CHAPTER 2.5.7.

EQUINE INFLUENZA (INFECTION WITH EQUINE INFLUENZA VIRUS)

SUMMARY

Description of the disease: Equine *influenza is an acute respiratory infection of horses, donkeys, mules and zebras caused by two distinct subtypes* (*H7N7, formerly equi-1, and H3N8, formerly equi-2*) *of influenza A virus within the genus* Influenzavirus *A of the family* Orthomyxoviridae*. Viruses of the H7N7 subtype have not been isolated since the late 1970s. Equine influenza viruses of both subtypes are considered to be of avian ancestry and highly pathogenic avian H5N1 has been associated with an outbreak of respiratory disease in donkeys in Egypt. In fully susceptible equidae, clinical signs include pyrexia and a harsh dry cough followed by a mucopurulent nasal discharge. In partially immune vaccinated animals, one or more of these signs may be absent. Vaccinated infected horses can still shed the virus and serve as a source of virus to their cohorts. Characteristically, influenza spreads rapidly in a susceptible population. The disease is endemic in many countries with substantial equine populations. In recent years, infection has been introduced into Australia and re-introduced into South Africa and Japan; to date New Zealand and Iceland are reported to be free of equine influenza virus.*

While normally confined to equidae, equine H3N8 influenza has crossed the species barrier to dogs. Extensive infection of dogs has been reported in North America where it normally produces mild fever and coughing but can cause fatal pneumonia. While equine influenza has not been shown to cause disease in humans, serological evidence of infection has been described primarily in individuals with an occupational exposure to the virus. During 2004–2006 influenza surveillance in central China (*People's Rep. of*) *two equine H3N8 influenza viruses were also isolated from pigs.*

Identification of the agent: *Embryonated hens' eggs and/or cell cultures can be used for virus isolation from nasopharyngeal swabs or nasal and tracheal washes. Isolates should always be sent immediately to an OIE Reference Laboratory. Infection may also be demonstrated by detection of viral nucleic acid or antigen in respiratory secretions using the reverse-transcription polymerase chain reaction* (*RT-PCR*) *or an antigen-capture enzyme-linked immunosorbent assay* (*ELISA*), *respectively.*

Serological tests: *Diagnosis of influenza virus infections is usually only accomplished by tests on paired sera; the first sample should be taken as soon as possible after the onset of clinical signs and the second approximately 2 weeks later. Antibody levels are determined by haemagglutination inhibition* (*HI*) *or single radial haemolysis* (*SRH*).

Requirements for vaccines: *Spread of infection and severity of disease may be reduced by the use of potent inactivated equine influenza vaccines containing epidemiologically relevant virus strains. Inactivated equine influenza vaccines contain whole viruses or their subunits. The vaccine viruses are propagated in embryonated hens' eggs or tissue culture, concentrated, and purified before inactivation with agents such as formalin or beta-propiolactone. Inactivated vaccines provide protection by inducing humoral antibody to the haemagglutinin protein. Responses are generally short-lived and multiple doses are required to maintain protective levels of antibody. An adjuvant is usually required to stimulate durable protective levels of antibody. Live attenuated virus and viral vectored vaccines have been licensed in some countries.*

Vaccine breakdown has been attributed to inadequate vaccine potency, inappropriate vaccination schedules, and outdated vaccine viruses that are compromised as a result of antigenic drift. An in-vitro potency test (single radial diffusion) can be used for in-process testing of the antigenic content of inactivated products before addition of an adjuvant. In process testing of live and vectored

vaccines relies on titration of infectious virus. International surveillance programmes monitor antigenic drift among equine influenza viruses and each year the Expert Surveillance Panel (ESP) for Equine Influenza makes recommendations for suitable vaccine strains. Following a change in recommendations, vaccines should be updated as quickly as possible to ensure optimal protection. This is particularly important for highly mobile horse populations and for any horse travelling internationally.

A. INTRODUCTION

Equine influenza is caused by two subtypes: H7N7 (formerly subtype 1) and H3N8 (formerly subtype 2) of influenza A viruses (genus *Influenzavirus* A of the family *Orthomyxoviridae*); however there have been very few reports of H7N7 subtype virus infections in the last 30 years (Webster, 1993).

In fully susceptible equidae, clinical signs include pyrexia, nasal discharge and a harsh dry cough; pneumonia in young foals and donkeys and encephalitis in horses have been described as rare events (Daly *et al*., 2006; Gerber, 1970). Clinical signs associated with infection in dogs also include fever and a cough; occasionally infection results in suppurative bronchopneumonia and peracute death (Crawford *et al.,* 2005). Characteristically, influenza spreads rapidly in a susceptible population. The virus is spread by the respiratory route, and indirectly by contaminated personnel, vehicles and fomites. The incubation period in susceptible horses may be less than 24 hours. In partially immune vaccinated animals the incubation period may be extended, one or more clinical signs may be absent and spread of the disease may be limited. This makes clinical diagnosis of equine influenza more difficult as other viral diseases, such as equine herpesvirus-associated respiratory disease, may clinically resemble a mild form of influenza. Horses infected with equine influenza virus become susceptible to secondary bacterial infection and may develop mucopurulent nasal discharge, which can lead to diagnosis of bacterial disease with the underlying cause being overlooked.

Equine influenza viruses are believed to be of avian ancestry, and more recent transmission of avian viruses to horses and donkeys has been recorded. The sequence analysis of an H3N8 virus isolated in 1989 from horses during a limited influenza epidemic in North Eastern China (People's Rep. of) established that the virus was more closely related to avian influenza viruses than to equine influenza viruses (Guo *et al*., 1992). Avian H5N1 has been associated with respiratory disease of donkeys in Egypt (Abdel-Moneim *et al*., 2010).

Equine influenza viruses have the potential to cross species barriers and have been associated with respiratory disease in dogs primarily in North America (Crawford *et al*., 2005). Isolated outbreaks of equine influenza have also occurred in dogs within the UK but the virus has not become established in the canine population. Close contact with infected horses was thought to be involved in each outbreak in the UK. Equine influenza viruses have also been isolated from pigs in central China (People's Rep. of) (Tu *et al*., 2009). Despite the occasional identification of seropositive persons with occupational exposure there is currently little evidence of zoonotic infection of people with equine influenza (Alexander & Brown, 2000).

