2.6.25. Avian live virus vaccines: extraneous agents in finished product

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.

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:

- Avian adenoviruses
- Avian encephalomyelitis virus
- Avian infectious bronchitis virus
- Avian infectious bursal disease virus types 1 and 2
- Avian infectious haemorrhagic enteritis virus
- Avian infectious laryngotracheitis virus
- Avian infectious bursal disease virus types 1 and 2
- Avian infectious bronchitis viruses
- Avian encephalomyelitis virus
- Avian paramyxoviruses 1 to 9
- Avian paramyxoviruses 1 to 9
- Avian paroviruses
- Avian pox virus
- Avian reticuloendotheliosis virus
- Chicken anaemia virus
- Duck enteritis virus
- Duck hepatitis virus type I
- Egg drop syndrome virus
- Fowl pox virus
- Influenza viruses
- Marek’s disease virus
- Turkey herpesvirus
- Turkey rhinotracheitis virus

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.

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.

EUROPEAN PHARMACOPOEIA 5.0

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

GENERAL PROVISIONS

a) In the following tests, chicks 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 vaccine test applied are not less than that prescribed here and the survival rate requirements are adapted accordingly.

d) In these tests, use the liquid vaccine or reconstitute a quantity of the freeze-dried preparation to be tested with the liquid stated on the label or another suitable diluent such as water for injections. Unless otherwise stated or justified, the test substance contains the equivalent of 10 doses in 0.1 ml of inoculum.

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. *Test for extraneous agents in seed lots.*

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.
2. TEST IN CHICKEN EMBRYO FIBROBLAST CELL S

Prepare 7 monolayers of primary or secondary chicken embryo fibroblasts, from the tissues of 9- to 11-day-old embryos, 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 vaccine, as described below. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers. Allow adsorption for 1 h and add culture medium. Incubate the culture for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made with pooled cells and fluids from all 5 monolayers after carrying out a freeze-thaw cycle. Inoculate 0.1 ml of pooled material onto each of 5 recently prepared monolayers of chicken embryo fibroblast cells, each monolayer having an area of about 25 cm² each as before. For the last subculture, grow the cells also on a suitable substrate so as to obtain an area of about 10 cm² of cells from each of the monolayers, for test A. The test is not valid if less than 80 per cent of the test monolayers, or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine. At the end of the total incubation period, carry out the following procedures:

A. Fix and stain (with Giemsa or haematoxylin and eosin) about 10 cm² of confluent cells from each of the 5 original monolayers. Examine the cells microscopically for any cytopathic effect, inclusion bodies, syncytial formation, or any other evidence of the presence of a contaminating agent from the test vaccine.

B. Drain and wash about 25 cm² of cells from each of the 5 monolayers. Cover these cells with a 0.5 per cent suspension of washed chicken red blood cells (using at least 1 ml of suspension for each 5 cm² of cells). Incubate the cells at 4 °C for 20 min and then wash gently in phosphate buffered saline pH 7.4. Examine the cells microscopically for haemadsorption attributable to the presence of a haemadsorbing agent in the test vaccine.

3. TEST FOR EGG DROP SYNDROME VIRUS

Prepare 11 monolayers of chicken embryo liver cells, from the tissues of 13- to 16-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 test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, add culture medium. Inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of chicken embryo liver cells, each monolayer having an area of about 25 cm² as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures at frequent intervals throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine. At the end of the total incubation period, carry out the following procedure: test separately, cell culture fluid from the test monolayers, positive control monolayers and negative control monolayers, using chicken red blood cells, for haemagglutination attributable to the presence of a contaminating agent.

The test is not valid if egg drop syndrome 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 monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of egg drop syndrome virus or any other extraneous agent.

4. TEST FOR MAREK’S DISEASE VIRUS

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-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 test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with a suitable strain of egg drop syndrome virus (not more than 10 CCID₅₀ in 0.1 ml) to serve as positive controls. Maintain 2 non-inoculated monolayers as negative controls.

C. Test pooled cell culture fluids using chicken red blood cells for haemagglutination attributable to the presence of a haemagglutinating agent in the test vaccine.

