CHAPTER 3.9.7.

INFLUENZA A VIRUSES OF SWINE

SUMMARY

Description and importance of disease: *Influenza A viruses of swine (IAV-S) cause a highly contagious respiratory disease characterised by coughing, sneezing, nasal discharge, fever, 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.*

Detection and identification of the agent: *Samples 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. Antigen enzyme-linked immunosorbent assays (ELISA) are commercially available for detection of type A influenza viruses and are typically directed against the conserved nucleoprotein (NP). Viral RNA can readily be detected by reverse-transcription polymerase chain reaction (RT-PCR) assays targeting the matrix (M) or the hemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) encoding genes in samples from respiratory tissues, such as trachea or lung and nasal swabs. Immunohistochemistry can be conducted on formalin-fixed tissue. Real-time RT-PCR is increasingly used as it is highly sensitive and can be performed on scale at relatively low unit cost. Oral fluids collected from cotton ropes hung in a pig pen may also be useful as a group or population sample. Virus isolation can be achieved using embryonated fowls' eggs, continuous cell lines or primary cell cultures. Viruses can be subtyped (HxNy) by identification of the hemagglutinin (HA) and neuraminidase (NA) viral envelope proteins using the haemagglutination inhibition (HI) and the neuraminidase inhibition tests on virus isolates, or by RT-PCR assays direct on clinical material or on isolates. Gene sequencing is now widely applied and can be used to determine virus subtype and, importantly, genotype, the latter providing invaluable data associated with genetic diversity of IAV-S driven by both genetic reassortment and continual genetic drift especially in the genes encoding HA and NA.*

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 HA 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 subtypes 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, some based on autologous strains. Vaccines may include antigens from a single IAV-S subtype or multiple IAV-S subtypes. Vaccines should ideally reflect the current antigenic profile of field viruses, containing subtypes and strains that are changed as needed to assure protection.*

A. INTRODUCTION

Influenza A viruses of swine (IAV-S) cause 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 the haemagglutinin (HA) and neuraminidase (NA) proteins in the viral envelope that contain the immunodominant epitopes. Subtypes (HxNy) of IAV-S that are most frequently identified in pigs include classical and avian (av) H1N1, human (hu) H1N1 and H1N2, reassortant (r) H3N2, and rH1N2. Other subtypes that have been infrequently identified in pig populations (although not necessarily maintained) include rH1N7, rH3N1, H2N3, avH4N6, avH3N3, and avH9N2. The H1N1, H1N2 and H3N2 viruses found in Europe are antigenically and genetically different from those found in America or Asia (Anderson *et al.,* 2016; Brown, 2013; Karasin *et al*., 2000; 2002; Lewis *et al.,* 2016; Olsen, 2002; Vincent *et al*., 2009; Watson *et al.,* 2015; Webby *et al*., 2004; Zhou *et al*., 1999). Due to the substantial genetic diversity in H1 viruses circulating in swine among geographical regions, haemagglutinin H1 can be classified according to genetic characteristics into clades; 1A classical lineage derived from the 1918 human pandemic viruses, 1B human seasonal lineage associated with 1990s human-to-swine transmission episodes, and 1C Eurasian avian lineage associated with viruses introduced to swine in Europe and Asia from avian species (Anderson *et al.,* 2016).

