cultures for the production of veterinary vaccines, with the exception of the karyotype test and the tumorigenicity test, which do not have to be carried out.

c) In tests using cell cultures, precise specifications are given for the number of replicates, monolayer surface areas and minimum survival rate of the cultures. Alternative numbers of replicates and cell surface areas are possible as well, provided that a minimum of 2 replicates are used, the total surface area and the total volume of test substance applied are not less than that prescribed here and the survival rate requirements are adapted accordingly.

d) For a freeze-dried preparation, reconstitute using a suitable liquid. Unless otherwise stated or justified, the test substance must contain a quantity of virus equivalent to at least 10 doses of vaccine in 0.1 ml of inoculum.

e) If the virus of the seed lot would interfere with the conduct and sensitivity of the test, neutralise the virus in the preparation with a monospecific antiserum.

f) Monospecific antiserum and serum of avian origin used for cell culture or any other purpose, in any of these tests, shall be free of antibodies against and free from inhibitory effects on the organisms listed hereafter under 7. Antibody specifications for sera used in extraneous agents testing.

g) Where specified in a monograph or otherwise justified, if neutralisation of the virus of the seed lot is required but difficult to achieve, the in vitro tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent.

h) Other types of tests than those indicated may be used provided they are at least as sensitive as those indicated and of appropriate specificity. Nucleic acid amplification techniques (2.6.21) give specific detection for many agents and can be used after validation for sensitivity and specificity.

1. TEST FOR EXTRANEOUS AGENTS USING EMBRYONATED HENS’ EGGS

Use a test substance, diluted if necessary, containing a quantity of neutralised virus equivalent to at least 10 doses of vaccine in 0.2 ml of inoculum. Suitable antibiotics may be added. Inoculate the test substance into 3 groups of 10 embryonated hens’ eggs as follows:

2.6.22. ACTIVATED COAGULATION FACTORS

Where applicable, determine the amount of heparin present (2.7.12) and neutralise the heparin by addition of protamine sulphate R (10 µg of protamine sulphate neutralises 1 IU of heparin). Prepare 1 to 10 and 1 to 100 dilutions of the preparation to be examined using
B. Drain and wash about 25 cm² of cells from each of the substrates. Obtain about 10 cm² of cells from for the last subculture, grow the cells also on a suitable

2. TEST IN CHICKEN KIDNEY CELLS

Prepare 7 monolayers of chicken kidney cells, each monolayer having an area of about 25 cm². Maintain 2 monolayers as negative controls and treat these in the same way as the 5 monolayers inoculated with the test substance, as described below. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test substance onto each of the 5 monolayers. Allow adsorption for 1 h, add culture medium. Inoculate 2 of the replicate monolayers with subgroup A avian leucosis virus (not more than 10 CCID₅₀ in 0.1 ml), 2 with subgroup B avian leucosis virus (not more than 10 CCID₅₀ in 0.1 ml) and 2 with subgroup J avian leucosis virus (not more than 10 CCID₅₀ in 0.1 ml) as positive controls. Maintain not fewer than 2 non-inoculated replicate monolayers as negative controls.

Inoculate the cells for a total of at least 9 days, subcultivating at 3- to 4-day intervals. Retain cells from each passage level and harvest the cells at the end of the total incubation period. Wash cells from each passage level from each replicate and resuspend the cells at 10⁶ cells per millilitre in barbital-buffered saline for subsequent testing by a Complement Fixation for Avian Leucosis (COFAL) test or in phosphate buffered saline for testing by Enzyme-Linked Immunosorbent Assay (ELISA). Then, carry out 3 cycles of freezing and thawing to release any group-specific antigen and perform a COFAL test or an ELISA test on each extract to detect group-specific avian leucosis antigen if present. The test is not valid if group-specific antigen is detected in fewer than 5 of the 6 positive control replicate monolayers or if a positive result is obtained in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test replicate monolayers are inconclusive, then further subcultures of reserved portions of the fibroblast monolayers shall be made and tested until an unequivocal result is obtained. If a positive result is obtained for any of the test monolayers, then the presence of avian leucosis virus in the test substance has been detected.

The seed lot complies with the test if there is no evidence of the presence of any avian leucosis virus.

4. TEST FOR AVIAN RETICULOENDOTHELIOSIS VIRUS

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day-old embryos that are known to be genetically susceptible to subgroups A, B and J of avian leucosis viruses and that support the growth of exogenous but not endogenous avian leucosis viruses (cells from C/E strain chickens are suitable). Each replicate shall have an area of about 50 cm².

Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of the test substance onto each of 5 of the replicate monolayers. Allow adsorption for 1 h, and add culture medium. Inoculate 2 of the replicate monolayers with subgroup A avian leucosis virus (not more than 10 CCID₅₀ in 0.1 ml), 2 with subgroup B avian leucosis virus (not more than 10 CCID₅₀ in 0.1 ml) and 2 with subgroup J avian leucosis virus (not more than 10 CCID₅₀ in 0.1 ml) as positive controls. Maintain not fewer than 2 non-inoculated replicate monolayers as negative controls.

Inoculate the cells for a total of at least 9 days, subculturing at 3- to 4-day intervals. Retain cells from each passage level and harvest the cells at the end of the total incubation period. Wash cells from each passage level from each replicate and resuspend the cells at 10⁶ cells per millilitre in barbital-buffered saline for subsequent testing by a Complement Fixation for Avian Leucosis (COFAL) test or in phosphate buffered saline for testing by Enzyme-Linked Immunosorbent Assay (ELISA). Then, carry out 3 cycles of freezing and thawing to release any group-specific antigen and perform a COFAL test or an ELISA test on each extract to detect group-specific avian leucosis antigen if present. The test is not valid if group-specific antigen is detected in fewer than 5 of the 6 positive control replicate monolayers or if a positive result is obtained in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test replicate monolayers are inconclusive, then further subcultures of reserved portions of the fibroblast monolayers shall be made and tested until an unequivocal result is obtained. If a positive result is obtained for any of the test monolayers, then the presence of avian leucosis virus in the test substance has been detected.

The seed lot complies with the test if there is no evidence of the presence of any avian leucosis virus.

4. TEST FOR AVIAN RETICULOENDOTHELIOSIS VIRUS

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day-old chick embryos or duck embryo fibroblasts from the tissues of 13- to 14-day-old embryos, each monolayer having an area of about 25 cm².

Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of the test substance onto each of 5 of the monolayers. Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with avian
reticuloendotheliosis virus as positive controls (not more than 10 CCID50 in 0.1 ml). Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cells for a total of at least 10 days, subculturing twice at 3- to 4-day intervals. The test is not valid if fewer than 3 of the 4 positive controls or fewer than 4 of the 5 test monolayers or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the fibroblasts on a suitable substrate so as to obtain an area of about 10 cm² of confluent fibroblasts from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent fibroblasts derived from each of the original 11 monolayers by immunostaining for the presence of avian reticuloendotheliosis virus. The test is not valid if avian reticuloendotheliosis virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test monolayers are inconclusive then further subcultures of reserved portions of the fibroblast monolayers shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of avian reticuloendotheliosis virus.

5. TEST FOR CHICKEN ANAEMIA VIRUS
Prepare eleven 20 ml suspensions of the MDCC-MSBI cell line or another cell line of equivalent sensitivity in 25 ml flasks containing about 5 × 10⁵ cells/ml. Inoculate 0.1 ml of test substance into each of 5 flasks. Inoculate 4 of the suspensions with 10 CCID₅₀ chicken anaemia virus as positive controls. Maintain not fewer than 2 non-inoculated suspensions. Maintain all the cell cultures for a total of at least 24 days, subculturing 8 times at 3- to 4-day intervals. During the subculturing the presence of chicken anaemia virus may be indicated by a metabolic colour change in the infected cultures, the culture fluids become red in comparison with the control cultures. Examine the cells microscopically for cytopathic effect. At this time or at the end of the incubation period, centrifuge the cells from each flask at low speed and resuspend at about 10⁶ cells/ml and place 25 µl in each of 10 wells of a multi-well slide. Examine the cells by immunostaining.

The test is not valid if chicken anaemia virus is detected in fewer than 3 of the 4 positive controls or in any of the non-inoculated controls. If the results for more than 1 of the test suspensions are inconclusive, then further subcultures of reserved portions of the test suspensions shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of chicken anaemia virus.

6. TEST FOR EXTRANEOUS AGENTS USING CHICKS
Inoculate each of at least 10 chicks, with the equivalent of 100 doses of vaccine by the intramuscular route and with the equivalent of 10 doses by eye-drop. Chicks that are 2 weeks of age are used in the test except that if the seed virus is pathogenic for birds of this age, older birds may be used, if required and justified. In exceptional cases, for inactivated vaccines, the virus may be neutralised by specific antiserum if the seed virus is pathogenic for birds at the age of administration. Repeat these inoculations 2 weeks later. Observe the chicks for a period of 5 weeks from the day of the first inoculation. No antimicrobial agents shall be administered to the chicks during the test period. The test is not valid if fewer than 80 per cent of the chicks survive to the end of the test period.

