CHAPTER 2.8.7.

INFLUENZA A VIRUS OF SWINE

SUMMARY

Influenza A viruses of swine (IAV-S) cause a highly contagious viral infection of pigs. IAV-S infections cause respiratory disease characterised by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, breathing difficulty, and depressed appetite. In some instances, IAV-S 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 IAV-S infections, while mortality rates are generally low. Secondary bacterial infections can exacerbate the clinical signs following infection with IAV-S. Transmission is through contact with IAV-S-containing secretions such as nasal discharges and aerosols created by coughing or sneezing.

Identification of the agent: *Samples for virus identification should be collected within 24–72 hours after development of clinical signs. The animal of choice is an untreated, acutely ill pig with compatible clinical signs. Virus can readily be detected in lung tissue and nasal swabs. Oral fluids collected from cotton ropes hung in a pig pen may also be useful as a group or population specimen. Virus isolation can be conducted in embryonated fowl eggs and on continuous cell lines or primary cell cultures. Viruses can be subtyped using the haemagglutination inhibition (HI) and the neuraminidase inhibition tests on virus isolates, or by reverse transcription-polymerase chain reaction assays direct on clinical material or on isolates. Immunohistochemistry can be conducted on formalin-fixed tissue. Enzyme-linked immunosorbent assays (ELISA) are commercially available for detection of type A influenza viruses.*

Serological tests: *Historically, the primary serological test for detection of IAV-S antibodies is the HI test conducted on paired sera. The HI test is subtype specific. The sera are ideally collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent IAV-S infection. Additional serological tests that have been described are the agar gel immunodiffusion test, indirect fluorescent antibody test, virus neutralisation, and ELISA. Due to the increasing amount of antigenic diversity in influenza A viruses of swine and the need to use multiple H types in HI assays, there is a general trend towards use of commercially available ELISAs that are not subtype specific.*

Requirements for vaccines: *Inactivated, adjuvanted IAV-S vaccines are commercially available. Vaccines may be in the form of a single IAV-S subtype or may contain multiple IAV-S subtypes. Vaccines should reflect the current antigenic profile of field viruses, containing subtypes and strains that are changed as needed to assure protection.*

A. INTRODUCTION

Influenza A virus of swine (IAV-S) is a highly contagious viral infection of pigs that can have significant economic impact on an affected herd. IAV-S is an enveloped virus with a segmented RNA genome. It belongs to the *Influenzavirus A* genus of the family *Orthomyxoviridae*. The type A viruses are further subdivided based on their haemagglutinin and neuraminidase proteins. Subtypes of IAV-S that are most frequently identified in pigs include classical and avian H1N1, human (hu) H1N1 and H1N2, reassortant (r) H3N2, and rH1N2. Other subtypes that have been infrequently identified in pigs include rH1N7, rH3N1, H2N3, avian (av) H4N6, avH3N3, and avH9N2. The H1N1, H1N2 and H3N2 viruses found in Europe are antigenically and genetically different from those found in America (Brown, 2013; Karasin *et al.*, 2000; 2002; Olsen, 2002; Vincent *et al.*, 2009; Webby *et al.*, 2004; Zhou *et al.*, 1999). Pigs have receptors in their respiratory tract that can bind influenza A viruses of swine, humans, and avian species. Consequently, pigs have been called 'mixing vessels' for the development of new influenza viruses when influenza A viruses of swine, avian, and/or humans undergo genetic reassortment in pigs. IAV-S 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 pleuro-pneumoniae, Mycoplasma hyopneumoniae* and other bacterial agents*.* However, many of these pathogens have other signs that do not mimic IAV-S infections. *Actinobacillus pleuropneumoniae,* in the acute form of the infection, has clinical signs most similar to IAV-S infections, such as dyspnoea, tachypnoea, abdominal breathing, coughing, fever, depression and anorexia. Clinical signs and nasal shedding of influenza A virus can occur within 24 hours of infection, and shedding typically ceases by day 7–10 after infection. Two forms of 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 typical clinical signs of infection. Morbidity rates can reach 100% with IAV-S 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 IAV-S containing secretions such as nasal discharges and aerosols created by coughing or sneezing. Human infections with IAV-S can occur and a limited number of deaths have been reported (Lindstrom *et al.,* 2012; Myers *et al.*, 2007). Precautions should be taken to prevent human infection as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Conversely, influenza A viruses can occasionally be transmitted from people to pigs. Similarly, influenza viruses can also be transmitted occasionally from poultry to pigs as well as from pigs to poultry, especially domestic turkeys. In the spring of 2009 a newly identified H1N1 virus (H1N1pdm09) was detected in people in the western hemisphere. This novel virus was composed entirely of genes from IAV-S, but with a complicated evolutionary history. The matrix and neuraminidase genes were from European H1N1 IAV-S of avian lineage and the remaining genes were from North American IAV-S of swine, avian, and human lineage. The H1N1pdm09 spread rapidly throughout the world through human-to-human transmission. In addition to continued independent circulation in humans, swine cases in both Northern and Southern Hemispheres occurred simultaneously and the virus became endemic in many swine populations worldwide. The H1N1pdm09 subsequently reassorted with other IAV-S and contributed to newly identified genomic constellations of viruses around the globe.

