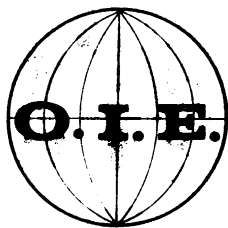

MANUAL OF RECOMMENDED DIAGNOSTIC TECHNIQUES AND REQUIREMENTS FOR BIOLOGICAL PRODUCTS FOR LISTS A & B DISEASES

This manual has been edited by the Standards Commission and approved by the International Committee of the OIE

VOLUME II

(1990)

OIE - 12, rue de Prony - 75017 PARIS - France
Tel: (1) 42.27.45.74 - Fax: (1) 42.67.09.87 - Telex: EPIZOTI 642 285 F

CONTENTS OF VOLUME II

Page

FOREWORD iii

PREFACE v

ACKNOWLEDGMENTS ix

CONTRIBUTORS xi

GENERAL INFORMATION

I. Sampling methods xv
II. Tests for sterility and freedom from contamination of biological materials xix

LIST A* Divider

A1 Foot and mouth disease 1
A2 Vesicular stomatitis 2
A4 Rinderpest 3
A12 African swine fever 4
A13 Hog cholera (classical swine fever) 5

LIST B*

B5 Heartwater 6
B6 Leptospirosis 7
B7 Q fever 8
B12 Bovine brucellosis 9
B13 Bovine genital campylobacteriosis 10
B16 Dermatophilosis 11
B19 Infectious bovine rhinotracheitis 12
B25 Caprine arthritis-encephalitis 13
B26 Contagious agalactia 14
B28 Enzootic abortion of ewes 15
B29 Sheep pulmonary adenomatosis 16
B34 Contagious equine metritis 17
B35 Dourine 18
B36 Epizootic lymphangitis 19
B39 Equine influenza 20
B41 Equine rhinopneumonitis 21
B44 Equine viral arteritis 22
B50 Atrophic rhinitis of pigs 23
B60 Fowl cholera 24
B63 Infectious bursal disease 25
B64 Marek's disease 26
B66 Avian chlamydiosis 27
B68 Myxomatosis 28
B80 Malignant catarrhal fever 29
B81 Bovine virus diarrhoea 30

* *The remaining Lists A and B diseases will appear in Volumes III and IV*

EQUINE INFLUENZA
(B/039)

SUMMARY

Equine influenza is caused by two members of the genus Influenzavirus *of the family* Orthomyxoviridae. *Diagnosis of influenzavirus infections of horses is based on virus isolation from horses with acute respiratory illness or on the demonstration of increased antibody titres of paired sera taken in the acute and convalescent stages of the disease. Ideally, both methods are used.*

Identification of the agent: Embryonated chicken eggs or cell cultures can be used for virus isolation from nasal swabs or washings from guttural pouches. Subtype 2 strains grow equally well in both systems but subtype 1 strains are quite refractory to propagation in cell cultures.

Viral growth is monitored by haemagglutination (HA) or in cell cultures by haemadsorption (HAD) using chicken or guinea pig erythrocytes (RBC). If HA or HAD are positive, isolates can be typed by haemagglutination-inhibition (HI) using strain-specific antisera. Isolates should always be sent to International Reference Laboratories (OIE or WHO) for typing and strain collection. Samples which yield negative results (i.e. showing no HA or HAD after one passage) should be re-passaged.

Serological tests: Diagnosis of influenzavirus infections can usually only be done by tests on paired sera; the first sample should be taken as soon as possible after the onset of clinical signs and the second about two weeks later. Sera are heat-inactivated and pre-treated to reduce non-specific reactions. Antibody titres are determined by HI or single radial haemolysis (SRH). For SRH, serial dilution of sera is usually not required. HI and SRH give comparable results but SRH is superior if large numbers of sera are to be tested.

Requirements for biological products: Equine influenza virus vaccines contain inactivated strains of subtypes 1 and 2 and induce humoral antibody responses after vaccination.

A. DIAGNOSTIC TECHNIQUES

Equine influenza is caused by two members of the genus *Influenzavirus* of the

family *Orthomyxoviridae* and consists of two subtypes, subtype 1 (H_7N_7) and subtype 2 (H_3N_8). These are not genuine pathogens for man, although horses can be infected with human influenza virus subtypes. These infections are unusual but can represent a potential biohazard to laboratory personnel. All influenza viruses are highly contagious for susceptible hosts, including embryonated hens' eggs and cell cultures. Care must therefore be taken during the handling of infected eggs or cultures (1).

Reference strains should not be propagated in a diagnostic laboratory, and never at the same time or in the same place. All working areas must be efficiently disinfected before and after virus manipulations, which should preferably be conducted within biohazard containment.