Collect serum from each chick at the end of the test period. Test each serum sample for antibodies against each of the agents listed below (with the exception of the virus type of the seed lot) using one of the methods indicated for testing for the agent.

A. Standard tests

<table>
<thead>
<tr>
<th>AGENT</th>
<th>TYPE OF TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian adenoviruses, group 1</td>
<td>SN, EIA, AGP</td>
</tr>
<tr>
<td>Avian encephalomyelitis virus</td>
<td>AGP, EIA</td>
</tr>
<tr>
<td>Avian infectious bronchitis virus</td>
<td>EIA, HI</td>
</tr>
<tr>
<td>Avian infectious laryngotracheitis virus</td>
<td>SN, EIA, IS</td>
</tr>
<tr>
<td>Avian leucosis viruses</td>
<td>SN, EIA</td>
</tr>
<tr>
<td>Avian nephritis virus</td>
<td>IS</td>
</tr>
<tr>
<td>Avian reoviruses</td>
<td>IS, EIA</td>
</tr>
<tr>
<td>Avian reticuloendotheliosis virus</td>
<td>AGP, IS, EIA</td>
</tr>
<tr>
<td>Chicken anemia virus</td>
<td>IS, EIA, SN</td>
</tr>
<tr>
<td>Egg drop syndrome virus</td>
<td>HI, EIA</td>
</tr>
<tr>
<td>Avian infectious bursal disease virus</td>
<td>AGP, EIA</td>
</tr>
<tr>
<td>Influenza A virus</td>
<td>AGP, EIA</td>
</tr>
<tr>
<td>Marek’s disease virus</td>
<td>AGP</td>
</tr>
<tr>
<td>Newcastle disease virus</td>
<td>HI, EIA</td>
</tr>
<tr>
<td>Turkey rhinotracheitis virus</td>
<td>EIA</td>
</tr>
<tr>
<td><em>Salmonella pullorum</em></td>
<td>Agg</td>
</tr>
</tbody>
</table>

A. Additional tests for turkey extraneous agents

If the seed virus is of turkey origin or was propagated in turkey substrates, tests for antibodies against the following agents are also carried out.

<table>
<thead>
<tr>
<th>AGENT</th>
<th>TYPE OF TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydia spp.</td>
<td>EIA</td>
</tr>
<tr>
<td>Avian infectious haemorrhagic enteritis virus</td>
<td>AGP</td>
</tr>
<tr>
<td>Avian paramyxovirus 3</td>
<td>HI</td>
</tr>
<tr>
<td>Avian infectious bursal disease virus type 2</td>
<td>SN</td>
</tr>
</tbody>
</table>

A test for freedom from turkey lympho-proliferative disease virus is carried out by intraperitoneal inoculation of twenty 4-week-old turkey poultets. Observe the poultets for 40 days. The test is not valid if more than 20 per cent of the poultets die from non-specific causes. The seed lot complies with the test if sections of spleen and thymus taken from 10 poultets 2 weeks after inoculation show no macroscopic or microscopic lesions (other than those attributable to the seed lot virus) and no poult dies from causes attributable to the seed lot.

C. Additional tests for duck extraneous agents

If the seed virus is of duck origin or was propagated in duck substrates, tests for antibodies against the following agents are also carried out.
2.6.25. Avian live virus vaccines: extraneous agents in finished product

### EXTRANEOUS AGENTS TESTING

#### 7. ANTIBODY SPECIFICATIONS FOR SERA USED IN EXTRANEOUS AGENTS TESTING

All batches of serum to be used in extraneous agents testing either to neutralise the vaccine virus (seed lot or batch of finished product) and all batches of avian serum used as a supplement for culture media used for tissue culture propagation, shall be shown to be free of antibodies against and free from inhibitory effects on the following micro-organisms by suitably sensitive tests:

<table>
<thead>
<tr>
<th>AGENT</th>
<th>TYPE OF TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydia spp.</td>
<td>EIA</td>
</tr>
<tr>
<td>Duck and goose parvoviruses</td>
<td>SN</td>
</tr>
<tr>
<td>Duck enteritis virus</td>
<td>SN</td>
</tr>
<tr>
<td>Duck hepatitis virus type I</td>
<td>SN</td>
</tr>
</tbody>
</table>

The seed lot complies with the test if there is no evidence of the presence of any extraneous agent. The test is not valid if antibodies are detected in the chicks to any of the test agents before inoculation.

