

SWINE INFLUENZA

SUMMARY

Swine influenza is a highly contagious viral infection of pigs. Swine influenza virus (SIV) infections cause respiratory disease characterised by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, breathing difficulty, and depressed appetite. In some instances, SIV infections are associated with reproductive disorders such as abortion. Clinical signs and nasal shedding of virus can occur within 24 hours of infection. Morbidity rates can reach 100% with SIV infections, while mortality rates are generally low. Secondary bacterial infections can exacerbate the clinical signs following infection with SIV. Transmission is through contact with SIV-containing secretions such as nasal discharges and aerosols created by coughing or sneezing.

Identification of the agent: Virus identification is best accomplished by collection of samples within 24–48 hours after development of clinical signs. The animal of choice is an untreated, acutely ill pig with an elevated rectal temperature. Virus can readily be detected in lung tissue and nasal swabs. Virus isolation can be conducted in embryonated chicken eggs and on continuous cell lines. Isolated viruses can be subtyped using the haemagglutination inhibition (HI) and the neuraminidase inhibition tests, or by reverse transcription-polymerase chain reaction assays. Immunohistochemistry can be conducted on formalin-fixed tissue and a fluorescent antibody test can be conducted on fresh tissue. Enzyme-linked immunosorbent assays (ELISA) are commercially available for detection of type A influenza viruses.

Serological tests: The primary serological test for detection of SIV antibodies is the HI test conducted on paired sera. The HI test is subtype specific. The sera are generally collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent SIV infection. Additional serological tests that have been described are the agar gel immunodiffusion test, indirect fluorescent antibody test, virus neutralisation, and ELISA.

Requirements for vaccines and diagnostic biologicals: Inactivated, adjuvanted SIV vaccines are commercially available. Vaccines may be in the form of a single SIV subtype or may contain multiple SIV subtypes. Vaccines should reflect the current antigenic profile of field viruses, containing subtypes and strains that are changed as needed to assure protection. The finished vaccine must be shown to be pure, safe, potent, and efficacious.

A. INTRODUCTION

Swine influenza is a highly contagious viral infection of pigs that can have significant economic impact on an affected herd (26, 32). The swine influenza virus (SIV) is a type A orthomyxovirus with a segmented RNA genome. The type A swine influenza viruses are further subdivided based on their haemagglutinin and neuraminidase proteins. Subtypes of SIV that are most frequently identified in pigs include classical and avian H1N1, reassortant (r) H3N2, and rH1N2 (5, 9, 10, 21, 25, 29). Other subtypes that have been identified in pigs include rH1N7, rH3N1, avian (av) H4N6, avH3N3, and avH9N2 (2, 13, 16, 26, 28). The H1N1, H1N2 and H3N2 viruses found in Europe are antigenically and genetically different from those found in America (1, 3, 4, 7, 13–16, 23, 24, 31, 37–39). Pigs have receptors in their respiratory tract that will bind swine, human, and avian influenza viruses. Consequently, pigs have been called 'mixing vessels' for the development of new influenza viruses when swine, avian, and/or human influenza viruses undergo genetic reassortment in pigs (12). SIV infections are described as causing respiratory disease characterised by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, breathing difficulty and depressed appetite. Other agents that may cause respiratory disease in pigs include porcine reproductive and respiratory syndrome virus, Aujeszky's disease (pseudorabies) virus, porcine respiratory coronavirus, *Actinobacillus pleuropneumoniae* and other bacterial agents, and *Mycoplasma hyopneumoniae*. However, most of these have other signs that do not mimic swine influenza (20). *Actinobacillus pleuropneumoniae*, in the acute form of the infection, has clinical signs most similar to swine

influenza, such as dyspnoea, tachypnoea, abdominal breathing, coughing, fever, depression and anorexia. Clinical signs and nasal shedding of SIV can occur within 24 hours of infection, and shedding typically ceases by day 7–10 after infection. Two forms of the disease occur in swine, epidemic or endemic. In the epidemic form, the virus quickly moves through all phases of a swine unit with rapid recovery, provided there are not complicating factors such as secondary bacterial infections. In the endemic form, clinical signs may be less obvious and not all pigs may demonstrate traditional clinical signs of infection. Morbidity rates can reach 100% with SIV infections, while mortality rates are generally low. The primary economic impact is related to retarded weight gain resulting in an increase in the number of days to reach market weight. Transmission is through contact with SIV containing secretions such as nasal discharges and aerosols created by coughing or sneezing. Human infections with SIV can occur and a limited number of deaths have been reported (9, 10, 22, 25). Precautions should be taken to prevent human infection as described in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities. Conversely, human influenza viruses can be transmitted from animal caretakers to pigs. Similarly, influenza virus can also be transmitted from poultry to pigs as well as from pigs to poultry.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Because SIV is a potential human pathogen, all work with infectious tissues, swabs, embryonated eggs, and cell cultures should be done in a class II biological safety cabinet.