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for diagnosis of IAV-S and their purpose*

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	+++	+++	+++	+++	+++	–
Conventional PCR	–	–	–	++	–	–
Detection of immune response						
HI	+	+	+	++	++	+++
ELISA	+++	+++	+++	+	+++	+

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; n/a = not applicable.
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; HI = haemagglutination inhibition; ELISA = enzyme-linked immunosorbent assay; Note that antigen ELISA assays are designed for use in clinically ill animals. Their reliability in clinically healthy animals is questionable.

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1   A combination of agent identification methods applied on the same clinical sample may be needed in some situations.

1. Identification of the agent

Because IAV-S is a potential human pathogen, all work with potentially infectious diagnostic specimens, embryonated eggs, and cell cultures should be done in a class II biological safety cabinet. Additional safety precautions (personal protective equipment) should be used when working with infected pigs such as respirators and eye protection.

1.1. Culture

1.1.1. Sample processing

Lungs can be processed for virus isolation in a variety of ways, for example by lavage with sterile media listed below, or with tissue maceration by 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. Oral fluids may require adjustments to sample processing method used for nasal swabs due to the viscous nature of the specimen and increased propensity for bacterial contamination. Samples should ideally be shipped to a diagnostic laboratory overnight on wet ice, not frozen (see http://offlu.net for guidance on sample collection and sample shipment). 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 or colder. Lung supernatant is inoculated without further dilution. Swab and oral fluid 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. For filtration, low protein adsorption membrane, such as PVDF membrane, is recommended to minimise virus loss. 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.

1.1.2. Cell culture virus isolation

i)   Virus isolation can be conducted in cell lines and primary cells susceptible to influenza A virus infection. Madin–Darby canine kidney (MDCK) cells are broadly permissive for various subtypes and strains of IAV-S and are therefore the preferred cell line, but primary swine kidney, swine testicle, swine lung, or swine tracheal cells can also 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 TPCK[2]-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 lavage fluid, tissue suspension, oral fluids, 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 0.5–2 ml into a 25 cm^2 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% CO_2.

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 3–7 days with periodic examination for cytopathic effect (CPE). If CPE is not observed at the end of the incubation period, the cell culture container can be frozen at –70°C or colder, 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

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2   TPCK: tosylphenylalanylchloromethane

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.1.5 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 (Chiapponi *et al.,* 2012; Nagarajan *et al.,* 2010;). However, validation using endemically circulating strains in the region should be done to ensure fitness for purpose of tests since endemic strains of IAV-S may vary genetically between regions.

1.1.3. Egg inoculation (Senne, 1998)

i) Use 9- to 11-day-old embryonated fowl eggs (Senne, 1998).

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 35–37°C for up to 5 days and candle daily. Eggs with embryos that die within 24 hours of inoculation are discarded (assumed to be trauma-induced deaths associated with the inoculation process).

iv) Refrigerate eggs with embryos that died more 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 IAV-S 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 IAV-S with the haemagglutination (HA) test (see below).

vii) Repass (up to 1–2 passages) fluids negative for haemagglutinating activity (negative for IAV-S) in eggs or on cell lines as described above. Isolation may be improved by making tenfold dilutions of the fluid in cell culture medium.

1.1.4. Haemagglutination test

i) Prepare a 0.5% erythrocyte suspension from male turkey or chicken blood. Dispense whole blood into a tube and add PBS. For example, 10–20 ml whole blood in a 50 ml centrifuge tube to which PBS is added to fill the tube. Gently invert the tube several times to wash the erythrocytes. Centrifuge at 800 *g* for 10 minutes in a refrigerated centrifuge. Aspirate PBS and buffy coat (white blood cell layer) from the tube. Refill the tube with fresh PBS and resuspend erythrocytes thoroughly. Repeat the washing and centrifugation cycle two additional times. Once washing is complete, add sufficient erythrocytes to PBS to make a 0.5% solution. Certain virus 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. 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 (24°C) or 4°C until a distinct button has formed (30–60 minutes) in the negative control well.

vii)   Wells with complete haemagglutination (positive HA, IAV-S 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 IAV-S). 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.