In endemic countries the economic losses due to equine influenza can be minimised by vaccination and many racing authorities and equestrian bodies have mandatory vaccination policies. Vaccination does not produce sterile immunity; vaccinated horses may shed virus and contribute silently to the spread of the disease. Appropriate risk management strategies to deal with this possibility should be developed.

B. DIAGNOSTIC TECHNIQUES

Test methods available for the diagnosis of equine influenza and their purpose are summarised in Table 1. Laboratory diagnosis of acute equine influenza virus infections is based on virus detection in nasal swabs collected from horses with acute respiratory illness. Alternatively, the demonstration of a serological response to infection may be attempted with paired serum samples. Ideally, both methods are used. Equine influenza virus may be isolated in embryonated hens' eggs or cell culture. Infection may also be demonstrated by detection of viral antigen in respiratory secretions using an antigen capture enzyme-linked immunosorbent assay (ELISA) or of viral genome using reverse-transcription polymerase chain reaction (RT-PCR) assays. All influenza viruses are highly contagious for susceptible hosts and care must therefore be taken during the handling of infected eggs or cultures to avoid accidental cross-contamination. Standard strains should not be propagated in the diagnostic laboratory, at least never at the same time or in the same place where diagnostic samples are being processed. All working areas must be efficiently disinfected before and after virus manipulations, which should preferably be conducted within biohazard containment level 2 and class II safety cabinets.

It is important to obtain samples as soon as possible after the onset of clinical signs, preferably within 3–5 days. These samples include nasopharyngeal swabs and nasal or tracheal washings, the latter taken by endoscopy. Swabs may consist of absorbent cotton wool sponge/gauze on wire, and should be long enough to be passed via

the ventral meatus into the nasopharynx. Swabs should be transferred to a tube containing transport medium immediately after use. This medium consists of phosphate buffered saline (PBS) containing either 40% glycerol or 2% tryptose phosphate broth with 2% antibiotic solution (penicillin [10,000 units], streptomycin [10,000 units] in sterile distilled water [100 ml]), and 2% fungizone (250 mg/ml stock). 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 –70°C or below. Samples should be kept cool during transport to the laboratory.

Sample processing should follow the quality procedures outlined in Chapter 1.1.5 *Quality management in veterinary testing laboratories*, taking measures to prevent cross contamination. The liquid is expelled from the swab by squeezing with forceps and the swab is then disposed of suitably. Further antibiotics may be added if samples appear to be heavily contaminated with bacteria. The remainder of the fluid is stored at –70°C. Samples treated with antibiotics are allowed to stand on ice for 30–60 minutes and are then centrifuged at 1500 ***g*** for 15 minutes to remove bacteria and debris; the supernatant fluids are used for inoculation. The remainder of the fluid is stored at –70°C. Filtration of samples is not advised as influenza virus may adsorb on to the filter and be lost from the sample.

Table 1. *Test methods available for the diagnosis of* equine influenza

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
Virus isolation	–	+	–	++	–	–
Real time RT-PCR	–	+++	+++	+++	+++	–
RAD	–	+	–	++	+	–
Antigen-capture ELISA	–	++	++	+++	++	–
Detection of immune response						
HI	++	++[a]	–	+++[a]	+++	++
SRH	++	+[a]	–	+++[a]	+++	+++
ELISA	+	–	+[b]	+[a]	+	+

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
RT-PCR = reverse-transcription polymerase chain reaction; RAD = rapid antigen detection;
HI = haemagglutination inhibition; SRH = single radial haemolysis; ELISA = enzyme-linked immunosorbent assay.
[a]Testing of paired samples required;
[b]May be useful for DIVA (detection of infection in vaccinated animals) when used with appropriate vaccines.

1. Identification of the agent

Isolation of infectious virus may be carried out in embryonated hens' eggs or cell cultures. Traditionally, eggs have been preferred for isolation of equine influenza as many clinical isolates do not grow well in cells without serial passage. Comparison of H3N8 viruses isolated in eggs and Madin–Darby canine kidney (MDCK) cells indicated that MDCK cells are capable of selecting variant viruses that are not representative of the predominant virus in clinical specimens (Ilobi *et al.*, 1994). However, some viruses have been successfully isolated in MDCK cells but not in eggs and selection of variants has also occurred as a result of culture in eggs (Oxburgh & Klingborn, 1999). Ideally, isolation should be attempted using both substrates.

1 A combination of agent identification methods applied on the same clinical sample is recommended.

RT-PCR and real-time RT-PCR assays are being widely used in diagnostic laboratories as a more sensitive alternative to virus isolation (Quinlivan *et al.*, 2005). Influenza virus antigen in nasal secretions may also be detected directly by a sensitive antigen-capture ELISA for the H3N8 virus using a monoclonal antibody (MAb) against the equine influenza virus nucleoprotein (Livesay *et al.*, 1993).This assay is not commercially available, other than as a diagnostic service, but commercial self-contained kits for detecting human influenza are available and have been shown to detect equine influenza antigen (Chambers *et al.*, 1994; Yamanaka *et al.*, 2008). This approach is less sensitive than RT-PCR but provides a rapid result on which management decisions may be based. It should not be used to the exclusion of virus isolation. It is essential that new viruses be isolated and sent to reference laboratories for characterisation as part of the surveillance programme to monitor antigenic drift and emergence of new viruses and to provide isolates for inclusion in updated vaccines. Positive RT-PCR and ELISA results are useful in the selection of samples for virus isolation attempts if resources are limited, or for the selection of specimens to be sent to a reference laboratory for virus isolation and characterisation.

1.1. Virus isolation in embryonated hens' eggs

Fertile eggs are set in a humid incubator at 37–38°C and turned twice daily; after 10–11 days, they are examined by candling and live embryonated eggs are selected for use. The area above the air sac is cleansed with alcohol and a small hole is made through the shell. Inoculum may be introduced into the amniotic or the allantoic cavity. Several eggs/sample are inoculated (0.1 ml) in the amniotic cavity with no additional dilution of the sample (sample may also be diluted 1/10 and 1/100 in PBS containing antibiotics). The syringe is withdrawn approximately 1 cm and a further 0.1 ml is inoculated into the allantoic cavity. Alternatively, many laboratories opt to inoculate into the allantoic cavity alone through a second hole drilled just below the line of the air sac. The hole(s) is/are sealed with wax or Sellotape, and the eggs are incubated at 34–35°C for 3 days. The embryos that die within 24 hours following inoculation should be discarded. The eggs that contain embryos that die more than 24 hours after inoculation or contain live embryos after 3 days are examined for the presence of equine influenza virus.