a) Culture

- **Sample processing**

Lung tissue can be processed for virus isolation in a variety of ways, for example with a mortar and pestle, stomacher, homogeniser, or mincing with a scalpel blade or scissors. Processing of the tissue is done in cell culture medium with antibiotic supplement (e.g. 10 × working strength), at a final concentration of 10–20% weight to volume. Nasal swabs should be collected in cell culture medium or phosphate buffered saline (PBS), supplemented with antibiotics and bovine serum albumin (5 mg/ml). Fetal bovine serum should not be included. Samples should ideally be shipped to a diagnostic laboratory overnight on wet ice, not frozen. Upon receipt at the laboratory, the nasal swabs are vigorously agitated by hand or on a vortex mixer. The nasal swab and lung materials are centrifuged at 1500–1900 **g** for 15–30 minutes at 4°C. The supernatant is collected and maintained at 4°C until inoculated. If supernatant is to be held for longer than 24 hours before inoculation, it should be stored at –70°C. Lung supernatant is inoculated without further dilution. Nasal swab supernatant can also be inoculated without dilution or diluted 1/3 in cell culture medium. Antibiotics are added to the cell culture medium used for processing and/or the supernatant can be filtered to reduce bacterial contamination, but this may decrease virus titre. As an alternative, the virus preparation may be treated with antibiotics such as gentamicin (100 µg/ml) or penicillin (10,000 units/ml: streptomycin (10,000 units/ml) and 2% fungizone (250 mg/ml) for 30–60 minutes at 4°C prior to inoculating the embryos or cell culture.

- **Cell culture virus isolation**

- i) Virus isolation can be conducted in cell lines and primary cells susceptible to SIV infection. Madin–Darby canine kidney (MDCK) is the preferred cell line, but primary swine kidney, swine testicle, swine lung, or swine tracheal cells can be used.
- ii) Wash confluent cell monolayers (48–72 hours post-seeding) three times with cell culture medium containing a final concentration of 1 µg/ml of TPCK¹-treated trypsin; however, the concentration will depend on the type of trypsin and the cells used (0.3–10 µg/ml may be used). The cell culture medium can be supplemented with antibiotics, but is not supplemented with fetal bovine serum.
- iii) Inoculate cell cultures with an appropriate amount of tissue suspension or swab supernatant. Note: The volume of inoculum will vary with the size of the cell culture container. In general, 100–200 µl are inoculated in each well of a 24-well culture plate, 1 ml in each Leighton tube, and 1–2 ml into a 25 cm² flask.
- iv) Incubate inoculated cell cultures for 1–2 hours at 37°C with occasional rocking. When using cell culture containers that are open to the environment, such as culture plates, incubation should be done in a humidified incubator with 5% CO₂.
- v) Remove the inoculum and wash the cell monolayer three times with the cell culture medium containing trypsin.
- vi) Add an appropriate volume of the cell culture maintenance medium to all containers and incubate at 37°C for 5–7 days with periodic examination for cytopathic effect (CPE). If CPE is not observed at the

1 TPCK: tosylphenylalanylchloromethane

end of the incubation period, the cell culture container can be frozen at -70°C , thawed, and blind passaged as described above (step iii). If CPE is observed, an aliquot of the cell culture medium can be tested for haemagglutinating viruses or by reverse transcription-polymerase chain reaction (RT-PCR) for conserved influenza virus genes such as nucleoprotein or matrix, and can be collected and used as inoculum for confirmation by the fluorescent antibody technique (see Section B.1.e below). Cover-slips (Leighton tube, 24-well cell culture plate) or chamber slides with MDCK (or other appropriate cell) monolayer can be inoculated for this purpose. The isolation procedure is as described above (step iii). In some instances, it may be necessary to make tenfold dilutions of the cell culture virus in order to have appropriate CPE on the cover-slip. Influenza subtypes can be determined by the haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests, or by RT-PCR with primers validated for sensitive and specific amplification of individual HA and NA genes.

- **Egg inoculation (30)**

- i) Use 10–11-day-old embryonated chicken eggs.
- ii) Inoculate 0.1–0.3 ml of inoculum into the allantoic cavity and amniotic sac; many laboratories only inoculate via the allantoic route with similar sensitivity. Generally, 3–4 eggs are inoculated per sample.
- iii) Incubate eggs at $35\text{--}37^{\circ}\text{C}$ for 3–4 days and candle daily. Eggs with embryos that have died within 24 hours of inoculation are discarded.
- iv) Refrigerate eggs with embryos that have died later than 24 hours after inoculation. Harvest amniotic and allantoic fluids from eggs with dead embryos and from eggs with viable embryos at the end of the incubation period. All egg materials should be considered to be potentially infectious and should be treated accordingly to prevent SIV exposure to the laboratory worker.
- v) Centrifuge fluids at 1500–1900 **g** for 10–20 minutes at 4°C . Transfer the supernatant to another tube for testing.
- vi) Fluids are evaluated for the presence of SIV with the haemagglutination (HA) test (see below).
- vii) Repass fluids negative for haemagglutinating activity (negative for SIV) in eggs or on cell lines as described above. Isolation may be improved by making tenfold dilutions of the fluid in cell culture medium. Antibiotics may be added to the cell culture fluid.