Pig cells have receptors in their respiratory tract that allow attachment and infection by influenza A viruses of swine, humans, and avian species. Consequently, pigs have been called 'mixing vessels' for the development of novel influenza viruses through genetic reassortment in pigs. IAV-S infections cause respiratory disease characterised by coughing, sneezing, nasal discharge, fever, 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 (Mollett *et al.,* 2023). Thus, laboratory investigations are necessary to determine infection with IAV-S. 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 herds, epidemic or endemic (Li & Robertson, 2021). In the epidemic form, the virus quickly moves through all phases of a swine unit with rapid recovery, provided there are no 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. In some cases, the endemic form may affect successive batches of pigs at a given physiological stage (Rose *et al.,* 2013). In all cases, the endemic form is responsible for IAV-S persistence at the herd level. Morbidity rates can reach 100% with epidemic 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 discharge, droplets and aerosols created by coughing or sneezing. Human infections with IAV-S so-called variant (v) strains can occur and a limited number of deaths have been reported (Lindstrom *et al.,* 2012; Myers *et al*., 2007; Pulit-Penaloza *et al.,* 2019). 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, so called reverse zoonoses or anthroponoses, creating opportunity for further virus diversity. Influenza A 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 (M) and neuraminidase (NA) genes were from European H1N1 IAV-S of 1C avian lineage and the remaining genes were from North American IAV-S of a triple reassortant (swine, avian, and human) lineage (Zhou *et al.,* 1999). The H1N1pdm09 spread rapidly throughout the world through human-to-human transmission. In addition to continued independent circulation in humans, reverse zoonoses of 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 (Anderson *et al.,* Lewis et al., 2016; 2021; Watson *et al.,* 2015).

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	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	+	+	++	+++	+	–
Real-time RT-PCR	+++	+++	+++	+++	++	–
Conventional PCR	–	–	–	++	–	–
Detection of immune response						
HI	+	+	+	++	++	+++
ELISA	+++	+	++	+	+++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.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.
[(a)]A combination of agent identification methods applied on the same clinical sample may be needed in some situations

1. Identification of the agent

Clinical specimens and cultures of IAV-S should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Because IAV-S is a potential human pathogen, all work with infectious virus, potentially infectious diagnostic samples, embryonated eggs, and cell cultures should be done in a class II microbiological safety cabinet (MSC). Additional safety precautions (personal protective equipment) should be used when working with infected pigs such as respiratory personal protective equipment (RPE) and eye protection.

1.1. Culture

1.1.1. Sample processing

Respiratory tissues 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 a physiological solution such as 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 (e.g. 1% fetal bovine serum [FBS] or FBS-free medium) or phosphate-buffered saline (PBS), supplemented with antibiotics and bovine serum albumin (5 mg/ml). 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 specimen collection and shipment). Upon receipt at the laboratory, the nasal swabs are vigorously agitated by hand or on a vortex mixer. The nasal swab and tissue specimens are centrifuged at 1500–1900 ***g*** for 15–30 minutes at 4°C. The supernatant is collected and maintained at 4°C until inoculation. If supernatant is to be held for longer than 24 hours before inoculation, it should be stored at –70°C or colder. The clarified tissue suspension 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 (polyvinylidene fluoride) 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 (Feng *et al.,* 2011; Karakus *et al.,* 2018). Madin–Darby canine kidney (MDCK) cells are broadly permissive for various subtypes and strains of IAV-S and are therefore often a 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 without FBS and containing a final concentration of 1 μg/ml of TPCK[1]-treated trypsin. 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.

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, or 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 viruses or by reverse transcription-polymerase chain reaction (RT-PCR) for conserved influenza virus genes such as nucleoprotein (NP) or matrix (M) encoding genes, which have largely replaced antibody based confirmatory tests. However all culture medium can be used in an HA assay to detect viral particles. Alternatively, the culture supernatant can be used as inoculum for virus detection by ELISA (see Section B.1.5) or by the fluorescent antibody or immunohistochemistry (IHC) techniques (see Section B.1.3 and 1.4 below). Usually monolayer cultures (i.e. 24-well cell culture plate) with MDCK (or other appropriate cell) 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 produce appropriate CPE on the monolayer. Influenza subtypes can be determined by the haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests, or by real-time or conventional RT-PCR with primers validated for sensitive and specific amplification of individual HA and NA genes (for example Bonin *et al*., 2018; Chiapponi *et al*., 2012; Henritzi *et al.*, 2016; Nagarajan *et al.,* 2010; Ryt-Hansen *et al*., 2020) or by gene sequencing and comparing data obtained with those of previously characterised swIAV-s. 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

i) Use 9- to 11-day-old embryonated fowls' 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 however for primary

1 TPCK: tosylphenylalanylchloromethane

isolation, amniotic inoculation can increase sensitivity. Generally, 3–4 eggs are inoculated per specimen.