It is important to obtain samples as soon as possible after the onset of clinical signs. These samples include nasal swabs or washings, swabs taken at endoscopy or washings from the guttural pouches. Swabs consist of cotton wool on applicator sticks which should be transferred to a vial containing transport medium immediately after use. This medium consists of phosphate buffered saline (PBS) containing 40% glycerol. If the samples are to be inoculated within 1-2 days, they may be held at 4°C but, if kept for longer, they should be stored at -60°C or below. Samples should also be transported at these temperatures.

Only one sample is processed at a time. The liquid is expelled from the swab, which is suitably disposed of, and 0.1 ml of an antibiotic solution is added to 0.9 ml of the fluid. The remainder of the fluid is stored at -60°C. The antibiotic solution contains gentamycin (1 mg/ml), or penicillin (1000 units/ml) and streptomycin (500 µg/ml). Samples treated with antibiotics are centrifuged at 1500 g for 15 minutes to remove bacteria and debris; the supernatant fluids are used for inoculation.

1. Identification of the agent

Embryonated hens' eggs

Fertile eggs are set in a humidified incubator at 38°C and turned twice daily; after 10-11 days, they are examined by candling. Only live embryonated eggs are selected for use. The area above the air sac is cleansed with alcohol and a small hole drilled through the shell. Three eggs/sample are inoculated (0.1 ml) into the amniotic cavity. The syringe is withdrawn about 1 cm and a further 0.1 ml inoculated into the allantoic cavity. The hole is sealed with wax and the eggs incubated at 34-37°C for 2-3 days.

The eggs are then transferred to 4°C overnight to kill the embryos and to reduce bleeding at harvest. The shells are disinfected and the allantoic fluid is harvested by pipette, each harvest being kept separate. These are tested

for HA activity using a 0.5% suspension of chicken erythrocytes (RBC) or 0.4% guinea pig RBC. If there is no HA activity, aliquots of each harvest are pooled and re-passaged into further eggs. All HA positive samples are divided into aliquots and stored at -70°C; 1 aliquot is titrated for HA immediately and if it has a titre of 1:16 or more, the isolate is typed. Non-agglutinated RBC form a "stream" when the tube or plate is tilted.

Cell cultures

Cultures of the Madin-Darby canine kidney cell line (MDCK, ATCC CCL34) may be used to isolate equine influenza viruses. The cells are grown in tubes and infected in triplicate when grown to confluence with 0.25-0.5 ml of each sample, processed as described above. The cultures are maintained with medium containing 2 µg/ml trypsin, and examined daily for evidence of cytopathic effect (CPE). If positive, or after 7 days in any case, the supernatant fluids are tested for HA. Fluids with titres of 1:16 or more are typed immediately. Negative fluids are re-passaged.

Alternatively, the cells are screened for evidence of haemadsorption (HAD). This procedure detects expression of viral antigens at the cell surface. The medium is removed from the cultures and the tubes washed with PBS. One to two drops of a 50% suspension of chicken or guinea pig RBC are added, the tubes rotated carefully, and kept at room temperature for 30 minutes. Unbound RBC are washed off with PBS and the cultures examined microscopically for evidence of HAD.

Typing of new isolates of equine influenza viruses is best done by HI using specific antisera, prepared preferably in ferrets. Isolates may first be treated with Tween-ether, which destroys viral infectivity and reduces the risk of cross-contaminations.

For HI, both macro- and micro-tests may be used. The antigens are diluted to final HA titres of 1:4 or 1:8. Antisera, treated to reduce non-specific haemagglutinins, are serially diluted in PBS, antigen is added and the mixtures reacted for 30 minutes at room temperature. The RBC (0.5% chicken or 0.4% guinea pig) are added, gently mixed and allowed to stand at room temperature for 30 minutes. The volumes of the microtest are 0.025 ml for PBS, sera and antigen, respectively, followed by 0.05 ml for the RBC suspension. In the macrotest, 0.5 ml volumes are used throughout. The HI titres are read as the highest dilution of serum giving a complete inhibition of agglutination, i.e., the RBC form a "stream" when the plates are tilted. Incomplete inhibition is always scored as agglutination. All isolates should be sent to the International Reference Centres designated by OIE and WHO.

Standard antigens must be titrated in parallel with these tests and should include: subtype 1 (A/eq/Prague/56) and subtype 2 (A/eq/Miami/63;

A/eq/Fontainebleau/79; A/eq/Kentucky/81; or A/eq/Solvalla/79). Additionally, recent isolates from the same geographical area should be included if available. The standard antigens should be treated with Tween-ether to avoid cross-contamination. Test antigens and standard antigens are always back-titrated to confirm their antigen content.