- **Haemagglutination test**

- i) Prepare a 0.5% erythrocyte suspension from male turkey or chicken blood. Certain strains agglutinate turkey rather than chicken erythrocytes to greater or lesser degrees. Therefore, it may be necessary to choose the species of erythrocytes based on the strains circulating in a given area. Washed erythrocytes and 0.5% suspensions of erythrocytes can be stored at 4°C for up to 1 week. Discard if haemolysis is observed.
- ii) Dispense 50 μl PBS in a row of 8–12 wells on a 96-well V- or U-bottom microtitre plate for each unknown virus. U-bottom plates are generally preferred over V-bottom plates. One additional row of wells should be included for a positive control.
- iii) Add 50 μl of undiluted isolate to the first well of each corresponding row.
- iv) Serially dilute the isolate with a micropipette set to deliver 50 μl . The resulting dilutions will range from 1/2 (well 1) to 1/2048 (well 11). Well 12 contains PBS only and serves as a cell control.
- v) Add 50 μl of 0.5% erythrocyte suspension to each well and agitate the plate to mix thoroughly. Note: keep erythrocytes thoroughly suspended during the dispensing process.
- vi) Cover the plate with sealing tape and incubate at room temperature until a distinct button has formed (30–60 minutes) in the control well.
- vii) Wells with complete haemagglutination (positive HA, SIV present) will have erythrocytes spread throughout the well in a 'mat' type appearance. Wells with a distinct button of erythrocytes at the bottom of the well are negative for haemagglutinating activity (negative for SIV). Incomplete HA activity is demonstrated by partial buttons characterised by fuzzy margins or 'donut-like' appearance. When interpretation between negative and incomplete inhibition is doubtful, tilt the microtitre plate to about a 45-degree angle for 20–30 seconds and look for streaming, which produces a tear-drop appearance and translucency around the cells in wells with negative hemagglutination. Wells with partial inhibition will not produce a tear drop.

b) Typing SIV isolates

- **Haemagglutination inhibition test**

- i) Dilute reference HA antigens (H1, H3, etc.) to a concentration of 8 HA units (HAU) per 50 μl (4 HAU/25 μl) in 0.01 M PBS, pH 7.
- ii) Standardise unknown influenza A viruses to contain 8 HAU in 50 μl .

- iii) Conduct a back titration (HA test) for all unknown isolates and the H subtype antigens to assure that the correct HAUs are present. The back titration is performed as described in the HA procedures except that six well dilutions are used instead of eleven.
- iv) Treat each reference serum (specific for an individual HA subtype) with RDE (receptor-destroying enzyme); add 50 µl serum to 200 µl RDE (1/10 dilution in calcium saline solution equalling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 µl 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes. Combine 200 µl treated sample and 25 µl PBS. Note: RDE treatment is recommended as it will reduce nonspecific reactions and will enhance the identification of H1N2 and H3N2 isolates.
- v) Remove natural serum agglutinins from the sera by treating diluted serum with 0.1 ml packed, washed erythrocytes per 1 ml diluted serum. Incubate for 30 minutes at room temperature with occasional mixing to keep the erythrocytes suspended. Centrifuge the treated serum at 800 **g** for 10 minutes and then retain the serum.
- vi) Dispense 25 µl of standardised antigen (unknown isolate or positive control antigen) into three wells of a 96-well V- or U-bottom microtitre plate. Add 50 µl of PBS to several wells to serve as an erythrocyte cell control. Note: 25 µl of PBS can be used in place of the 25 µl of standardised antigen.
- vii) Add 25 µl of the appropriate standardised antiserum to the first well of the H subtype being evaluated. Serially dilute the antiserum in 25 µl volumes in the antigen wells with a pipette set to deliver 25 µl. Repeat this procedure for each H subtype being evaluated. Note: If 25 µl of PBS was used in place of the 25 µl of standardised antigen in step vi, add 25 µl of standardised antigen to each well containing the standardised antiserum.
- viii) Cover plate(s) and incubate at room temperature for 10–30 minutes.
- ix) Add 50 µl 0.5% erythrocyte suspension to each well and shake/agitate the plate(s) to mix thoroughly. Keep the erythrocytes thoroughly suspended during the dispensing process.
- x) Cover the plate(s) with sealing tape and incubate at room temperature until a distinct button has formed in the positive control wells (usually 30–60 minutes). Observe the plates after about 20 minutes' incubation for evidence of haemagglutination as some isolates may begin to elute (detach from erythrocytes) in 30 minutes.
- xi) Read test results as described above for the HA test. A sample is considered positive for a specific H subtype if haemagglutination is inhibited. The test is considered valid if the positive reference antigen and its homologous antiserum demonstrate the expected HI titre and the back titration of each antigen (unknown and positive control) is 8 HAUs. If these conditions are not met, the test should be repeated.
- xii) If erythrocytes in the cell control wells do not settle into a well-defined button, check the following as possible causes: incorrect formulation of PBS, excessive evaporation from the plates, erythrocytes too old, or incorrect concentration of erythrocytes.