1.2. Typing influenza A viruses of swine (IAV-S) isolates

1.2.1. 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 7.2–7.4. Reference antigens should represent what is actively circulating in the region where the pigs are located. For guidance, the OIE Reference Laboratory in the region should be consulted regarding reference antigens.

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 ensure 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, and representative of actively circulating viruses in the region) 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 reference serum 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 reference serum.

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 (24°C) or 4°C 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 4 or 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.

1.2.2. Neuraminidase inhibition test

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

1.3. Fluorescent antibody test

1.3.1. Test procedure

i) This technique can be used for tissue sections, cover-slips/slides, or 96-well plates of infected cell monolayers (Vincent *et al.*, 1997). Positive and negative controls should be included with all staining procedures.

ii) Note this technique is highly dependent on use of reference reagents representative of circulating viruses in the region and on skilled readers who can differentiate between positive results and background staining (specificity). This method of virus detection is of lower sensitivity compared with other available assays such as PCR.

iii) 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.

iv) Prepare frozen tissue sections on glass slides. Fix the glass slides in acetone for 5–10 minutes and air-dry.

v) Apply conjugate (fluorescein-labelled IAV-S antibody) and incubate in a humid chamber at 37°C for 30 minutes. Preferably the conjugate contains Evans blue for counter staining.

vi) Rinse in PBS, pH 7.2, soak for 5–10 minutes in fresh PBS, rinse in distilled water, and air-dry.

vii) 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.

viii) Observe stained slides in a darkened room with the use of an ultraviolet microscope. Cells infected IAV-S 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. Known positive and negative slides should be included when testing unknowns to verify the test procedure worked and to use as a basis for differentiating between positive (IAV-S) staining and negative (background) staining. 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).

1.4. Immunohistochemistry (Vincent *et al.*, 1997)

1.4.1. Test procedure

i) Slice formalin-fixed, paraffin-embedded lung in 4-µm thick sections and place on poly-L-lysine-coated slides (alternatively, commercially available charged slides can be used and are deemed superior to lysine-coated slides by some). 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- IAV-S 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) IAV-S -infected tissues are identified by the presence of brown staining in bronchiolar epithelium and pneumocytes.

1.5. Antigen-capture enzyme-linked immunosorbent assays

Type A antigen-capture enzyme-linked immunosorbent assays (ELISAs) and membrane immunoassays are commercially available for detection of human and animal influenza viruses. These types of assays have been used for detection of IAV-S in lung tissue and nasal swabs (Swenson *et al.*, 2001). The assays are generally available through human health and animal health care companies. These assays tend to be of lower sensitivity compared with other assays such as PCR.

1.6. Polymerase chain reaction

The highly conserved matrix protein and the nucleoprotein are the best targets for screening for infection with IAV-S by RT-PCR. Following the identification of the novel (pandemic) H1N1 in 2009, molecular assays based on an avian influenza matrix real-time PCR (Spackman *et al.*, 2002) were adapted for use in swine (Brookes *et al.,* 2009; Slomka *et al.,* 2010). Modifications to the assay vary by country and a swine influenza reference laboratory may be consulted for the most suitable matrix PCR assay.

The IAV-S real-time RT-PCR procedure described in this chapter targets the matrix (M) gene of Influenza A viruses. The matrix primer/probe set is a quasi-multiplex real-time RT-PCR that uses a single forward primer, probe and two reverse primers. The two reverse primers can generically detect the Eurasian, North American and pandemic 2009 H1N1 matrix lineages.

The real-time RT-PCR uses a one-step procedure. Specific primers are designed to amplify the target region (see Table 2). Non-extendable fluorogenic hydrolysis probes measure the target PCR product formation during each cycle of the PCR reaction. The probes are labelled at the 5' end with a reporter dye, and non-fluorescing quencher at the 3' end. Once the probe hybridises to the target sequence, the 5' nuclease activity of Taq polymerase will hydrolyse the probe, and separate the quencher from the reporter dye. This results in the fluorescence of the separated reporter dye, which is detected spectrophotometrically and recorded. The amount of fluorescence recorded and the cycle number of detection is proportional to the amount of target template in the samples.

For this procedure, it is critical to have separate preparation areas and equipment for nucleic acid extraction, RNA transfer, and master mix preparation. A "clean" area is needed to prepare reagents used for PCR that is free of amplified c-DNA or sample RNA.