Neuraminidase

Typing of neuraminidase requires specific antisera and no routine technique is available. Such typing is therefore best done in reference laboratories.

2. Serological tests

Infections are detected by conducting serological tests on paired sera to show a rise in antibody titre. These tests should be carried out whether virus isolation has been attempted or not. Two methods exist, each equally efficient and widely used. The complement fixation (CF) test can also be applied but the technique is not standardized.

a) Haemagglutination-inhibition (HI) test

The standard antigens are described above. If available, isolates from recent cases should be included. The antigen is first treated with Tween-ether. Lyophilised treated antigens for equine influenza viruses are available from the OIE Reference Centre. The test is best done in microtitre plates using the appropriate dilution equipment.

Sera are pre-treated to remove non-specific haemagglutinins and are inactivated by heating at 56°C for 30 minutes. Pre-treatments include the use of one of the following: (i) Kaolin and RBC absorption, (ii) Potassium periodate, or (iii) *V. cholerae* receptor-destroying enzyme (RDE). All three procedures yield similar results. The treated sera are diluted in PBS, antigen with a titre of 1:4 or 1:8 is added, and these are kept at room temperature for 30 minutes after gentle mixing. RBC are added and the test is read 30 minutes later. The HI titres are the highest dilutions of serum that completely inhibit HA. Titre increases of 4-fold or more between paired sera indicate recent infections.

b) Single radial haemolysis (SRH)

In this test, viral antigens are coupled to fixed RBC that are suspended in agarose containing guinea pig complement (C'). Wells are punched in the agarose and filled with test sera. Influenza antibodies and C' lyse the antigen-coated RBC, resulting in a clear, haemolytic zone around the well; the size of this zone can be measured.

Special immunodiffusion plates (Hyland; Miles Scientific) may be used for the assay, but simple Petri dishes are also suitable. Sheep RBC are collected into Alsever's solution and washed 3 times. The C' can be obtained commercially, or normal guinea pig serum can be used. The antigens are allantoic fluids or purified preparations; the strains used are the same as for the HI tests. The viruses are coupled to RBC by potassium periodate or, in the case of purified preparations, by chromic chloride. The coupled antigen-RBC preparations are mixed with C', together with a 1% solution of agarose (low melting grade) in PBS. Care must be taken that the temperature not be allowed to rise above 42°C at any time. The mixture is poured into plates and left overnight at 4°C. Wells of 2-3 mm in diameter and 12 mm apart are punched in the solidified agarose 6 mm from the edge of the plates. Such plates may be stored at 4°C for several weeks. Plates are prepared for each antigen and pre-tested with known positive and negative antisera.

For safety reason, all sera are heat-inactivated (at 56°C for 30 minutes) but no further treatment is necessary. Paired sera should be assayed in duplicate on the same plate; all sera are tested in a plate containing all components except virus. Sera that show haemolytic activity for sheep RBC must be pre-absorbed with sheep RBC. Zones of lysis should be clear, and not hazy or translucent. All clear zones should be measured and the area of haemolysis calculated. Differences in diameter between duplicate samples must be less than 1.5-fold. In paired sera, differences in diameters of 2-fold or more are considered significant for infection. Sera with differences between 1.5-fold and 2-fold should be re-tested.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Equine influenza virus vaccines consist of inactivated virus subtypes 1 and 2, or their subunits, that induce sufficient humoral antibody responses in seronegative horses that are susceptible to these agents (2).

1. Seed management

a) Characteristics

The vaccine must contain the following influenza virus strains:

Subtype 1: A subtype 1 strain, such as A/eq/Prague/1/56; but other subtype 1 strains may be used.

Subtype 2: Two subtype 2 strains are available:

i) One classical strain (i.e. isolated before 1979), such as A/eq/Miami/1/63; but other strains may be used if a very close antigenic relationship to the above strain has been demonstrated.

ii) One strain isolated following the antigenic drift of 1979-81, such as A/eq/Fontainebleau/79, A/eq/Kentucky/81 or A/eq/Solvalla/79. Other strains may be used if they are shown to be either closely related to the three preceding strains or of local importance.

The prevalence and antigenic variation of current isolates of equine influenza viruses must be monitored continuously, preferably by OIE or WHO Reference Laboratories. If the results of such studies indicate a significant antigenic drift, drifted strains should also be included.

b) Culture

Virus strains may be obtained from OIE or WHO Centres or from the ATCC (American Type Culture Collection, Rockville, MD 26852, USA). The strains are propagated in the allantoic cavity of 10-day-old embryonated hens' eggs. All manipulations must be conducted separately for each strain. Viral growth is monitored by HA tests. Passaged virus is identified by serological tests, such as HI or SRH. Seed virus is divided into aliquots and stored in freeze-dried form or at -70°C.