- **Neuraminidase inhibition test**

Subtype identification based on the NI test is beyond the scope of many laboratories. Reference laboratories should be consulted for N typing of isolates.

c) Fluorescent antibody test

- i) This technique can be used for tissue sections, cover-slips/slides, or 96-well plates of infected cell monolayers. Positive and negative controls should be included with all staining procedures.
- ii) Inoculated cells are incubated for an appropriate length of time to allow 10–25% of the cells to become productively infected with virus. Rinse the cover-slip or slide once in PBS, place in 100% acetone for 5–10 minutes and air-dry. Acetone should be used in a vented hood.
- iii) Prepare frozen tissue sections on glass slides. Fix the glass slides in acetone for 5–10 minutes and air-dry.
- iv) Apply conjugate (fluorescein-labelled swine influenza antibody) and incubate in a humid chamber at 37°C for 30 minutes. Preferably the conjugate contains Evans blue for counter staining.
- v) Rinse in PBS, pH 7.2, soak for 5–10 minutes in fresh PBS, rinse in distilled water, and air-dry.
- vi) Place cover-slips on glass slides, cell side down, with mounting fluid. Remove the rubber gasket from chamber slides and add mounting fluid followed by a glass cover-slip. Mounting fluid followed by a glass cover-slip is also placed over tissue sections on the slide. If 96-well plates are used, mounting medium and cover-slips are not required.
- vii) Observe stained slides in a darkened room with the use of an ultraviolet microscope. Cells infected with SIV are identified by the presence of bright apple-green fluorescence. It is recommended that the person examining the slides receive training in reading fluorescein-labelled slides as they can be

difficult to interpret. It is also important to use an antibody that recognises all possible viruses circulating in the area (e.g. a pan-anti-influenza A nucleoprotein antibody).

d) Immunohistochemistry (36)

- i) Slice formalin-fixed, paraffin-embedded lung in 4- μ m thick sections and place on poly-L-lysine-coated slides. Positive and negative control tissues should be included with all tests.
- ii) Heat slides at 60°C for 15 minutes, deparaffinise, and rehydrate through immersions in decreasing concentrations of ethanol and then in distilled water.
- iii) Treat samples with 3% hydrogen peroxide for 10 minutes and rinse twice in distilled water.
- iv) Digest samples with 0.05% protease for 2 minutes and rinse twice for 2 minutes in 0.1 M Tris/PBS buffer, pH 7.2, at room temperature.
- v) Apply primary mouse anti-SIV monoclonal antibody (directed against the viral nucleoprotein) to each slide and incubate at room temperature for 1 hour or overnight at 4°C. Rinse slides with Tris/PBS buffer.
- vi) Apply secondary antibody (biotinylated goat anti-mouse antibody) for 10 minutes at room temperature. Rinse with Tris/PBS buffer.
- vii) Apply tertiary antibody (peroxidase-conjugated streptavidin) for 10 minutes at room temperature. Rinse with Tris/PBS buffer.
- viii) Apply diaminobenzidine tetrahydrochloride solution for 5 minutes at room temperature. Rinse twice in distilled water.
- ix) Counterstain slides in Gill's haematoxylin for 10–30 seconds, wash in water for 2 minutes, dehydrate, clear, and add cover-slips.
- x) SIV-infected tissues are identified by the presence of brown staining in bronchiolar epithelium and pneumocytes.

e) Antigen-capture enzyme-linked immunosorbent assays

Type A antigen-capture enzyme-linked immunosorbent assays (ELISAs) are commercially available for detection of human and animal influenza viruses. These types of assays have been used for detection of SIV in lung tissue and nasal swabs (19, 33). The assays are generally available through human health care companies.

f) Polymerase chain reaction

RT-PCR tests have been developed for the diagnosis of swine influenza (8, 17). Population-wide validation data for these tests are not currently available.

2. Serological tests

The primary serological test for detection of SIV antibodies is the HI test and it is subtype specific. It should be conducted on paired sera collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent SIV infection. Additional serological tests that have been described but not commonly used are the virus neutralisation, agar gel immunodiffusion test, and indirect fluorescent antibody test. ELISA technology for detection of SIV antibodies has been described in the literature and commercial kits have been marketed. The validation of the ELISA kits is ongoing.

• **Haemagglutination inhibition test**

- i) Dilute reference HA antigens (H1, H3, etc.) to a concentration of 4–8 HAU/25 μ l in 0.01 M PBS, pH 7.2.
- ii) *H1N1 test*: Heat inactivated sera for 30 minutes at 56°C. Dilute 1/10 in PBS. Add 0.1 ml packed, washed erythrocytes to 1 ml of heat-inactivated, diluted serum and mix. Incubate at room temperature for 30 minutes with periodic shaking every 10–15 minutes. Centrifuge at 800 **g** for 10 minutes at 4°C. Note: Sera can be treated with RDE and erythrocytes as described below in step iii as an alternative to heat inactivation and treating with packed erythrocytes.
- iii) *H1N2 and H3N2 test*: Add 50 μ l serum to 200 μ l RDE (1/10 dilution in calcium saline solution equalling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 μ l 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes. Combine 200 μ l treated sample and 25 μ l PBS. Add 50 μ l of 50% erythrocytes. Shake and incubate for 30 minutes at room temperature or overnight at 4°C. Centrifuge at 800 **g** for 10 minutes at 4°C.