The eggs are transferred to 4°C for 4 hours or overnight to kill the embryos and to reduce bleeding at harvest. The shells are disinfected, and the amniotic and/or allantoic fluid is harvested by pipette, each harvest being kept separate. These are tested for haemagglutination (HA) activity by mixing twofold dilutions of the harvested fluid in equal volumes (0.025 ml) with chicken red blood cells (RBCs) (0.5% [v/v] packed cells in PBS) in V- or U-bottomed microtitre plates or 0.4% guinea-pig RBCs (0.4% [v/v] packed cells in PBS) in V- or U-bottomed plates. Plates are incubated for approximately 30 minutes preferably at 4°C to prevent neuraminidase activity. If chicken RBCs are used, the plates may be read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. Non-agglutinated guinea-pig cells appear as a button at the bottom of the well and may take longer to settle. If there is no HA activity, aliquots of each harvest are pooled and passaged into further eggs. All HA positive samples are divided into aliquots and stored at –70°C; one aliquot is titrated for HA immediately. The HA titre is the reciprocal of the greatest dilution to show agglutination. If the HA titre is 1/16 or more, the isolate is characterised immediately. If titres are low, positive samples should be passaged. Care should be taken to avoid generation of defective interfering particles by prediluting the inoculum 1/10, 1/100, 1/1,000. Positive samples arising from the highest dilution should be selected as stocks for storage. It may be necessary to undertake as many as five passages to isolate the virus, particularly from vaccinated horses. If virus has not been recovered by the fifth passage, further passages are unlikely to be successful.

1.2. Virus isolation in cell cultures

Cultures of the MDCK cell line (MDCK, ATCC CCL34) may be used to isolate equine influenza viruses. The cells are grown to confluence in tubes and then infected in triplicate with 0.25–0.5 ml of each sample, processed as described above. Prior to inoculation, the cell monolayer is washed at least once with tissue culture medium containing trypsin (2 µg/ml) without serum. The cultures are maintained with serum-free medium containing 0.5–2 µg/ml trypsin (treated with TPCK [L-1-tosylamine-2-phenylethyl chloromethyl ketone] to remove chymotrypsin, available pretreated, e.g. from Sigma), and examined daily for evidence of cytopathic effects (CPE). If positive, or after 7 days in any case, the supernatant fluids are tested for HA. Fluids with titres of ≥1/16 are characterised immediately. Negative fluids and those with titres <1/16 are repassaged up to five passages.

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 are washed with PBS. One or two drops of a 50% suspension of chicken or guinea-pig RBCs are added, the tubes are rotated carefully, and kept at room temperature (23°C ± 2°C) for 30 minutes. Unbound RBCs are washed off with PBS, and the cultures are examined microscopically for evidence of HAD.

1.3. Haemagglutinin subtyping

The HA subtype of new isolates of equine influenza viruses may be determined by haemagglutination inhibition (HI; Section B.2.1) using H7N7- and H3N8-specific antisera. Isolates may first be treated with Tween 80/ether, which destroys viral infectivity and reduces the risk of cross-contamination. In the case of H3N8 viruses particularly, this treatment enhances the HA activity (John & Fulginiti, 1966). However, treatment with Tween 80/ether also decreases specificity and may increase the variability of the results obtained. Standard antigens must be titrated in parallel with tests to identify viruses and should include H7N7 strains (e.g. A/eq/Prague/56, A/eq/Newmarket/77) and H3N8 strains (e.g. A/eq/Newmarket/2/93, and A/eq/South Africa/4/03 and A/eq/Richmond/1/07). Virus strains may be obtained from OIE Reference Laboratories (see Table given in Part 4 of this *Terrestrial Manual*). Additionally, recent isolates from the same geographical area should be included if available. The standard antigens should be treated with Tween 80/ether to avoid cross-contamination. Test antigens and standard antigens are always back-titrated to confirm their antigen content.

Since the 1980s, only subtype H3N8 viruses have been isolated from horses. The HA sequence of equine H3 isolates can be determined rapidly by RT-PCR and sequencing, as described by Rash *et al.* (2014), and is encouraged for surveillance purposes.

New isolates of equine influenza viruses may be further characterised by HI using strain-specific antisera. The species in which antibodies are raised will influence the cross-reactivity of the antiserum, with ferrets providing the most strain-specific antibody (Mumford, 1992). The specificity and cross reactivity of the sera are also influenced by the immunisation schedule. Sera obtained 3 weeks after a single antigen application are considered to be most discriminative.

All isolates should be sent immediately to an International Reference Laboratory designated by OIE or the World Health Organization (WHO) for inclusion in the strain surveillance programme to monitor antigenic drift and emergence of new viruses.

1.4. Neuraminidase subtyping

Subtyping of neuraminidase requires specific antisera and no routine technique is available. Subtyping can also be done using specific PCR primers. Since the 1980s only subtype H3N8 viruses have been isolated from horses. The NA sequence of equine H3 isolates can be determined rapidly by RT-PCR and sequencing, as described by Rash *et al.* (2014), and is encouraged for surveillance purposes.

1.5. Polymerase chain reaction

RT-PCR assays both conventional and real-time, are increasingly used for the detection of equine influenza genome in nasal secretions as they are more sensitive than virus culture in eggs or detection of nucleoprotein using rapid antigen detection (RAD) kits for the detection of human influenza. A probe-based real-time RT-PCR assay based on the matrix gene and developed for the detection of a wide range of influenza type A strains including avian H5N1,(Heine et al., 2007) was combined with an automated DNA extraction system to establish a high throughput assay used in the mass screening of horses during the eradication programme in Australia in 2007 (Foord *et al.*, 2009). This type of paninfluenza assay has been used effectively for diagnosis and surveillance of equine influenza by OIE Reference Laboratories (Gildea *et al.,* 2013a). TaqMan® real-time RT-PCR assays specific for equine-2 influenza (H3N8) and equine-1 (H7N7) viruses have also been described and a commercial RT-PCR kit for the detection of equine influenza has become available (Lu *et al.*, 2009). No RT-PCR assay has yet been validated in line with the OIE Validation Template but some are accredited to ISO17025.

