

1 Procedure

1.1 Exposing virus to disinfectant

- 1.1.1 Each disinfectant test at the dilution requested by the manufacturer is performed in WHO hard water. When testing a disinfectant against FMDV this is done in the presence of 1% FBS in WHO hard water. When testing a disinfectant against SVDV this is done only in the presence of WHO hard water. Subsequently in the document, this will be referred to as '+/- FBS'.
- 1.1.2 Prepare the sample diluent. Place 0.9 ml of diluent into sterile glass bijoux. Place the aliquoted diluents in the fridge to cool.
- 1.1.3 Prepare a ten times disinfectant solution in WHO hard water with or without 1 % FBS. This is expressed as parts disinfectant to parts water. This should be prepared immediately prior to the test.
- 1.1.4 To sterile 1.5 ml microcentrifuge tubes, add 800 µl of sterile WHO hard water (+/- FBS) and 100 µl of 10 x disinfectant dilution or 100µl formaldehyde as an internal control. To a sterile microcentrifuge tube, add 900 µl of sterile WHO hard water (+/- FBS). Cool to 4°C.
- 1.1.5 To the tubes containing disinfectant sample and internal control, add 100 µl of virus working stock. To a tube containing hard water and disinfectant add 100µl media as a disinfectant control. For the positive virus control, add 100 µl of virus working stock to the tube containing 900 µl of sterile WHO hard water (+/- FBS). Place all tubes at 4 °C for 30 minutes.
- 1.1.6 Immediately after the 30 minute incubation period add 0.1 ml of each sample and control into 0.9 ml of diluent. Cap and mix each tube. Serial dilute.

1.2 Plaque assay to measure FMDV/SVDV titres in each dilution.

- 1.2.1 Add 0.2 ml of each serially diluted disinfectant-virus sample to triplicate wells.
- 1.2.2 Add 0.2 ml of sample diluent to triplicate wells as a negative control.
- 1.2.3 Add 0.2 ml of internal control to triplicate wells
- 1.2.4 Add 0.2 ml of disinfectant control to duplicate wells.
- 1.2.5 Add 0.2 ml of the positive control to each of the triplicate wells.
- 1.2.6 Incubate at 35 to 39°C for one hour. Prepare the Eagles overlay with Agar Noble and store at 40 to 44 °C until required.
- 1.2.7 Add 2 ml of the overlay mixture to each well, incubate for 48 hours at 35 to 39°C.
- 1.2.8 Add 2 ml of 10% (w/v) TCA to each well for 10-20 minutes as a cell fixative. Add 0.5 -1 ml of Crystal violet stain to each well for 5 to 10 minutes to stain the cells. Wash the stain off with a gentle water stream.

1.3 Results for FMDV and SVDV

- 1.3.1 Examine each well visually and mark the presence of each plaque on the underside of the plate with a marker pen. Calculate the titre. Then use a linear mixed model to estimate the reduction in titre and the confidence intervals.
- 1.3.2 If the disinfectant has shown a reduction of four logs or more from the virus control at the manufacturers requested test dilution, it is considered to have passed.



This document provides stakeholders such as manufacturers and regulators with details of the test method for understanding the Defra Approvals test under the Diseases of Poultry Order and The Avian Influenza and Influenza of Avian Origin in Mammals Order.

The test organism used is Newcastle Disease Virus (NDV), strain Herts 1933.

The test includes a soiling agent or “Interfering Substance” (Yeast) and is carried out at +4°C. The contact time is 30 minutes. At the end of this time a dilution is made in 5% horse serum. Disinfectants must reduce the virus titre by at least 4 log₁₀ (10,000 fold). Those that pass the test are eligible for inclusion on the Defra Approvals list.

1. PROCEDURE/METHOD

1.1 Test Reliability

- 1.1.1 Test Controls: The diluted virus stock is back titrated in each test to ensure viability and sufficient titre. Results of each back titration are monitored to allow trend analysis.
- 1.1.2 A product toxicity test is run on each test occasion to monitor the effect on the test system.

1.2 Dilution of Disinfectants

- 1.2.1 Prepare the disinfectant to the manufacturer’s specified dilution(s) and according to the specific gravity (if applicable), in WHO hard water using the largest practical volumes.