- iv) Dispense 50 µl treated serum into two wells of a V- or U-bottom 96-well plate. Dispense 25 µl of treated serum into two wells to be used as a serum control. Positive and negative control sera are treated in the same way as the unknown sera.
- v) Dispense 25 µl PBS in the serum control wells and all empty wells except two wells identified as the cell control wells. Add 50 µl PBS in the cell control wells.
- vi) Make serial twofold dilutions of the serum in 25 µl volumes in the plate and then add 25 µl of appropriate antigen to all test wells except the serum control wells and the cell control wells.
- vii) Incubate covered plates at room temperature for 30–60 minutes.
- viii) Add 50 µl of 0.5% erythrocyte suspension to each well, shake, and incubate at room temperature for 20–30 minutes until a distinct button forms at the bottom of the cell control wells. Keep erythrocytes thoroughly suspended during the dispensing process.
- ix) Conduct a HA test using the HI test antigens prior to and simultaneously to conducting the HI test to verify that antigen concentrations are appropriate.
- x) For the test to be valid, there should be no haemagglutination in the serum control well, no inhibition of haemagglutination with the negative serum, the positive serum should have its anticipated HI titre and the HA back titration should indicate 4–8 HAU per 25 µl.

b) Enzyme-linked immunosorbent assay (18)

ELISA technology for detection of SIV antibodies has been described in the literature and ELISAs are available as commercially produced kits.

C. REQUIREMENT FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

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 and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Identity of the seed should be well documented, including the source and passage history of the organism. All defining characteristics such as haemagglutinin and neuraminidase subtype should be established. Haemagglutination inhibition and neuraminidase inhibition by subtype-specific antisera or RT-PCR and sequencing can be used to establish the H and N subtypes. Also, aliquots of the master seed virus (MSV) can be neutralised with specific antiserum, e.g. antiserum produced against H1N1 or H3N2 SIV, then inoculated into the allantoic sac of 10-day old embryonated chicken eggs or on to susceptible cell lines such as the MDCK cell line. Allantoic fluid or cell culture supernatant is harvested 72–96 hours post-inoculation and tested for HA activity. Identity is demonstrated by the lack of HA activity in the neutralised seed, and the presence of HA activity in the non-neutralised seed. Significant antigenic differences present in a given strain that set it apart from other members of its subtype, and that purportedly have a beneficial impact on its use as a vaccine, should be confirmed.

b) Method of culture

SIV seed can be grown in eggs or in cell culture. Selection of a culture method is dependent on the degree of virus adaptation, growth in medium, rate of mutation, and viral yield in the specific culture system. SIV vaccine products should be limited to five passages from the MSV to avoid genetic/antigenic variation.

c) Validation of culture

The purity of the seed and cells to be used for vaccine production must be demonstrated. The MSV should be free from adventitious agents, bacteria, or *Mycoplasma*, using tests known to be sensitive for detection of these microorganisms. The test aliquot should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against SIV and the virus/antibody mixture is cultured on several types of cell line monolayers. Cultures are subpassaged at 7-day intervals for a total of at least 14 days, then tested for cytopathogenic and haemadsorbing agents. Cells are also examined for adventitious viruses that may have infected the cells or seed during previous passages. Potential contaminants include bovine viral diarrhoea virus, reovirus, rabies virus, Aujeszky's disease (pseudorabies) virus, transmissible gastroenteritis virus, porcine respiratory coronavirus, porcine parvovirus, porcine adenovirus, haemagglutinating encephalomyelitis virus, porcine rotavirus, porcine circovirus, and porcine reproductive and respiratory syndrome virus. Cell lines on which the seed is tested

include: an African green monkey kidney (Vero) cell line (rabies and reoviruses), a porcine cell line, a cell line of the species of cells used to propagate the seed, if not of porcine origin, and cell lines for any other species through which the seed has been passaged. Additionally, a cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended. Bovine viral diarrhoea virus is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems.

Factors that may contribute to instability during production, such as replication on an unusual cell line, should be investigated. If production is approved for five passages from the master seed, then sequencing of the genes for H and N at the maximum passage may be warranted to confirm the stability of the viral seed.

d) Validation as a vaccine

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.