Although the genetic sequence of isolates can also be derived from PCR assays it remains essential to isolate infectious virus in order to examine the antigenic properties of new isolates and evaluate antigenic drift in the field.

2. Serological tests

Influenza infections can be detected by performing serological tests on paired sera to show a rise in specific antibody. These tests should be carried out whether virus isolation has been attempted or not. They are robust and may prove positive in the absence of virus isolation. Two simple methods exist, HI and single radial haemolysis (SRH), each equally efficient and widely used. ELISAs for antibodies against influenza nucleoprotein are available but are less commonly used (Galvin *et al.,* 2013).The complement fixation (CF) test can also be applied, but is not in general use. The paired serum samples, i.e. the acute and convalescent samples, should be tested together to minimise the impact of inter-assay variability. The standard antigens are described above

(Section B.1.3). If available, isolates from recent cases should be included. Freeze-dried post-infection equine antisera to A/eq/Newmarket/77 (H7N7), A/eq/Newmarket/1/93 (American lineage H3N8), A/eq/Newmarket/2/93 (European lineage H3N8), A/eq/South Africa/4/03 (Florida Clade 1, sublineage of American lineage) and an influenza-negative equine serum, are available from the European Directorate for the Quality of Medicines (EDQM)[2]. These sera have been assigned SRH values through an international collaborative study and can be used as primary reference sera for the assay (Daly *et al.*, 2007; Mumford, 2000).

2.1. Haemagglutination inhibition test

The antigen is first treated with Tween 80/ether in order to increase the sensitivity of the test, particularly for H3N8 viruses. The HI test can also be performed without ether treatment although at reduced sensitivity. The test is best done in microtitre plates using the appropriate dilution equipment. A macrotest may be used, for which antigen is diluted to a final HA titre of 1/8 per well and the volumes for PBS, sera and antigen are 0.5 ml. Sera are pretreated to remove nonspecific haemagglutinins, then inactivated at 56°C for 30 minutes. Pretreatments include the use of one of the following: (a) kaolin and RBCs absorption, not recommended for H7N7 HI, (b) potassium periodate, or (c) *Vibrio cholerae* receptor-destroying enzyme. Potassium periodate or *V. cholerae* receptor-destroying enzyme is the treatment of choice. The treated sera are diluted in PBS, a standard dose of antigen is added (HA titre of 1/4 per well for microtitration assay), and these are kept at room temperature (23°C ± 2°C) for 30 minutes. After gentle mixing, RBCs are added and the test is read 30 minutes later. The HI titres are read as the highest dilution of serum giving complete inhibition of agglutination. Either chicken RBCs (1% [v/v] packed cells) in V-bottomed microtitre plates or guinea-pig RBCs (0.5% [v/v] packed cells) in V- or U-bottomed plates may be used. If chicken RBCs are used, the plates may read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. Non-agglutinated guinea-pig cells appear as a 'button' in the bottom of the well and may take longer to settle. The HI titre is the reciprocal of the greatest dilution showing complete inhibition of agglutination. Currently, a cut-off point for positive samples has not been determined for the HI test and thus, low titres should be investigated further. Titre increases of fourfold or more between paired sera indicate recent infection.

2.1.1. Tween 80/ether treatment of the virus

i) To 39.5 ml of infective allantoic fluid, add 0.5 ml of a 10% (v/v) suspension of Tween 80 in PBS to give a 0.125% (v/v) concentration of Tween 80.

ii) After mixing gently at room temperature for 5 minutes, add 20 ml of diethyl ether to give a final concentration of 33.3% by volume, and mix the suspension well at 4°C for 15 minutes.

iii) After allowing the layers to separate by standing, remove the aqueous layer containing the disrupted virus particles to a glass bottle with a loose lid and allow the excess ether to evaporate off overnight (John & Fulginiti, 1966). Safety precautions while handling ether must be strictly observed and work should be confined to a fume hood.

iv) Store treated virus in aliquots at –70°C.

2.1.2. Titration of haemagglutination

i) Add 25 µl of PBS to all wells in a row of a microtitre plate.

ii) Add 25 µl of virus to first well then generate a twofold dilution series across the plate leaving the last well as a negative control.

iii) Add an extra 25 µl of PBS to all wells.

iv) Add 50 µl of RBCs to all wells. Leave at room temperature or at 4°C (particularly if ambient temperatures are high), for 30 minutes. The HA titre is taken as the last virus dilution giving partial HA.

2.1.3. Potassium periodate pretreatment of sera

i) Mix one volume (150 µl) of serum with two volumes (300 µl) of freshly prepared 0.016 M potassium periodate (0.38 g in 100 ml PBS), and leave at 22°C (± 2°C) for 15 minutes.

ii) Add a further one volume of 3% glycerol in PBS to neutralise any excess periodate solution, mix and leave at room temperature (23°C ± 2°C) for 15 minutes.

iii) Inactivate in a 56°C water bath for 30 minutes.

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2.1.4. Test procedure

i) Dispense 25 µl of PBS to all wells of a microtitre plate.

ii) Add serum (25 µl) to the first well of a row of 12, then generate twofold serial dilutions (1/8 to at least 1/512, allowing for dilution of 1/4 from treatment of serum), leaving the last well as a control.

iii) Dilute the antigen to give a dose of 4 HA units (4 × minimum agglutinating dose, i.e. titre/4).

iv) Add 25 µl to each well, and incubate at room temperature (22°C ± 2°C) for 30 minutes.

v) Add 50 µl of RBCs to each well. Leave at room temperature or at 4°C (particularly if ambient temperatures are high), for 30 minutes.

vi) The plates may be read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. No agglutination is recorded as a positive result.

2.2. Single radial haemolysis

In this test, viral antigens are coupled to fixed RBCs 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 RBCs, resulting in a clear, haemolytic zone around the well; the size of this zone is directly proportional to the level of strain-specific antibody in the serum sample (Morley *et al.*, 1995).