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 intercurrent deaths associated with the inoculation process).

iv) Refrigerate eggs with embryos that died more than 24 hours after inoculation or 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) Harvest amniotic and allantoic fluids, centrifuging if necessary 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, or by ELISA (see Section B.1.5) or by (M- or NP-gene) RT-PCR (see Section B.1.6).

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 or guinea-pig blood (Takemae *et al.,* 2010). 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 or guinea-pig 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 of 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) Complete serial two-fold dilutions of 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 diffuse '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 WOAH 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 noting multiple sera for same subtype may be required to take account of antigenic diversity, 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 highly recommended as it will reduce nonspecific reactions and will enhance the identification of H1N2 and H3N2 isolates.

v) Remove natural serum agglutinins from the reference sera by treating diluted sera 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 in cell control wells.

vii) Add 25 µl of the appropriate reference serum to the first well of the H subtype being evaluated. Serially twofold 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 serum 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 (within twofold) 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 (Kaplan & Vincent, 2020). Increasingly PCR or NA gene sequencing methodologies are replacing such tests.

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 operators 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 RT-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 a fixative (i.e. 4% paraformaldehyde, 100% acetone or 10% normal buffered formalin) for 5–10 minutes and air-dry. Many fixatives 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. Note it is important to use an antibody that recognises all possible viruses circulating in the area (e.g. a pan-anti-influenza A nucleoprotein antibody).

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.

1.4. Immunohistochemistry

1.4.1. Test procedure (Vincent *et al*., 1997)

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). 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) at a predetermined dilution 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 development reagent (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.

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 RT-PCR.

1.6. Reverse-transcription polymerase chain reaction

At present, conventional virus isolation and other characterisation techniques for the diagnosis of IAV-S infections remain a key method and provide virus for more detailed analyses including *in-vivo* assessment and gene sequencing. Furthermore, they may be invaluable in confirming or disproving the presence of infectious virus when other test results including conventional and real-time RT-PCR are all weakly positive. However, conventional methods tend to be costly, labour intensive and slow. There have been enormous developments and improvements in molecular and other diagnostic techniques, many of which are now routinely applied as a first choice for the diagnosis of IAV-S infections.

Molecular techniques have been used preferentially for diagnosis for some time now. Furthermore, there have recently been developments in their application to the detection and characterisation of IAV-S directly from clinical samples from infected pigs. It is imperative that when using highly sensitive molecular detection methods that allow rapid direct detection of viral RNA for confirmatory laboratory diagnosis of IAV-S infections, stringent protocols are in place to prevent the risk of cross-contamination between clinical samples. In addition, RNA detection test methodologies should be validated to the WOAH standard (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*) using clinical material to demonstrate the tests as being 'fit for purpose' for application in a field diagnostic setting, which may include the use of internal test standards. The control reactions enable greater confidence in the integrity of the molecular reactions, clinical samples and results.

Furthermore, these evaluations enable the appropriate setting of test thresholds for interpretation between positive and negative samples. The increased sensitivity of real-time RT-PCR leads to the detection of viral RNA in samples in the absence of infectious virus and care should be taken when interpreting outputs with small detection limits that may not be indicative of active infection. This problem can be overcome, through the testing of multiple samples from the same cohort of infected pigs.

In settings with more limited facilities, RT-PCR techniques on clinical samples can, with the correctly defined primers, result in rapid detection and subtype identification including a cDNA or PCR product that can be used for nucleotide sequencing. Real-time RT-PCR, usually based around the hydrolysis probe method for generation of the target-specific fluorescence signal, has become the method of choice in many laboratories for at least partial diagnosis directly from clinical samples. The method offers rapid results, with sensitivity and specificity comparable with virus isolation. These are ideal qualities for IAV-S disease management, where the period of time in which an unequivocal diagnosis can be obtained is crucial for decision making. The approach to diagnosis using real-time RT-PCR adopted in most laboratories has been based on initial generic detection IAV-S in clinical samples, primarily by initially targeting the matrix (M) or the nucleoprotein (NP) encoding genes, which are highly conserved for all influenza A viruses and the best targets for screening for infection with IAV-S by RT-PCR, followed by specific real-time RT-PCR testing for H1 and H3 subtype viruses. Genomic sequencing is required to further classify the H1 clades (e.g. gamma vs beta H1) and H3 clusters (e.g. cluster IV or 2010.1).