Strains used in vaccine production should be antigenically relevant to SIV strains circulating in the field (6, 11, 34, 35). Haemagglutination inhibition and neutralisation tests demonstrating cross-reactivity between antisera from animals vaccinated with the candidate vaccine strain and current field isolates can be used for the selection. A vaccination/challenge study in swine, using homologous and heterologous challenge strains, will indicate the degree of protection afforded by the vaccine. Swine used in vaccination/challenge studies should be free of antibodies against SIV at the start of the experiments. Vaccination/challenge studies should be conducted using virus produced by the intended production method, at the maximum viral passage permitted, and using swine of the minimum recommended age listed on the label. Initially, lots are formulated to contain varying amounts of viral antigen. The test lot containing the least amount of antigen that demonstrates protection becomes the standard against which future production lots are measured. The most valuable criterion for blind trial evaluations of treatment groups is a statistically significant reduction of virus (titres and duration of shedding) in the respiratory tract of vaccinated pigs. Differences in clinical observations and lung lesions are also among the criteria used in evaluation of a successful trial. If *in-vivo* or *in-vitro* test methods are to be used to determine the potency of each production lot of vaccine, those assays should be conducted concurrent with the minimum antigen studies in order to establish the release criteria. Combination vaccines containing more than one strain of SIV are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

2. Method of manufacture

Once the vaccine is shown to be efficacious, and the proposed conditions for production are acceptable to regulatory authorities, approval may be granted to manufacture vaccine. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. When the virus has reached its maximum titre, as determined by HA, CPE, fluorescent antibody assay, or other approved technique, the virus is clarified, filtered, and inactivated. Several inactivating agents have been used successfully, including formalin or binary ethylenimine. An inactivation kinetics study should be conducted using the approved inactivating agent on a viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, then inoculated on to a susceptible cell line or into the allantoic sac of embryonated eggs, should indicate a linear and complete loss of titre by the end of the inactivation process. This is represented as less than one infectious particle per 10^4 litres of fluids following inactivation. Typically, adjuvant is added to enhance the immune response.

3. In-process control

Cell cultures should be checked macroscopically for abnormalities or signs of contamination and discarded if unsatisfactory. A lot is ready to harvest when viral CPE has reached 80–90%. Virus concentration can be assessed using antigenic mass or infectivity assays.

4. Batch control

a) Sterility

During production, tests for bacteria, *Mycoplasma*, and fungal contamination should be conducted on both inactivated and live vaccine harvest lots and confirmed on the completed product (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Safety

Mice or guinea-pigs can be used to evaluate the safety of an inactivated product. In one model, eight mice are inoculated intraperitoneally or subcutaneously with 0.5 ml and observed for 7 days. The mouse safety test may not be applicable when certain adjuvants are used, especially saponin-based products. In the other

model, two guinea-pigs are each injected with a 2-ml dose either intramuscularly or subcutaneously and observed for 7 days. Adverse clinical signs or mortality attributable to the vaccine is indicative of a lot that is not acceptable for use. The completeness of viral inactivation in a killed product can be determined by multiple passes in cell culture or eggs of the post-inactivation, pre-adjuvant production fluids, followed by HA testing for the presence of virus.

Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended. If the vaccine is to be used in swine destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21–60 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

c) Potency

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation and prior to further processing. Relative potency ELISA, HA, and HI are among the assays that can be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

d) Duration of immunity

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label. Once a suitable potency assay has been identified, should antigenic drift require replacement of strains within the vaccine, strains of the same subtype can be evaluated in either the host animal or a correlated laboratory animal model. However, circulating strains may show significant antigenic differences from the vaccine strain, but the vaccine strain may still provide protection (34). Also, the vaccine may not protect against a new strain that appears to be antigenically similar to the vaccine. Other factors that play a role include the adjuvant and the antigenic dose. Consequently, it would appear that the efficacy of a vaccine will always have to be evaluated in swine.

e) Stability

Vaccines should be stored with minimal exposure to light at $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$, or as approved by the designated regulatory authorities. The shelf life should be determined by use of the approved potency test (Section C.5.b) over the proposed period of viability.

f) Preservatives

The most common preservative is thimerosal, at a final concentration not to exceed 0.01% (1/10,000). Addition of thimerosal or other mercury-based compounds should be avoided if possible. Antibiotics may be used as preservatives in SIV vaccines but are limited as to kinds and amounts. Also restricted are residual antibiotics from cell culture media that may be present in the final product. For example, the total amount of preservative and residual gentamicin is not to exceed 30 mcg per ml of vaccine.

g) Precautions

Inactivated SIV vaccines present no special danger to the user, although accidental inoculation may result in an adverse reaction due to the adjuvant and secondary components of the vaccine. Generally, healthy pigs of weaning age or older and pregnant sows at any stage of gestation may be safely vaccinated with inactivated SIV vaccines.

5. Tests on the final product

a) Safety

Final container samples of completed product from inactivated vaccines should be tested in young mice as described above in Section C.4.b.

b) Potency

The potency assay established at the time of the minimum antigen protection study should be used to evaluate new lots for release. The assay needs to be specific and reproducible. It must reliably detect vaccines that are not sufficiently potent. If laboratory animal serology is used instead of swine serology, it should first be demonstrated that vaccination of the laboratory animal induces a specific, sensitive, dose-dependent response as measured in the potency assay and is correlated to protection in swine (Section C.1.d).