Special immunodiffusion plates (MP Biomedical) may be used for the assay, but simple Petri dishes are also suitable. Sheep RBCs collected into Alsever's solution are washed three times. The C' can be obtained commercially, or normal guinea-pig serum can be used. The viral antigens are egg-grown stocks or purified preparations; the strains used are the same as for the HI tests. The viruses are coupled to RBCs by potassium periodate or by chromic chloride. The coupled antigen/RBCs preparations are mixed with C', together with a 1% solution of agarose (low melting grade) in PBS. Care must be taken to ensure that the temperature is not allowed to rise above 42°C at any time. The mixture is poured into plates and left to set. Wells of 3 mm in diameter and 12 mm apart are punched in the solidified agarose, at least 6 mm from the edge of the plates. Such plates may be stored at 4°C for 12 weeks. Plates are prepared for each antigen.

Sera are inactivated at 56°C for 30 minutes, but no further treatment is necessary. Paired sera should be assayed on the same plate. As a minimum, a subtype-specific antiserum should be included as a control serum in one well on each plate. All sera are tested in a control plate containing all components except virus to check for nonspecific lysis. Alternatively, an unrelated virus, such as A/PR/8/34 (H1N1), may be used in the control plate. Sera that show haemolytic activity for sheep RBCs must be pre-absorbed with sheep RBCs. Zones of lysis should be clear and not hazy or translucent. All clear zones should be measured and the area of haemolysis calculated.

2.2.1. Preparation of reagents

i) *Saline/HEPES:* 0.85% NaCl (4.25 g/500 ml); 0.05 M HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid; 5.95 g/500 ml); and 0.02% sodium azide. Make to pH 6.5 with NaOH.

ii) *Saline/HEPES/BSA:* as saline/HEPES with 0.2% (w/v) bovine serum albumin (BSA).

iii) *$CrCl_3$ stock solution* (*2.25 M*) *6 g/10 ml:* Make fresh 1/400 dilution in 0.85% NaCl for each assay.

iv) *PBS* (*London)/PBS 'A':* NaCl (10.00 g); KCl (0.25 g); Na_2PO_4 (1.45 g); KH_2PO_4 (0.25 g); and Na azide (0.20 g). Make up to 1 litre with distilled water.

v) *Agarose in PBS:* Place flask containing PBS 'A' on a stirrer. Slowly add 10 g agarose to the stirring solution. Liquefy in a pressure cooker. Dispense into glass bottles for storage at 22°C (± 2°C).

vi) *Virus antigen:* Allantoic fluid containing infectious virus is harvested and stored at –70°C. A short titration curve determines the optimum ratio of virus antigen to RBCs to be used when preparing sensitised sheep RBCs. The H7N7 influenza strains always produce clear zones; the H3N8 strains sometimes produce hazy zones, in which case it is necessary to concentrate the virus by centrifugation.

vii) *Sheep blood:* Collect blood into an equal volume of Alsever's solution and store at 4°C. It may be necessary to test bleed several sheep, as characteristics of RBCs from individual sheep vary. Keep the blood for 2 days before use, it may then be usable for up to 3 weeks, providing sterility is maintained.

viii) *Complement:* Use commercially available guinea-pig complement or collect serum from young guinea-pigs of 300–350 g body weight and store in small volumes at –70°C. For use, thaw in cold water and hold at 4°C prior to mixing.

ix) *Treatment of sera:* Use undiluted sera heat inactivated at 56°C for 30 minutes. Avoid repeated freeze–thaw cycles.

2.2.2. Test procedure

i) Wash sheep RBCs at least three times in saline/HEPES.

ii) Prepare an appropriate volume of 8% RBCs (v/v packed cells) in saline/HEPES, having first calculated the number of plates required and allowing 1 ml per 6 × 11 cm immunoplate and 1–2 ml extra.

iii) Add a predetermined volume[3] of virus antigen to the 8% RBCs solution. Hold the mixture at 4°C for 10 minutes. Haemagglutination may be observed.

iv) SLOWLY add CrCl3 (1/400 in 0.85% NaCl) at half the total volume of virus/RBCs suspension. Hold at 22°C (±2°C) for 5 minutes with occasional mixing.

v) Sediment the sensitised RBCs by centrifugation at 1500 ***g*** for 5 minutes.

vi) Gently resuspend in saline/HEPES/BSA and centrifuge at 1500 ***g*** for 5 minutes.

vii) Resuspend RBCs to an 8% suspension in PBS 'A'.

During the sensitisation process, melt the agarose. Shortly before use, pipette 7.8 ml volumes to Universal bottles and retain at 42°C. Check that the agar has cooled to 42°C before use.

2.2.3. Preparation of plates

i) Add 0.9 ml of virus-sensitised sheep RBCs to 7.8 ml of agarose (42°C). Mix quickly, but gently.

ii) Add 0.3 ml of undiluted guinea-pig serum. Mix again and pour into immunoplates on a levelling table. Allow to set and air dry without a lid for 5 minutes.

iii) Place lids on plates and store at 4°C in a humid box until used.

iv) Prepare control plates with unsensitised cells or cells sensitised with an unrelated virus. Batches of prepared plates can be stored for several weeks.

v) Punch 3 mm holes in the set gels to a prepared template, allow for 16 test sera and a positive control serum. On antigen control plates, prepare five rows of eight wells.

vi) Pipette 10 µl of heat-inactivated (56°C for 30 minutes) test sera and a positive control serum to appropriate wells. Incubate at 34°C for 20 hours in a humid box.

vii) Measure zone diameters, and calculate areas of haemolysis after the area of the well has been deducted.