Due to the high diversity of swine HA and NA gene sequences, it can be difficult to use real-time RT-PCR to differentiate between subtypes and specially to differentiate between different lineages within a subtype. Therefore, sequencing is often more precise.

Numerous assays have been reported for highly sensitive detection of M (or NP) gene fulfilling the criteria for a suitable screening test. Following the identification of the novel (pandemic) H1N1 in 2009, molecular assays based on an avian influenza M gene real-time RT-PCR (Spackman *et al*., 2002) were adapted for use in swine (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 RT-PCR assay for the region in which it is being applied.

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.

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 RT-PCR that is free of amplified c-DNA or sample RNA.

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

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

*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 3).

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 laboratory and must have a C_t value in the range of 21–30 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 at a predetermined level typically in the range 25–30

    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 3. Example real-time RT-PCR master mix for a one step reaction

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 4. 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 (or from clinical samples dependent on C_t value) can be subtyped using conventional methods or by real-time PCR assays that can differentiate the genetically distinct HxNy viruses (Bonin *et al.,* 2018; Chiapponi *et al.,* 2012; Henritzi *et al.,* 2016; Larsen *et al.,* 2020). Increasingly, differential HA and NA real-time PCRs are being used in many regions. Matrix RT-PCR positive diagnostic samples can also be subtyped through use of HA and NA subtyping RT-PCRs. Samples with high matrix C_t's may not be detectable by subtyping RT-PCRs and it may be necessary to attempt virus isolation prior to identifying the subtype. Screening and subtyping RT-PCR reagents are commercially available; however, laboratories need to ensure they will detect currently circulating influenza viruses in their area. In many instances, due to the increased genetic variability of HA and NA genes, 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 sequencing of several or all gene segments is increasingly being used to determine and monitor virus diversity. A recently developed universal nomenclature web-based tool provides an accurate method to assign clade designations to H1-HA sequences; http://www.fludb.org (Anderson *et al.,* 2016). High throughput sequencing can be applied to obtain genomic information on the isolate or directly from field samples to speed up characterisation of the influenza virus (Lee *et al.,* 2016).

Tests should be validated for the region in which they are to be applied given the worldwide genetic and antigenic diversity of IAV-S.

1.7. Gene sequencing

In 2023, real-time RT-PCR is the preferred method of virus surveillance because the test provides rapid sensitive diagnostics for IAV-S with high throughputs. However, greater use of sequencing technologies particularly as unit costs reduce with improvement in technology, offer powerful opportunities to simultaneously detect and sequence from clinical samples in a laboratory or field setting, for example applying nanopore technology.

Increasingly gene sequencing is being applied not only to detailed characterisation of viruses for use in molecular epidemiology but also in virus subtyping and defining markers for host range including zoonotic risk (Chauhan & Gordon, 2022). Sanger sequencing methodology has been widely used for decades and enables the rapid determination of typically a single (HA) target gene in 24–36 hours and still has widespread utility. However, as genomic data can be rapidly determined using whole genome sequencing technology it enables a broader analysis using a range of bioinformatics tools (Zhang *et al.,* 2017). For example, with the advent of greater access to sequencing methodology either through specialised laboratories or commercial providers it is now possible to determine the genomic sequences of IAV-S to provide a level of characterisation important in rapid pathogen identification and disease investigations. Conventionally, nucleotide sequences have been used in outbreak epidemiology to infer virus origin and precise relationships between different viruses associated within the same event (by phylogeny) to support outbreak management. Virus gene sequences of haemagglutinin and neuraminidase can rapidly be compared to known sequences of all subtypes in gene databases and used to reveal the closest match thereby identifying the virus subtype and phylogenetic relationships. This often avoids the need to culture the virus for rapid identification although reliability and quality of data reduces with increasing cycle threshold values in samples from real-time RT-PCR testing.