REFERENCES

1. BROWN I.H., HARRIS P.A., MCCAULEY J.M. & ALEXANDER D.J. (1998). Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of the H1N2 virus of novel genotype. *J. Gen. Virol.*, **79**, 2947–2955.
2. BROWN I.H., HILL M.L., HARRIS P.A., ALEXANDER D.J. & MCCAULEY J.W. (1997). Genetic characterization of an influenza A virus of unusual subtype (H1N7) isolated from pigs in England. *Arch. Virol.*, **142**, 1045–1050.
3. CASTRUCCI M.R., DONATELLI I., SIDOLI L., BARIGAZZI G., KAWAOKA Y. & WEBSTER R.G. (1993). Genetic reassortment between avian and human influenza A viruses in Italian pigs. *Virology*, **193**, 503–506.
4. CHOI Y.K., GOYAL S.M., FARNHAM M.W. & JOO H.S. (2002). Phylogenetic analysis of H1N2 isolates of influenza A virus from pigs in the United States. *Virus Res.*, **87**, 173–179.
5. CHOI Y.K., LEE J.H., ERICKSON G., GOYAL S.M., JOO H.S., WEBSTER R.G. & WEBBY R. J. (2004). H3N2 influenza virus transmission from swine to turkeys, United states. *Emerg. Infect. Dis.*, **10**, 2156–2160.
6. DE JONG J.C., VAN NIEUWSTADT A.P., KIMMAN T.G., LOEFFEN W.L., BESTEBROER T.M., BIJLSMA K., VERWEIJ C., OSTERHAUS A.D. & CLASS E.C. (1999). Antigenic drift in swine influenza H3 haemagglutinins with implications for vaccination policy. *Vaccine*, **17**, 1321–1328.
7. DONE S.H. & BROWN I.H. (1997). Swine influenza in the United Kingdom, past and present. *Large Anim. Pract.*, **2**, 20–28.
8. FOUCHIER R.A., BESTEBROER T.M., HERFST S., VAN DER KEMP L., RIMMELZWAAN G.F. & OSTERHAUS A.D. (2000). Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. *J. Clin. Microbiol.*, **38**, 4096–4101.
9. GREGORY V., LIM W., CAMERON K., BENNETT M., MAROZIN S., KLIMOV A., HALL H., COX N., HAY A. & LIN Y. P. (2001). Infection of a child in Hong-Kong by an influenza A H3N2 virus closely related to viruses circulating in European pigs. *J. Gen. Virol.*, **82**, 1397–1406.
10. GREGORY V., BENNETT M., THOMAS Y., KAISER L., WUNDERLI W., MATTER H., HAY A. & LIN Y.P. (2003). Human infection by a swine influenza a (H1N1) virus in Switzerland. *Arch. Virol.*, **148**, 793–802.
11. HEINEN P.P., VAN NIEUWSTADT A.P., DE BOER-LUIJTZE E.A. & BIANCHI A.T.J. (2001). Analysis of the quality of protection induced by a porcine influenza A vaccine to challenge with an H3N2 virus. *Vet. Immunol. Immunopathol.*, **82**, 39–56.
12. ITO T., COUCEIRO J.N.S.S., KELM S., BAUM L.G., KRAUSS S., CASTRUCCI M.R., DONATELLI I., KIDA H., PAULSON J.C., WEBSTER R.G. & KAWAOKA Y. (1998). Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J. Virol.*, **72**, 7367–7373.
13. KARASIN A.I., BROWN I.H., CARMAN S. & OLSEN C.W. (2000). Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. *J. Virol.*, **74**, 9322–9327.
14. KARASIN A.I., LANDGRAF J., SWENSON S., ERICKSON G., GOYAL S., WOODRUFF M., SCHERBA G., ANDERSON G. & OLSEN C.W. (2002). Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. *J. Clin. Microbiol.*, **40**, 1073–1079.
15. KARASIN A.I., SCHUTTEN M.M., COOPER L.A., SMITH C.B., SUBBARAO K., ANDERSON G.A., CARMAN S. & OLSEN C.W. (2000). Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977–1999: evidence for wholly human and reassortant virus genotypes. *Virus Res.*, **68**, 71–85.
16. KARASIN A.I., WEST K., CARMAN S. & OLSEN C.W. (2004) Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. *J. Clin. Microbiol.*, **42**, 4349–4354.
17. LANDOLT G., KARASIN A.I. HOFER C., MAHANEY J., SVAREN J. & OLSEN C.W. (2004). Use of real-time reverse transcriptase polymerase chain reaction assay and cell culture methods for detection of swine influenza A viruses. *Am. J. Vet. Res.*, **66**, 119–124.
18. LEE B.W., BEY R.F., BAARSCH M.J. & EMERY D.A. (1993). Subtype specific ELISA for the detection of antibodies against influenza A H1N1 and H3N2 in swine. *J. Virol. Methods*, **45**, 121–136.