2.2.4. Interpretation of the results

For results to be valid, positive and negative control sera should give results assigned through the international collaborative study (Daly *et al.*, 2007; Mumford, 2000) or if not using these international standards, results consistent with those expected on the basis of prior experience. Areas of haemolysis for the control sera should be clear and intra-laboratory variation should be no more than 5% for the control serum. Results may be expressed as mm^2 or as a ratio of the control serum value. Sera giving positive results in the control plate should be adsorbed with sheep RBCs. For diagnostic purposes, acute and convalescent sera should be tested in duplicate on the same plate. Increase in zone areas produced by convalescent serum compared with acute serum is evidence of infection. An increase in area of 25 mm^2 or 50% whichever is smaller, is routinely deemed to be significant but this depends on the

3 Prepare three plates by adding 0.6, 1.2 or 1.8 ml of virus antigen to 2 ml RBCs. Add 1.3, 1.6 and 1.9 ml $CrCl_3$ respectively and resuspend to 2 ml in PBS 'A'. Optimum volume of virus antigen is that which results in the largest and clearest zones with appropriate reference serum.

reproducibility of the test within the laboratory, and should be equivalent to a twofold or more increase in antibody concentration. This area can be calculated from a standard curve generated from a dilution series of a standard antiserum.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Equine influenza is a self-limiting disease and its economic significance is primarily due to the contagious nature of the virus and the disruption to equestrian activities. Rapid diagnosis, movement restrictions and vaccination are the key control measures for equine influenza. Vaccination decreases clinical signs and virus shedding. Influenza vaccines are widely available and are routinely used in competition horses in Europe, the Americas, and Asia. In some countries, vaccination is mandatory for sport horses and racehorses that are competing under rules of equestrian organisations. Following a primary course of three doses at intervals of around 0, 1 and 6 months, an annual booster is the usual minimum requirement. More frequent vaccination is recommended for young horses and some equestrian organisations require biannual boosters.

Equine influenza virus vaccines typically consist of inactivated whole viruses or their glycoprotein subunits, with or without adjuvant. Live attenuated virus and canary pox vectored vaccines have become available commercially in some countries. Immunity generated by inactivated vaccines administered via the intramuscular route is reliant on stimulation of circulating antibody to the HA, which neutralises virus; some products have been shown to stimulate antibody in respiratory secretions. Critically the integrity and conformation of the HA should be maintained during inactivation procedures to ensure that the vaccine stimulates appropriate neutralising antibody. This can be tested by use of an immunological assay such as SRD (single radial diffusion), which measures immunologically active HA capable of reacting with specific anti-HA antibodies. The immunogenicity of the vaccine can be confirmed by measurement of HA antibody stimulated in small animal models or the target species. Several equine influenza vaccines including inactivated whole virus, subunit and a canary pox-vectored vaccine have also been shown to have the potential for cell-mediated immunity (CMI) priming (Gildea *et al.,* 2013b).

Live virus-vectored vaccines, which contain the influenza virus gene encoding HA rather than antigen, cannot be potency tested by SRD. Instead, the infectious titre of the recombinant is used as an *in-vitro* measure of potency and immunogenicity is assessed by measurement of antibody stimulated in the target species.

Antibody to HA as measured by SRH, stimulated by inactivated whole virus, subunit or canary pox-vectored vaccine correlates well with protection against infection in an experimental challenge model system (Edlund Toulemonde *et al.*, 2005; Mumford, 1983; 1994). In contrast, a cold-adapted temperature-sensitive mutant used as a live attenuated vaccine replicates in the upper respiratory tract and does not stimulate high levels of circulating antibody to HA but nevertheless provides protection against challenge infection. Immunity is presumed to be mediated through mucosal or cellular responses rather than circulating antibody. As with the vectored vaccine, in-process control testing is reliant on measurement of the infectious virus titre.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature as manufacturers are obliged to meet European Pharmacopeia, USDA or other national and regional requirements.

All equine influenza vaccines should contain epidemiologically relevant strains

A formal equine influenza global surveillance programme has been in place since 1995. The OIE Reference Laboratories and other collaborating laboratories collect data on outbreaks of equine influenza and strain variation that is reviewed annually by an Expert Surveillance Panel (ESP) including representatives from OIE and WHO. This panel makes recommendations on the need to update vaccines, and these are published annually in the OIE *Bulletin* and on the OIE website. The criteria for updating equine influenza vaccines are similar to those for human influenza vaccines and based on analysis of antigenic changes, genetic changes and, when possible, supporting experimental challenge data.

H7N7: Many vaccines still contain an H7N7 strain. However, the Expert Surveillance Panel has recommended that the H7N7 component should be omitted as reports of infections with this subtype have not been substantiated during the past 30 years.

H3N8: Antigenic variants of H3N8 viruses co-circulate (Bryant *et al.*, 2009) and it is important to include a strain or strains that are epidemiologically relevant as recommended by the OIE Expert Surveillance Panel and published in the OIE *Bulletin* and on the OIE website. OIE Reference Laboratories can provide help in selecting suitable vaccine strains.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

For each vaccine strain, a prototype batch should be prepared to establish its suitability as a vaccine strain, i.e. purity and safety should be confirmed by standard techniques. The ability of seed-lot viruses to grow to high titre and generate sufficient antigenic mass to stimulate adequate antibody responses in the target species, should be confirmed. Additionally, vaccine virus derived in MDCK cells should be fully characterised to ensure that antigenic variants have not arisen during the culture process, such that the vaccine virus is no longer representative of the original isolate.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Virus strains may be obtained from OIE Reference Laboratories (see Table given in Part 4 of this *Terrestrial Manual*). Viruses selected as vaccine strains should be described in terms of origin and passage history. The strains are propagated in the allantoic cavity of 10-day-old embryonated hens' eggs or cell cultures, such as MDCK. 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 or by PCR using specific primers. If vaccine virus is grown in cell culture, antigenic studies with ferret sera and MAbs should be undertaken to ensure that variant viruses have not been selected during passage to prepare master and working seed viruses. Master and working seed viruses are divided into aliquots and stored in freeze-dried form at –20°C or at –70°C following testing for extraneous agents. Records of storage conditions should be maintained.

The master seed lot of each vaccine strain selected should be processed at one time to assure a uniform composition, tested for extraneous agents, and fully characterised. Antisera or MAbs for use in HI tests to characterise vaccine strains may be obtained from OIE and WHO Reference Laboratories.

Working seed lots are derived from a master seed lot and should be of uniform composition, free from extraneous agents, and fully characterised. Aliquots of the working seed are used for production of vaccine.

Master and working seed lots should be prepared in specific pathogen free eggs or, as a minimum, in eggs derived from a healthy flock.

If MDCK cells are used to propagate vaccine virus, master cell lines should be established and stored in liquid nitrogen, and should be tested for freedom from extraneous agents according to National Control Authority Guidelines.

Examination of seed viruses for extraneous agents including mycoplasmas and other equine viruses should be performed by appropriate techniques, including inoculation of susceptible tissue cultures and examination for cytopathic effect or application of fluorescent antibodies for antigen detection.