Increasingly such analyses are being applied at the whole genome level to reveal virus genotypes and provide greater analytical specificity to the analyses. Such approaches are especially valuable to track as virus evolution can be more precisely mapped including change through genetic reassortment, a key mechanism associated with virus diversity and fitness for pigs. This approach is especially valuable for early or first incursions in a new event as it enables greater precision in determining virus origin and the mechanisms leading to the emergence of virus. Translation of nucleotide sequences of all genomic segments into amino acid sequences enables data mining for other virus characteristics or traits such as tropism, host range markers including zoonotic and predicted antiviral drug susceptibility, which are invaluable for informing outbreak management (Garrido-Mantilla *et al.,* 2019; Noronha *et al.,* 2012; Pulit-Penaloza *et al.,* 2019).

2. Serological tests

The primary serological test for detection of IAV-S antibodies is the HI test and it is HA subtype specific. Reference antigens should reflect the contemporary virus genetic lineages 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 samples is suggestive of a recent IAV-S. Additional serological tests that have been described but not commonly used are the virus neutralisation (Gauger & Vincent, 2020), agar gel immunodiffusion test, and indirect fluorescent antibody test. ELISA technology for detection of IAV-S antibodies is well described in the literature and commercial kits are available (Barbe *et al.,* 2009; Ciacci-Zanella *et al.,* 2010).

2.1. Haemagglutination inhibition test

see Section B.1.2.1.

2.2. Enzyme-linked immunosorbent assay

ELISA technology for detection of (IAV-S) antibodies has been described in the literature and ELISAs are available as commercially produced kits (Barbe *et al.,* 2009; Ciacci-Zanella *et al.,* 2010).

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 and duration of viral shedding, an important aspect for reducing virus transmission while minimising the risk of exposure for pigs and people. Generally these vaccines are applied to breeding sows but piglet vaccination is becoming more widespread as IAV-S prevalence increases. The utility and efficacy of vaccination is highly variable at the global scale with many factors requiring consideration (Sandbulte *et al.,* 2015). To date, the majority of IAV-S vaccines used in pigs have been conventional inactivated whole virus vaccines prepared from infective allantoic fluid of embryonated chicken eggs or cell culture fluids, inactivated by beta-propiolactone or formalin and emulsified with mineral oil adjuvants. Because of the potential for reassortment leading to increased virulence, live conventional influenza vaccines have been used on a limited scale. Live attenuated vaccines tend to result in broader immune responses by eliciting both, humoral and cellular immune responses and some may also have the advantage of being effective in the presence of maternally derived antibodies. Biotechnology holds great potential for generating live IAV-S vaccines with altered gene segments that reduce the risk of reassortment, limit replication and abrogate negative aspects of live IAV-S vaccines.

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 matched to IAV-S strains circulating in the field where vaccination is to be applied. 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. Antigenic characteristics such as haemagglutinin (HA) and neuraminidase (NA) 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 HA and NA subtypes. Also, aliquots of the master seed virus (MSV) can be neutralised with specific antiserum (Gauger & Vincent, 2020), 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 HA and NA 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 dependent on local requirements. 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, beta-propiolactone or binary ethylenimine. Typically, an approved 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 FBS 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 for sterility and freedom from contamination of biological materials intended for veterinary use*).

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. A standard alternative is HAU or guinea-pig neutralisation units or GMNU (geometric mean of neutralising units induced in guinea-pigs after two immunisations with 0.5 ml of vaccine).

2.3. Requirements for regulatory approval

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-S signs.

iii) Environmental consideration

Inactivated IAV-S vaccines present no particular 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 μg/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 WOAH Reference Laboratories for swine influenza (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for swine influenza

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2023.