19. LEE B.W., BEY R.F., BAARSCH M.J. & SIMONSON R.R. (1993). ELISA method for detection of influenza A infection in swine. *J. Vet. Diagn. Invest.*, **5**, 510–515.
20. LOEFFEN W.L., KAMP E.M., STOCKHOFE-ZURWIEDEN N., VAN NIEUWSTADT A.P., BONGERS J.H., HUNNEMAN W.A., ELBERS A.R., BAARS J., NELL T. & VAN ZIJDERVELD F.G. (1999). Survey of infectious agents involved in acute respiratory disease in finishing pigs. *Vet. Rec.*, **145**, 123–129.
21. MAROZIN S., GREGORY V., CAMERON K., VALETTE M., AYMARD M., FONI E., BARIGAZZI G., LIN Y. & HAY A. (2002) Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. *J. Gen. Virol.*, **83**, 735–745.
22. MYERS K.P., OLSEN C.W., & GRAY G.C. (2007). Cases of swine influenza in humans: a review of the literature. *Clin. Infect. Dis.*, **44**, 1084-1088.
23. NOBLE S., MCGREGOR M.S., WENTWORTH D.E. & HINSHAW V.S. (1993). Antigenic and genetic conservation of the haemagglutinin in H1N1 swine influenza viruses. *J. Gen. Virol.*, **74**, 8243–8251.
24. OLSEN C.W. (2002). Emergence of novel strains of swine influenza virus in North America, *In: Trends in Emerging Viral Infections of Swine*, Morilla A., Yoon K.J. & Zimmerman J.J., eds. Iowa State University Press, Iowa, USA, 37–43.
25. OLSEN C.W., BRAMMER L., EASTERDAY B.C., ARDEN N., BELAY E., BAKER I. & COX N. J. (2002). Serologic evidence of H1 swine influenza virus infection in swine farm residents and employees. *Emerg. Infect. Dis.*, **8**, 814–815.
26. OLSEN C.W., BROWN I., EASTERDAY B.C. & VAN REETH K. (2005). Swine Influenza. *In: Diseases of Swine*, Straw B., D’Allaire S., Zimmerman J. & Taylor D., eds. Iowa State University Press, Iowa, USA, 469–482.
27. OLSEN C.W., KARASIN A. & ERICKSON G. (2003). Characterization of a swine-like reassortant H1N2 influenza virus isolated from a wild duck in the United States. *Virus Res.*, **93**, 115–121.
28. PEIRIS J. S. M., GUAN Y., MARKWELL D., GHOSE P., WEBSTER R. G. & SHORTRIDGE K. F. (2001) Cocirculation of avian H9N2 and contemporary “human” H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment. *J. Virol.*, **75**, 9679–9686.
29. SCHRADER C. & SÜSS J. (2004) Molecular epidemiology of porcine H3N2 influenza viruses isolated in Germany between 1982 and 2001. *Intervirology*, **47**, 72–77.
30. SENNE D.A. (1998). Virus propagation in embryonating eggs. *In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens*, Fourth Edition, Swayne D.E., Glisson J.R., Jackwood M.W., Pearson J.E. & Reed W.M., eds. American Association of Avian Pathologists, Kennett Square, Pennsylvania, USA, 235–240.
31. SHEERAR M.G., EASTERDAY B.C. & HINSHAW V.S. (1989). Antigenic conservation of H1N1 swine influenza viruses. *J. Gen. Virol.*, **70**, 3297–3303.
32. SHOPE R.E. (1931). Swine influenza. III. Filtration experiments and etiology. *J. Exp. Med.*, **54**, 373–380.
33. SWENSON S.L., VINCENT L.L., LUTE B.M., JANKE B.H., LECHTENBERG K.E., LANDGRAF J.G., SCHMITT B.J., KINKER D.R. & McMILLEN J.K. (2001). A comparison of diagnostic assays for the detection of type A swine influenza virus from nasal swabs and lungs. *J. Vet. Diagn. Invest.*, **13**, 36–42.
34. VAN REETH K., LABARQUE G., DE CLERCQ S. & PENSART M. (2001). Efficacy of vaccination of pigs with different H1N1 swine influenza viruses using a recent challenge strain and different parameters of protection. *Vaccine*, **19**, 4479–4486.
35. VAN REETH K., VAN GUCHT S. & PENSART M. (2003). Investigations of the efficacy of European H1N1- and H3N2-based swine influenza vaccines against the novel H1N2 subtype. *Vet. Rec.*, **153**, 9–13.
36. VINCENT L.L., JANKE B.H., PAUL P.S. & HALBUR P.G. (1997). A monoclonal-antibody-based immunohistochemical method for the detection of swine influenza virus in formalin-fixed, paraffin-embedded tissues. *J. Vet. Diagn. Invest.*, **9**, 191–195.
37. WEBBY R.J., SWENSON S.L., KRAUSS S.L., GERRISH P.J., GOYAL S.M. & WEBSTER R.G. (2000). Evolution of swine H3N2 influenza viruses in the United States. *J. Virol.*, **74**, 8243–8251.

38. WEBBY R.J., ROSSOW K., ERICKSON G., SIMS Y. & WEBSTER R. (2004). Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. *Virus Res.*, **103**, 67–73.
39. ZHOU N.N., SENNE D.A., LANDGRAF J.S., SWENSON S.L., ERICKSON G., ROSSOW K., LIU L., YOON K.J., KRAUSS S. & WEBSTER R.G. (1999). Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. *J. Virol.*, **73**, 8851–8856.

*
* *