Table 2. IAV-S matrix hydrolysis probe and primer sequences

Specificity	Description	Sequence
Matrix (any influenza A virus)	M+25* 5' Primer	5'-AgA TgA gTC TTC TAA CCg Agg TCg-3'
	M+64* Probe	5'-FAM-TCA ggC CCC CTC AAA gCC gA-BHQ-1 -3'
	M-124* 3' Primer	5'-TgC AAA AAC ATC TTC AAg TCT CTg-3'
	M-124* SIV 3' Primer**	5'- TgC AAA gAC ACT TTC CAg TCT CTg -3'

*Refers to the nucleotide position where the 5' end of the probe or primer anneals to the genome
**Primer detects the 2009 H1N1 pandemic matrix

i) Extract nucleic acid from sample. A positive and negative extraction control (PEC and NEC, respectively) will need to be used to confirm that the extraction was successful.

ii) Prepare RT-PCR master mix in a "clean" PCR room (Table 2).

iii) Aliquot 17 µl of reaction mix to each well in a 96-well plate. Transfer 8 µl of RNA template to each reaction in a designated RNA transfer room. When using a 96-well plate, use a support base to protect the bottom of the plate from scratches, finger prints, or picking up particles that could interfere with the optical system and alter the background fluorescence.

a) The following controls will need to be included in the PCR run to verify that the PCR and RNA extraction were successful: positive extraction control (PEC), negative extraction control (NEC), positive amplification (template) control (PAC), and negative amplification (template) control (NAC). PACs are diluted by each diagnostic lab, and must have a C_t value in the range of 21–29 for the run to be valid.

iv) Place samples in thermocycler and run at appropriate parameters.

v) Analyse results. The PCR run will be valid if:

a) The PAC C_t value is 21–29

b) The PEC is positive

c) Both NEC and NAC are negative

d) All samples and controls that are positive have "sigmoidal curve"

e) If the above conditions are not met, the test will need to be repeated.

Table 2. Example Real-time RT-PCR Master Mix for a one step kit

Component	Final Concentration	Volume per reaction (µl)
H_2O	–	0.83
2× RT-PCR buffer	1×	12.5
M+25 5' primer (20 µM)	200 nM	0.25
M-124 3' primer (20 µM)	200 nM	0.25
M-124 SIV 3' primer (20 µM)	200 nM	0.25
25× RT-PCR enzyme mix	1×	1
M+64 probe (6 µM)	60 nM	0.25
Detection enhancer (15×)	1×	1.67
Template	–	8
Total Reaction Volume	**–**	**25**

Table 3. Example thermocycler parameters

Stage	Cycles	Step	Time	Temperature
1	1		10 minutes	45°C
2	1		10 minutes	95°C
3	45	denaturation	1 second	94°C
		annealing*	30 seconds	60°C
		extension	15 seconds	72°C

*Collection of fluorescence

Viral isolates can be subtyped using conventional methods or by real-time PCR assays that can differentiate the genetically distinct H1 viruses from other known strains (Chiapponi *et al.,* 2012). Increasingly, differential real-time PCRs are being used in many regions. Matrix PCR diagnostic specimens can also be subtyped through use of subtyping PCRs. Samples with high matrix CT's may not be detectable by subtyping PCRs and it may be necessary to attempt virus isolation prior to identifying the subtype. Screening and subtyping PCR reagents are commercially available; however, laboratories need to ensure they will detect currently circulating influenza viruses in their area. In many instances it is necessary to conduct partial or complete gene sequencing of one or more of the IAV-S genes (i.e. neuraminidase, haemagglutinin) to ascertain the subtype of detected virus. Furthermore, virus genotyping based on gene sequencing several or all gene segments is increasingly being used to determine and monitor virus diversity. Tests should be validated for the region in which they are to be applied given the worldwide variability in IAV-S.

2.   Serological tests

The primary serological test for detection of IAV-S antibodies is the HI test and it is subtype specific. Reference antigens should reflect what is circulating in the region and as broadly cross reactive as possible with the specific subtype. 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 IAV-S 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 IAV-S antibodies has been described in the literature and commercial kits are available (Barbé *et al.,* 2009; Ciacci-Zanella *et al.,* 2010).