The presence of other common equine respiratory pathogens, e.g. equine herpesviruses 1, 2, 4, equine picornaviruses, equine viral arteritis, and equine adenoviruses, should be specifically excluded.

The absence of bacteria should be confirmed by standard sterility tests and toxicity tests in small animals.

2.2. Method of manufacture

2.2.1. Procedure

Production is based on a seed-lot system that has been validated with respect to the characteristics of the vaccine strains. Where eggs are used, each strain of virus is inoculated separately into the allantoic cavity of 9–11-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. Alternatively, each strain is inoculated separately into MDCk cell cultures. 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.

Monovalent virus pools should be inactivated as soon as possible after their preparation, by a method approved by the National Control Authority. If formalin (37% formaldehyde) or beta-propiolactone (2-oxetanone) is used, the concentration by volume should not exceed 0.2%. Ideally, pools should be held at 4°C and should be inactivated within 5 days of harvest. Inactivation of the vaccine must be demonstrated. A suitable method consists of inoculating 0.2 ml of undiluted monovalent pool and 1/10 and 1/100 dilutions of the monovalent pool into the allantoic cavities of groups of fertile eggs (10 eggs in each group), and incubating the eggs at 33–37°C for 3 days. At least 8 of the 10 eggs should survive at each dosage level. A volume of 0.5 ml of allantoic fluid is harvested from each surviving egg. The fluid harvested from each group is pooled, and 0.2 ml of each of the three pools is inoculated, undiluted, into a further group of 10 fertile eggs. Haemagglutinin activity should not be detected in these new groups of eggs. In some countries, the requirement that 80% of the eggs should survive during incubation may be impossible to satisfy, in which case the National Control Authority should then specify a modified requirement to be satisfied. Before inactivation, samples should be collected for bacterial and fungal sterility tests.

Monovalent material may be concentrated and purified by high-speed centrifugation or other suitable methods approved by the National Control Authority, either before or after the inactivation procedure. It is important to concentrate and purify the virus under optimum conditions, e.g. temperatures that preserve its antigenic properties.

The monovalent virus pool shall be shown not to contain viable influenza virus when tested by inoculation of embryonated hens' eggs, by a method approved by the National Control Authority. Alternatively, the satisfactory inactivation can also be demonstrated by inoculating monolayers of MDCK cells.

2.2.2. Requirements for substrates and media

Vaccines should be produced in eggs or in a cell line that meets the requirements of the National Control Authority. Wherever practicable the use of substances of animal origin for example, serum and trypsin should be kept to a minimum. Substances of animal origin used during production should be subjected to a suitable, validated sterilisation or inactivation procedure or be tested for the absence of extraneous organisms.

2.2.3. In-process controls

Relevant in-process controls should be applied before and after inactivation and before and after concentration and purification.

In-process controls include: (a) identity of virus strains (tested by HI); (b) sterility; (c) virus titre; (d) haemagglutinin content (tested by chicken RBCs agglutinating units, CCA [chick cell agglutination]); and (e) immunologically active HA (tested by SRD or another suitable immunochemical method).

2.2.4. Single radial diffusion test

SRD is a reliable method for measuring immunologically active HA in terms of µg HA, and is used routinely for potency testing of human influenza vaccines (Wood *et al.*, 1983b).

The potency of inactivated equine influenza vaccine depends on the concentration of immunologically active haemagglutinin (Wood *et al.*, 1983a).

Assessment of the antigenic content of the vaccine by CCA alone may be misleading, as the sensitivity of this assay is a reflection of the ability of virus strains to agglutinate RBCs. Disruption of virus may lead to an apparent increase in HA as measured by CCA. The CCA assay does not provide a measure of the antigenic properties of the HA (HA may retain its properties to bind to RBCs while losing its ability to stimulate antibody).

Most equine influenza vaccines contain more than one variant of the H3N8 subtype. In this situation, it is not possible to judge the potency of individual H3N8 components from serological tests performed on sera collected from horses or small animals vaccinated with the final product, because of cross-reactivity between the two isolates of the same subtype. Thus, it is important that a reliable method, such as SRD, be used to measure the potency of individual components before and after inactivation and prior to mixing and formulation with adjuvant.

In the SRD test, virus preparations are compared with a calibrated reference preparation of known HA content. Antigens are allowed to diffuse through a gel containing an antiserum specific for a particular HA. The distance diffused by the antigen before precipitation by the antibody incorporated in the gel is directly related to the concentration of haemagglutinin in the antigen preparations.

Standard reagents for SRD testing are available from the WHO International Laboratory for Biological Standards[4]. Reagents for the H3N8 strains A/eq/Miami/63, A/eq/Kentucky/81, A/eq/Newmarket/1/93 (American lineage) and A/eq/Newmarket/2/93 (European lineage) are currently available.

2.2.5. Final product batch tests

i) Sterility and purity

Tests of biological materials for sterility and freedom from contamination may be found in chapter 1.1.9.

ii) Safety

a) For inactivated or subunit vaccines absence of viral infectivity should be demonstrated by two passages in 10 embryonated hen eggs. The allantoic fluid should have no haemagglutinating activity.

b) Using no fewer than three horses, each horse is inoculated intramuscularly (at two different sites) with the dose of vaccine specified by the manufacturer; these inoculations are repeated 2–4 weeks later. The animals are kept under observation for 10 days after the second set of injections. No abnormal local or systemic reaction should ensue.

c) If vaccine is to be used in mares, safety should be demonstrated by giving two doses of vaccine to no fewer than two pregnant mares at the prescribed interval within the trimester for which the vaccine is recommended. Once safety has been demonstrated on a prototype batch, safety testing in pregnant mares may be omitted for routine testing of subsequent batches of the final product.

iii) Batch potency

Following mixing of viral antigens and adjuvants, aliquots should be potency tested *in vivo* using horses and guinea-pigs or a suitable alternative immunochemical assay. Adjuvants cause interference in quantitative *in-vitro* tests, such as CCA and SRD, although SRD may be used on the final product as a qualitative assay to demonstrate the presence of antigen for each vaccine strain. For repeated batch tests, only guinea-pigs or a suitable alternative immunochemical assay are used, subject to agreement of the National Control Authority.