Clinical signs of disease in the chicks during the test period (other than signs attributable to the virus of the seed lot) and the detection of antibodies in the chicks after inoculation, (with the exception of antibodies to the virus of the seed lot) are classed as evidence of the presence of an extraneous agent in the seed lot.

It is recommended that sera from these birds is retained so that additional testing may be carried out if requirements change.

##### 5.2.2.3. AGENT TYPE OF TEST

Other types of tests than those indicated may be used on the organisms listed under 7. Antibody specifications for sera used for neutralisation of the vaccine virus is required but difficult to achieve, the in vitro tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent. Alternatively, or in addition to in vitro tests conducted on the batch, a test for extraneous agents may be conducted on chick sera obtained from testing the batch of vaccine, as described under 6. Test for extraneous agents using chicks of chapter 2.6.24. Test for extraneous agents in seed lots.

##### 5.2.2.4. Test for extraneous agents in seed lots

- If the vaccine virus would interfere with the conduct and sensitivity of the test, neutralise the virus in the preparation with a monospecific antiserum.

- Where specified in a monograph or otherwise justified, if neutralisation of the vaccine virus is required but difficult to achieve, the in vitro tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent. Alternatively, or in addition to in vitro tests conducted on the batch, a test for extraneous agents may be conducted on chick sera obtained from testing the batch of vaccine, as described under 6. Test for extraneous agents using chicks of chapter 2.6.24.

#### BATCHES OF FINISHED PRODUCT

Batches of sera prepared for neutralising the vaccine virus must not be prepared from any passage level derived from the virus isolate used to prepare the master seed lot or from an isolate cultured in the same cell line.

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### 2.6.25. AVIAN LIVE VIRUS VACCINES: TESTS FOR EXTRANEOUS AGENTS IN BATCHES OF FINISHED PRODUCT

#### GENERAL PROVISIONS

- a) In the following tests, chickens and/or chicken material such as eggs and cell cultures shall be derived from chicken flocks free from specified pathogens (SPF) (5.2.2.2).
- b) Cell cultures for the testing of extraneous agents comply with the requirements for the master cell seed of chapter 5.2.4. Cell cultures for the production of veterinary vaccines, with the exception of the karyotype test and the tumorigenicity test, which do not have to be carried out.
- c) In tests using cell cultures, precise specifications are given for the number of replicates, monolayer surface areas and minimum survival rate of the cultures. Alternative numbers of replicates and cell surface areas are possible as well.

- Provided that a minimum of 2 replicates are used, the total surface area and the total volume of vaccine test applied are not less than that prescribed here and the survival rate requirements are adapted accordingly.

- d) Where specified in a monograph or otherwise justified, if neutralisation of the vaccine virus is required but difficult to achieve, the in vitro tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent. Alternatively, or in addition to in vitro tests conducted on the batch, a test for extraneous agents may be conducted on chick sera obtained from testing the batch of vaccine, as described under 6. Test for extraneous agents using chicks of chapter 2.6.24.

- e) If the vaccine virus would interfere with the conduct and sensitivity of the test, neutralise the virus in the preparation with a monospecific antiserum.

- f) Where specified in a monograph or otherwise justified, if neutralisation of the vaccine virus is required but difficult to achieve, the in vitro tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent. Alternatively, or in addition to in vitro tests conducted on the batch, a test for extraneous agents may be conducted on chick sera obtained from testing the batch of vaccine, as described under 6. Test for extraneous agents using chicks of chapter 2.6.24.

- g) Monospecific antiserum and serum of avian origin used for cell culture and any other purpose, in any of these tests, shall be free of antibodies against and free from inhibitory effects on the organisms listed under 7. Antibody specifications for sera used in extraneous agents testing (2.6.24).

- h) Other types of tests than those indicated may be used provided they are at least as sensitive as those indicated and of appropriate specificity. Nucleic acid amplification techniques (2.6.21) give specific detection for many agents and can be used after validation for sensitivity and specificity.

#### 1. TEST FOR EXTRANEOUS AGENTS USING EMBRYONATED HENS’ EGGS

Prepare the test vaccine, diluted if necessary, to contain neutralised virus equivalent to 10 doses of vaccine in 0.2 ml of inoculum. Suitable antibiotics may be added. Inoculate the test vaccine into 3 groups of 10 embryonated hens’ eggs as follows:

- group 1: 0.2 ml into the allantoic cavity of each 9- to 11-day-old embryonated egg.