1.3 Toxicity Test

- 1.3.1 Pre-cool a solution of 5% v/v horse serum in distilled water at +4°C.
- 1.3.2 Add 2.5ml of each dilution of disinfectant prepared in 1.2.1 to 2.5ml of 5% (w/v) Baker’s Yeast in WHO hard water.
- 1.3.3 Add 2.5ml of WHO hard water to 2.5ml of 5% (w/v) Baker’s Yeast in WHO hard water.
- 1.3.4 Mix all the solutions by shaking and place in a controlled temperature waterbath set at +4°C for 30 minutes. During this contact period shake the solutions at 10 minute intervals.
- 1.3.5 At the end of the contact period dilute the mixtures 1 in 200 in the pre-cooled solution of 5% v/v horse serum in distilled water.
- 1.3.6 Inoculate 0.2ml of each dilution into the chorioallantoic sac (CAS) of each of 10, nine to eleven-day-old embryonating eggs. (A minimum of six eggs may be used for the Toxicity Test if there are insufficient eggs for the full test).

- 1.3.7 Incubate the eggs at +37°C for seven days.
- 1.3.8 Candle the eggs regularly, preferably daily. Discard as non-specific deaths any embryos that die during the first 24 hours.

1.4 Disinfectant test

- 1.4.1 Pre-cool a solution of 5% v/v horse serum in distilled water at +4°C.
- 1.4.2 Dilute the stock NDV 1 in 25 using 5% (w/v) Baker's Yeast in WHO hard water.
- 1.4.3 Mix 2.5 ml volumes of virus suspension from 1.4.2 with 2.5ml of each disinfectant dilution prepared in 1.2.
- 1.4.4 Mix all the solutions by shaking and place in a controlled temperature waterbath set at +4°C for 30 minutes. During this contact period shake the solutions at 10 minute intervals.
- 1.4.5 At the end of the contact period prepare a 1 in 200 dilution of each virus/disinfectant mixture in the pre-cooled 5% v/v horse serum in distilled water.
- 1.4.6 Prepare serial tenfold dilutions, in the 5% v/v horse serum in distilled water, from each mixture prepared in 1.4.5 to give a range of dilutions from neat to 10^{-2} .
- 1.4.7 Inoculate 0.2 ml of each dilution into the CAS of each of 7, nine to eleven-day old embryonating eggs.
- 1.4.8 Incubate the eggs at +37°C for seven days.
- 1.4.9 Candle the eggs regularly, preferably daily. Discard as non-specific deaths any embryos that die during the first 24 hours.
- 1.4.10 Retain at +4°C any eggs in which the embryo dies or which is chilled on welfare grounds between day 2 and day 6 of incubation.
- 1.4.11 At the end of the incubation period, chill all the surviving embryos at +4°C.

1.5 Virus Back-Titration

- 1.5.1 Using a fresh, labelled container, mix 2.5ml of virus dilution from 1.4.2 with 2.5ml of WHO hard water.
- 1.5.2 Mix the virus/water solution by shaking and place in a controlled temperature waterbath set at +4°C for 30 minutes. During this contact period shake the solutions at 10 minute intervals.
- 1.5.3 Prepare a 1 in 200 dilution of this virus suspension in 5% v/v horse serum in distilled water.
- 1.5.4 Prepare serial tenfold dilutions in 5% v/v horse serum in distilled water from the mixture prepared in 1.5.3 to give a range of dilutions from neat to 10^{-8} .
- 1.5.5 Inoculate 0.2 ml of the 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} and 10^{-8} dilutions into the CAS of each of 7, nine to eleven-day-old embryonating eggs.
- 1.5.6 Incubate the eggs at +37°C for seven days.
- 1.5.7 Candle the eggs regularly, preferably daily. Discard as non-specific deaths any embryos that die during the first 24 hours.

- 1.5.8 Retain at +4°C any eggs in which the embryo dies or which is chilled on welfare grounds between day 2 and day 6 of incubation.
- 1.5.9 At the end of the incubation period, chill all the surviving embryos at +4°C.

1.6 Haemagglutination Test for NDV

- 1.6.1 Confirm the presence of NDV whenever the death of an embryo occurs unexpectedly by performing a slide haemagglutination (HA) test.
- 1.6.2 Harvest the chorioallantoic fluid (CAF) from each suspect egg separately.
- 1.6.3 Perform a HA test on each CAF.

2. RESULTS

- 2.1 In the toxicity test, a disinfectant dilution which kills 5 or more (3 or more if the minimum of six eggs are used) embryos after 24 hours incubation, is defined as toxic to the test system.
- 2.2 Calculate the virus titre for each mixture using the Spearman-Kärber method. Eggs that were chilled for welfare reasons (1.4.10) should be regarded as “dead” when calculating titres.
- 2.3 A disinfectant dilution which reduces the virus titre by 10^4 median egg infective doses (EID₅₀) plus an allowance for the uncertainty of measurement compared to the untreated virus/yeast control mixture passes the test.
- 2.4 The titre of the untreated virus/yeast control mixture must be at least $10^{4.5}$ EID₅₀ per volume inoculated.