4 National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.

a) Serological responses in horses

For a valid *in-vivo* potency test, naive seronegative horses must be selected for vaccination. Young horses or ponies (not less than 6 months old) should be screened for the presence of antibody using H7N7 and H3N8 viruses including recently isolated viruses relevant to the area in which the horses were reared. If HI tests are used for screening, H3N8 viruses should be treated with Tween 80/ether to maximise the sensitivity of the test. Alternatively, SRH may be used to establish the seronegative status of animals.

To test a vaccine for efficacy in horses, inject a volume corresponding to one vaccine dose by the recommended route into each of five susceptible seronegative horses. After the period recommended between the first and second doses, as stated on the label, a volume of vaccine corresponding to the second dose of vaccine is injected into each horse.

Three blood samples are collected from each animal, the first at the time of the first vaccination, the second 1 week after the first vaccination to check for anamnestic response, and the third 2 weeks after the second vaccination.

The serological assay used to measure the antibody response to the viruses contained in the vaccine must be standardised for a valid *in-vivo* potency test. The SRH assay (see Section B.2.2) is preferred as standard reference sera are available for quality control purposes from the European Pharmacopoeia[5]. These sera should be tested in parallel with the test sera to ensure that the test is valid with respect to sensitivity; the values obtained should not vary by more than 20% from the SRH values assigned in an international collaborative study (Daly *et al.*, 2007; Mumford, 2000). Due to poor repeatability and reproducibility of the HI test between different laboratories, no HI titre could be assigned to these sera.

Following vaccination the antibody value measured by SRH should not be less than 150 mm^2 This is higher than the value required in the European Pharmacopoeia Monograph for inactivated equine influenza vaccines (85 mm^2) as this value is not considered to be protective. If the value found for any horse after the first vaccination indicates that there has been an anamnestic response, the result is not taken into account. A supplementary test is carried out, as described above, replacing the horses that showed an anamnestic response with an equal number of new animals.

If the HI test is used, the antibody titre of each serum taken after the second vaccination in each test should not be less than 1/64 (calculated for the original serum, taking into account the predilution of 1/8). Alternatively, the antibody levels stimulated by the vaccine under test should be shown to be at least equal to the antibody levels stimulated by a standard vaccine tested in parallel that has been shown previously to protect horses against challenge infection.

b) Challenge studies in horses

Challenge studies may be carried out by exposing vaccinated horses/ponies to an aerosol of virulent influenza virus no fewer than 2 weeks and preferably more than 3 months after the second dose of vaccine. Comparisons of clinical signs, virus excretion and serological responses are made with a group of unvaccinated control animals challenged at the same time. The timing of the challenge procedure will reflect the claims to be made on the data sheet regarding duration of immunity. Protection at 2 weeks post-vaccination when antibody levels are at their peak level does not necessarily indicate good duration of immunity under field conditions.

If tests for potency in horses have been carried out with satisfactory results on a representative batch of vaccine, these tests 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.

c) Serological responses of guinea-pigs

Inject each of no fewer than five guinea-pigs free from specific antibodies with one vaccine dose. Collect blood samples 21 days later, and test the serum by SRH or HI

5 Serum to A/eq/Newmarket/1/77 (Catalogue number E0850010), A/eq/Newmarket/1/93 (E0850021) and A/eq/Newmarket/2/93 (E0850022).

(see Sections B.2.1 and B.2.2). Perform the tests of each serum using, respectively, the antigen(s) prepared from the strain(s) used in the production of the vaccine. The antibody titre of each serum in each test should not be less than the titre stimulated by a standard vaccine that has been shown to stimulate protective levels of antibody in horses.

2.3. Requirements for authorisation of vaccines

2.3.1. Safety requirements

The safety requirements may vary with the National Authority but usually include the assessment of the administration of an overdose and of the repeated administration of one dose to young horses and pregnant mares, if the vaccine is intended for use in pregnant mares. For live vaccines the dissemination by the vaccinated horse and the spread of the vaccine strain from vaccinated to unvaccinated horses along with the possible consequences must be investigated. Reversion to virulence studies should be performed by serial passaging of the live vaccine.

2.3.2. Efficacy requirements

The efficacy requirements may vary with the National Authority but usually include the assessment of the serological response in horses and virus challenge studies in susceptible horses (see Section C.2.2.5.iii.b).

Where claims for duration of immunity are made on the data sheet, these should be supported with data on the duration of protective levels of antibody maintained in horses vaccinated according to the recommended schedule. Antibody levels quoted as protective should be validated in challenge studies or by comparison with published reports.

2.3.3. Stability

Vaccines should be stored at 5±3°C and protected from light. The shelf life quoted on the data sheet should be demonstrated by testing the potency of aliquots over time using the guinea-pig potency test (see Section C.2.2.5.iii.c).

2.4. Requirements for authorisation of strain updates to vaccines

To enable vaccine manufacturers to respond quickly to recommendations from the Expert Surveillance Panel, existing vaccines that have been updated in accordance with the OIE recommendations should be authorised if they meet the requirements for final product batch testing (see Section C.2.2.5).

The Committee for Veterinary Medicinal Products (CVMP) for the European Agency for the Evaluation of Medicinal Products (EMEA) has developed Guidelines on the compliance of authorised equine influenza vaccines with OIE recommendations. In the USA, viral strain changes in equine influenza vaccines are addressed in the USDA/CVB/Veterinary Services Memorandum No. 800.111, which heed OIE recommendations.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

A canary pox recombinant vaccine is available in some countries and was used in the equine influenza control and eradication programme in Australia in 2007. A nucleoprotein ELISA was used to differentiate horses vaccinated with the recombinant vaccine from horses that had been exposed to virus by natural infection (DIVA) This DIVA was possible because the canary pox recombinant expresses only the haemagglutinin gene of equine influenza. There is some evidence that this vaccine may be used to prime the immune system in the face of maternally derived antibodies (Minke *et al.*, 2008).

3.2. Special requirements

National Authorities frequently require an environmental risk assessment. This may include an assessment of the potential direct and indirect, immediate or delayed adverse effects of the genetically modified vaccine on human and animal health and on the environment. The phenotypic and genetic stability of the vaccine and its potential interactions with other organisms may need to be evaluated.

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NB: There are OIE Reference Laboratories for Equine influenza
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for equine influenza