2.1. Haemagglutination inhibition test

2.1.1. Test procedure

i)   Dilute reference HA antigens (H1, H3, etc.) to a concentration of 4–8 HAU/25 µl 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. While the use of RDE is encouraged, there may be regional variability in its use for treatment of sera depending on serum specificity for some antigens used in the HI assay.

iii)   *H1N2* and *H3N2 test:* 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. Add 50 µl 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 (24°C) or 4°C for 30–60 minutes.

viii) Add 50 µl of 0.5% erythrocyte suspension to each well, shake, and incubate at room temperature (24°C) or 4°C 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.

2.2. Enzyme-linked immunosorbent assay (Barbé *et al.,* 2009; Ciacci-Zanella *et al.,* 2010)

ELISA technology for detection of (IAV-S) antibodies has been described in the literature and ELISAs are available as commercially produced kits.

C. REQUIREMENT FOR VACCINES

1. Background

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.

1.1. Rationale and intended use of the product

IAV-S infections can cause significant economic impact for producers because of reduced feed intake during illness resulting in decreased weight gain, increased days to market, and decreased feed efficiency. Where vaccination is practiced, vaccine is used to reduce the economic impact of disease by reducing the severity and duration of clinical signs. In addition, vaccines can reduce the level of viral shedding and the duration of viral shedding. Decreasing the amount of virus shed and duration of shedding can be important in reducing virus transmission while minimising the risk of exposure for pigs and people.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Strains used in vaccine production should be antigenically relevant to IAV-S strains circulating in the field. Haemagglutination inhibition and neutralisation tests demonstrating cross-reactivity between antisera from pigs vaccinated with the candidate vaccine strain and current field isolates can be used for the selection.

Identity of the seed should be well documented, including the source and passage history of the virus. All defining characteristics such as haemagglutinin and neuramindase subtype should be established. Haemagglutination inhibition and neuraminidase inhibition by subtype-specific antisera or real-time 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 H1 or H3 IAV-S, then inoculated into the allantoic sac of 10-day old embryonated chicken eggs or on 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.

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.

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

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 IAV-S 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.

2.2. Method of manufacture

2.2.1. Procedure

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. IAV-S 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. IAV-S vaccine products should be limited to five passages from the MSV to avoid genetic/antigenic variation. 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. Typically, adjuvant is added to enhance the immune response.

2.2.2. Requirements for substrates and media

Cells are 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.

2.2.3. In-process controls

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.

2.2.4. Final product batch tests

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

i)   Sterility and purity

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 of biological materials for sterility and freedom from contamination*).

ii)   Safety

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, and then inoculated on to a susceptible cell line or into the allantoic sac of embyonated 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.

iii) Batch 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.

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

2.3. Requirements for authorisation

2.3.1. Safety requirements

i)   Target and non-target animal safety

Final container samples of completed product from inactivated vaccines should be tested in young mice for safety. Generally, healthy pigs of weaning age or older and pregnant sows at any stage of gestation may be safely vaccinated with inactivated IAV-S vaccines. 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.

ii)   Reversion-to-virulence for attenuated/live vaccines

Reversion-to-virulence for live viral vaccines is often demonstrated by back passage through susceptible species. Virus is isolated from the vaccinated animal and the isolated virus is then used to inoculate additional animals. Sequential passage through animals should show that animals remain clinically healthy with no demonstration of typical IAV-S signs.

iii)   Environmental consideration

Inactivated IAV-S vaccines present no special danger to the user, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. Modified live virus vaccines may pose a hazard to the user depending on the level of inactivation of the virus and the susceptibility of humans to the swine-adapted virus.

Preservatives should be avoided if possible, and where not possible, should be limited to the lowest concentration possible. The most common preservative is thimerosol, at a final concentration not to exceed 0.01% (1/10,000). Antibiotics may be used as preservatives in IAV-S 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.

Vaccine bottles, syringes, and needles may pose an environmental hazard for vaccines using adjuvants or preservatives and for modified live virus vaccines. Instructions for disposal should be included within the vaccine packaging information and based on current environmental regulations in the country of use.

2.3.2. Efficacy requirements

i)   For animal production

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 IAV-S 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 IAV-S 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.

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. 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.

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 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

ii)   For control and eradication

The same principles apply as for animal production usage. In addition, it should be noted that antibody responses in vaccinated animals may not be differentiated from animals exposed to field virus. Therefore, vaccinated animals will need to be clearly identified if serological methods will be used in conjunction with compatible clinical signs to assess field virus exposure.

2.3.3. Stability

Vaccines should be stored with minimal exposure to light at 4°C±2°C, or as approved by the designated regulatory authorities. The shelf life should be determined by use of the approved potency test over the proposed period of viability.

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NB: There are OIE Reference Laboratories for Swine 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 swine influenza