2. Manufacture

Production is based on a seed-lot sytem that has been validated with respect to the characteristics of the vaccine strains. Each strain of virus is inoculated separately into the allantoic cavity of 9- to 11-day-old embryonated hens' eggs from a healthy flock. The eggs are incubated at a suitable temperature for 2-3 days and the allantoic fluid is collected. The viral suspensions of each strain are collected separately and inactivated. If necessary, they may be purified. Suitable adjuvants and antimicrobial preservatives may be added.

3. In-process control

In-process controls include:

a) Identity of virus strains (tested by HI),
b) Sterility,
c) Virus titre (titration in embryonated eggs), and
d) Haemagglutination (tested by HA).

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on general information.

b) Safety

Using not less than 2 horses, each horse is inoculated intramuscularly at 2 different sites with the dose of vaccine specified by the manufacturer; these are repeated 4 weeks later. The animals are kept under observation for 10 days after the second injections. No abnormal local or systemic reactions should ensue.

To test for completeness of inactivation, inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 embryonated hens's eggs, 9-11 days old. Incubate at a suitable temperature for 3 days. The death of any embryo within 24 hours of inoculation is considered to be non-specific and the embryo is discarded. The allantoic fluids are collected, pooled and passaged into further eggs in the same way. After 4 days' incubation, the allantoic fluids of these eggs should possess no HA activity.

c) Potency

Horses

To test a vaccine for efficacy in horses, inject a volume correponding to 1 vaccine dose by the recommended route into each of 5 susceptible seronegative horses. After the period stated on the label as that between the first and second injection, inject a volume corresponding to the second dose of vaccine.

Collect 2 blood samples from each animal, the first 1 week after the first vaccination and the second 2 weeks after the second vaccination. Separate the serum from each sample. Inactivate each serum by heating at 56°C for 30 minutes. To 1 volume of each serum add 3 volumes of PBS pH 7.4 and 4 volumes of a 25% (w/v) suspension of kaolin R in the same buffer solution. Shake each mixture at room termperature for 10 minutes. Centrifuge, collect the supernatant fluid and mix it with a concentrated suspension of chicken RBC. Allow to stand at 37°C for 1 hour and centrifuge. The dilution of serum that results is now 1:8. Other treatments of sera may also be used (see section 2).

Perform tests on each serum using, respectivly, the antigen or antigens prepared from the strain or strains used in the production of the vaccine. Using each diluted serum, prepare a series of 2-fold dilutions. To 0.025 ml of each of the latter, add 0.025 ml of a suspension of each antigen, respectively treated with ether or Tween-ether and containing 4 haemagglutinating units (HAU). Allow the mixtures to stand at room temperature for 30 minutes and add 0.05 ml of a 0.5% suspension of chicken RBC. Allow to stand at room temperature for 1 hour and note the last dilution of serum that still completely inhibits HA.

The antibody titre of each serum taken after the second vaccination in each test calculated for the original serum is not less than 1:64, taking into account the pre-dilution of 1:8. If the titre found for any horse after the first vaccination indicates that there has been an anamnestic response, that result is not taken into account. A supplementary test is carried out, as described above, using the number of horses that showed an anamnestic response.

If tests for potency in horses have been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared using the same seed-lot system, subject to agreement by the National Control Authority.

Guinea pigs

Into each of 10 guinea pigs free from specific antibodies, inject the dose stated on the label. Twenty-one days later, collect blood samples and separate the serum. Inactivate each serum by heating at 56°C for 30 minutes and treat the sera as described above for horse sera.

Perform the tests on each serum using, respectively, the antigen or antigens prepared from the strain or strains used in the production of the vaccine. Using each diluted serum, prepare a series of 2-fold dilutions. To 0.025 ml of each dilution, add 0.025 ml of a suspension of each antigen respectively treated with ether or Tween-ether and containing 4 HAU. Allow the mixtures to stand at room temperature for 30 minutes and add 0.05 ml of a 0.5% suspension of chicken RBC. Allow to stand at room temperature for 1 hour and note the last serum dilution that still completely inhibits HA. The antibody titre of each serum in each test before mixture with the suspension of antigen and RBC should not be less than 1:16, taking into account pre-dilutions by treatment.

The European Pharmacopoeia may also be used as a reference for the control of equine influenza virus vaccines.

REFERENCES

Review articles

1. U.S. Department of Health and Human Services (1982).- Concepts and procedures for laboratory-based influenza surveillance. Centers for Disease Control, Atlanta, Georgia, pp. 81-835.

2. WHO (1979).- WHO Expert Committee on Biological Standardization. Technical Report No. 638, Annex 3, pp. 148-170.