3. REFERENCES

Explanatory note on the approval of disinfectant for the purposes of the Animal Health Act 1981- DEFRA document DCA 180.

Tuberculosis Orders – (TBO) test

Defra approval of disinfectants under the Diseases of Animals (Approved Disinfectants) (England) Order 2007 for the purposes of the Animal Health Act 1981;

The Animal Plant and Health Agency (APHA) delivers the disinfectant approval scheme on behalf of Defra. Disinfectants may be tested for efficacy for use under the following animal disease orders in accordance with UK statutory legislation;

The Animal Health Act 1981 allows Ministers to make Orders for prescribing the cleansing and disinfection of places used for the holding of markets, fairs, exhibitions or sales of animals or lairage, yards, sheds, stables, vessels, aircraft, vehicles, pens etc. Orders may also be made under the European Communities Act stating where animal disease control legislation requires the use of an approved disinfectant.

Tuberculosis Orders – (TBO) test

- The effective concentration of the disinfectant is that which when added to the yeast and organism mixture provides the required reduction (see below) of the bacterial concentration. The APHA will confirm efficacy at the concentration specified by the manufacturer.
- The test follows the British Standard BS 6734:2004 Antimicrobial efficacy of disinfectants for veterinary and agricultural use, using *Mycobacterium fortuitum* (Strain NCIMB10384) as the challenge organism.
- A solution of the disinfectant to be tested is prepared at 100% of the manufacturer's recommended use dilution in standard hard water. The test is carried out using the challenge organism in a yeast suspension (5% w/v) at 4°C and a contact time of 60 minutes.
- Using the BS 6734:2004 method, the TBO test requires at least a 10^4 cfu reduction of the bacterial concentration to give a pass result.
- Following agreement in the Stakeholder Meeting in 2003 each test is repeated 3 times and a pass result must be achieved in all 3 tests for the product to have deemed to have passed.
- From August 2012, the test has been modified to provide a 95% level of confidence on a pass result. Therefore products will only pass if there is growth in 1/5 tubes or less. Prior to this time growth in 2/5 tubes was considered a pass but without any level of confidence.

General Orders (GO) test

Defra approval of disinfectants under the Diseases of Animals (Approved Disinfectants) (England) Order 2007 for the purposes of the Animal Health Act 1981;

The Animal and Plant Health Agency (APHA) delivers the disinfectant approval scheme on behalf of Defra. Disinfectants may be tested for efficacy for use under the following animal disease orders in accordance with UK statutory legislation;

General Orders – (GO) the Animal Health Act 1981 allows Ministers to make Orders for prescribing the cleansing and disinfection of places used for the holding of markets, fairs, exhibitions or sales of animals or lairage, yards, sheds, stables, vessels, aircraft, vehicles, pens etc. Orders may also be made under the European Communities Act stating where animal disease control legislation requires the use of an approved disinfectant.

General Orders (GO) test

- The effective concentration of the disinfectant is that which when added to the yeast and organism mixture provides the required reduction (see below) of the bacterial concentration. The APHA will confirm efficacy at the concentration specified by the manufacturer.
- The test is a modification of the former British Standard BS 6734: 1986 Determination of the Antimicrobial Efficacy of Disinfectants for Veterinary and Agricultural Use.
- A solution of the disinfectant to be tested is prepared at 100% of the manufacturer's recommended use dilution in standard hard water. The test is carried out using the challenge organism, at passage day 4, in a yeast suspension (5% w/v) at 4°C and a contact time of 30 minutes.
- Using the BS 6734:1986 method, the GO test requires at least a 10^5 cfu reduction of the bacterial concentration to give a pass result.
- The test is modified to include some improvements that are specified in the BS 6734: 2004 edition. Please note that the scope of this edition is only for disinfectants for use against tuberculosis. These improvements enhance the tolerance definition and reproducibility of the test.
- From August 2012, the test has been modified to provide a 95% level of confidence on a pass result. Therefore products will only pass if there is growth in 1/5 tubes or less. Prior to this time growth in 2/5 tubes was considered a pass but without any level of confidence.
- From 2014, the method was modified to use a different challenge organism; *Salmonella* Enteritidis NCTC 13665, the original strain was *Salmonella* Choleraesuis NCTC 10653. Comparative studies undertaken prior to the introduction of the new strain, determined that the modification may have made the test marginally more difficult for some products to gain a pass.