The test is not valid if there are any signs of extraneous agents in the negative control cultures. The batch of vaccine complies with the test if there is no evidence of the presence of any extraneous agent.
Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: trypsinise the cells, prepare separate pools of the cells from the test monolayers, from the positive control monolayers and from the negative control monolayers. Mix an appropriate quantity of each with a suspension of freshly prepared primary or secondary chick embryo fibroblasts and prepare 5, 4 and 2 monolayers, as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of Marek’s disease virus. The test is not valid if Marek’s disease 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 both of the 2 test monolayers are inconclusive then further subcultures of reserved portions of the fibroblasts shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of turkey rhinotracheitis virus or any other extraneous agent.

5. TESTS FOR TURKEY RHINOTRACHEITIS VIRUS

A. In chicken embryo fibroblasts

**NOTE:** this test can be combined with Test 2 by using the same test monolayers and negative controls, for all stages up to the final specific test for turkey rhinotracheitis virus on cells prepared from the last subculture.

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-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 test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with a suitable strain of turkey rhinotracheitis virus as positive controls (not more than 10 CCID₅₀ in 0.1 ml). Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: carry out a freeze-thaw cycle; prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers; inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of chicken embryo fibroblasts cells, each monolayer having a size of about 25 cm² as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative control monolayers survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of turkey rhinotracheitis virus. The test is not valid if turkey rhinotracheitis 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 both of the 2 test monolayers are inconclusive then further subcultures of reserved portions of the fibroblasts shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of turkey rhinotracheitis virus or any other extraneous agent.

B. In Vero cells

Prepare 11 monolayers of Vero cells, each monolayer having an area of about 25 cm². Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with a suitable strain of turkey rhinotracheitis virus (not more than 10 CCID₅₀ in 0.1 ml) to serve as positive controls. Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers. Inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of Vero cells, each monolayer having an area of about 25 cm² as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of turkey rhinotracheitis virus. The test is not valid if turkey rhinotracheitis 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 monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of turkey rhinotracheitis virus or any other extraneous agent.

6. TEST FOR CHICKEN ANAEMIA VIRUS

Prepare eleven 20 ml suspensions of the MDCC-MSB1 cell line or another cell line of equivalent sensitivity in 25 ml flasks containing about 5 x 10⁶ cells/ml. Inoculate 0.1 ml of test vaccine into each of 5 of these flasks. Inoculate 4 other 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 becoming 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, resuspend at about 10⁶ cells per millilitre 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 batch of vaccine complies with the test if there is no evidence of the presence of chicken anaemia virus.

7. TEST FOR DUCK ENTERITIS VIRUS

This test is carried out for vaccines prepared on duck or goose substrates.

Prepare 11 monolayers of primary or secondary Muscovy duck embryo liver cells, from the tissues of 21- or 22-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 test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h and add culture medium. Inoculate 4 of the monolayers with a suitable strain of duck enteritis virus (not more than 10 CCID₅₀ in 0.1 ml) to serve as positive controls. Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: trypsinise the cells and prepare separate pools of the cells from the test monolayers, from the positive control monolayers and from the negative control monolayers. Mix a portion of each with a suspension of freshly prepared primary or secondary Muscovy duck embryo liver cells to prepare 5, 4 and 2 monolayers, as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of duck enteritis virus. The test is not valid if duck enteritis 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 1 test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of duck enteritis virus or any other extraneous agent.

8. TEST FOR DUCK AND GOOSE PARVOVIRUSES

This test is carried out for vaccines prepared on duck or goose substrates.

Prepare a suspension of sufficient primary or secondary Muscovy duck embryo fibroblasts from the tissues of 16- to 18-day-old embryos, to obtain not fewer than 11 monolayers, each having an area of about 25 cm². Inoculate 0.5 ml of test vaccine into an aliquot of cells for 5 monolayers and seed into 5 replicate containers to form 5 test monolayers. Inoculate 0.4 ml of a suitable strain of duck parvovirus (not more than 10 CCID₅₀ in 0.1 ml) into an aliquot of cells for 4 monolayers and seed into 4 replicate containers to form 4 positive control monolayers. Prepare 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: carry out a freeze-thaw cycle. Prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers. Inoculate 0.5 ml, 0.4 ml and 0.2 ml of the pooled materials into aliquots of a fresh suspension of sufficient primary or secondary Muscovy duck embryo fibroblast cells to prepare 5, 4 and 2 monolayers, as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of duck or goose parvovirus. The test is not valid if duck parvovirus 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 1 test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of duck (or goose) parvovirus or any other extraneous agent.