THAI AGRICULTURAL STANDARD

TAS 10050-2013

DIAGNOSIS OF SWINE INFLUENZA

National Bureau of Agricultural Commodity and Food Standards
Ministry of Agriculture and Cooperatives

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Published in the Royal Gazette, Announcement and General Publication Volume 130,
Special Section 1733 (Ngo),
Dated 25 JUNE B.E.2556 (2013)
Technical Committee on the Elaboration of Thai Agricultural Standard for Diagnosis of Swine Respiratory Diseases

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Swine influenza is a swine respiratory disease caused by influenza virus type A which has a risk of transmission to human, causing economic loss and public health problems. Moreover, the genetic changes of virus can produce a novel influenza A virus subtype affecting accuracy and precision of the diagnosis. Therefore, the Agricultural Standards Committee deems it necessary to establish the Thai Agricultural Standard on Diagnosis of Swine Influenza as a guideline for laboratory diagnosis and pig farm certification.

This standard is based on the following documents:


NOTIFICATION OF THE MINISTRY OF AGRICULTURE AND COOPERATIVES
ON THE ESTABLISHMENT OF : THAI AGRICULTURAL STANDARD:
DIAGNOSIS OF SWINE INFLUENZA
UNDER THE AGRICULTURAL STANDARDS ACT B.E. 2551 (2008)
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Whereas the Agricultural Standards Committee, by the decision of the First Session dated 6 March B.E. 2556 (2013), deems it necessary to establish an agricultural standard for Diagnosis of Swine Influenza as a voluntary standard in accordance with the Agricultural Standards Act B.E. 2551 (2008) to promote such agricultural commodity to meet its standard on quality and safety.

By virtue of Sections 5, 15 and 16 of the Agricultural Standards Act B.E. 2551 (2008), the Minister of Agriculture and Cooperatives hereby issues this Notification on the Establishment of Thai Agricultural Standard: Diagnosis of Swine Influenza (TAS 10050-2013), as voluntary standard, details of which are attached herewith.

Notified on 20 April B.E. 2556 (2013)

(Mr.Yukol Limlamthong)
Minister of Agriculture and Cooperatives
1. SCOPE

This agricultural standard provides essential details for the diagnosis of swine influenza caused by influenza virus type A, subtype H1 and H3.

2. DEFINITIONS

For the purpose of this standard:

2.1 Diagnosis means investigation of the etiology in conjunction with disease diagnosis.

2.2 Swine influenza means an acute respiratory disease of swine caused by influenza virus type A subtype H1 and H3 called swine influenza virus (SIV) in this standard.

2.3 Swine means domesticated and wild animals of the family Suidae, the cloven–hoofed mammals.

2.4 Positive control set means a test panel containing the standard microbes to be studied for comparison with the unknown samples.

2.5 Negative control set means a test panel that does not contain the standard microbes to be studied for comparison with the unknown samples.

2.6 Biosafety laboratory means a laboratory that can prevent hazard from the exposure of biological materials such as pathogens, blood, tissues, genetic materials and toxins contaminated in the laboratory by safe laboratory operating procedures in place to reduce risk of exposure and mitigate the spread of those biological materials.

3. DIAGNOSIS

Laboratory diagnoses for swine influenza are pathology, immunology, virology and molecular biology. The methods, described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals established by the World Organisation for Animal Health (OIE), shall be used. An alternative method shall be selected from published scientific journals with similar or better sensitivity and specificity with a modification to suit virus subtype in Thailand.

SIV adversely affects pig production and may be harmful to human (Appendix A) as well as high risk of spread in laboratory. Therefore, the diagnosis of SIV should be taken place in biosafety laboratory under the appropriate working spaces, equipment, tools, and operating
procedures. Personnel should have experiences and skills to perform good laboratory practices.

3.1 Sampling

Sampling procedures specified in Appendix B shall be followed.

3.2 Sample collection and transport

Sample shall be collected in appropriate quantity and method and rapidly sent to laboratory. Proper sample collection and transport can increase the accuracy of laboratory diagnostic tests. Sample used for SIV diagnosis can be collected from sick or suspected animals by using nasal swab or blood collection. In case of dead animal, lung tissue shall be sampled.

3.2.1 Sample collection from live animal

3.2.1.1 Secretion from respiratory tract

Collect secretion from respiratory tract for virus identification by swab from nasal cavity of infected animal, which shows clinical signs within 24-48 hours (h) (Appendix C). Such sample shall be kept in viral transport media or other medium with antibiotic supplement (Appendix D, Section D.2).

For swab sample, five swabs can be placed in one transport media tube and sent to laboratory immediately or within 24 h. The samples shall be kept in tightly sealed container to prevent viral spread and stored at 4°C (or 2-8°C), but not frozen.

3.2.1.2 Serum

For serological tests, 3-5 ml blood shall be collected from jugular vein. It shall be conducted on paired sera collected 10–21 days apart in order to evaluate the increase in antibody titre.

3.2.2 Sample collection from dead animal

3.2.2.1 Lung tissue, especially bronchioles without post mortem autolysis shall be sampled at the area with normal and lesion to obtain 3-4 grams (g) per piece of lung tissue. Sample shall be kept in double bags with clear label and stored at 0-4°C. Sample shall be sent to laboratory immediately or within 24 h. Lung sample from each animal shall be kept in individual bag.

3.3 Diagnostic techniques of swine influenza

Diagnosis of SIV shall be performed in a biosafety laboratory which can be divided into two techniques as follows:

(1) Virus identification
This technique comprises of virus isolation through egg inoculation or cell culture, detection of viral antigen or viral genomes. Prior to the virus isolation, haemagglutination test (HA) shall be carried out in order to identify type A influenza virus and to discriminate SIV from other viruses present with the same viral properties. If HA test is positive, several techniques shall be used to confirm the result, such as, haemagglutination inhibition test (HI test) (Appendix E) or molecular techniques, such as reverse transcription-polymerase chain reaction (RT-PCR) (Appendix F) or real-time RT-PCR.

Detection of viral antigen from tissue sample may also be performed by fluorescent antibody test, immunohistochemistry technique (Appendix G) or other techniques approved by the OIE.

(2) Serological tests

This technique is to measure antibody against haemagglutinin which is specific to each viral subtype. For example, HI test detects an increase of antibody. In addition, other techniques may be applied, for instance, agar gel immunodiffusion test, indirect fluorescent antibody test, virus neutralization test, or enzyme-linked immunosorbent assay (ELISA) approved by the OIE.

3.3.1 Virus isolation

3.3.1.1 Sample processing for virus isolation

(1) For secretion from respiratory tract sample (Section 3.2.1.1), centrifuge at 1500–1900 \( g \) (acceleration due to gravity) for 15-30 minutes at 4°C.

(2) For lung tissue sample (Section 3.2.2), prepare 1 g of tissue at lesion area containing bronchioles and cut into small pieces and then grind the tissue with a mortar and pestle. Aseptic sand can be added for fine grinding. Add 5-10 ml of PBS solution and mix well. Centrifuge at 1500–1900 \( g \) for 15-30 minutes at 4°C.

(3) Collect supernatant and add antibiotic(s), such as gentamicin (100 µg/ml), or penicillin (10,000 units/ml): streptomycin (10,000 units/ml), and 2% fungizone (250 mg/ml). Leave the supernatant at 4°C for 1 h in order to eliminate contaminated bacteria. The bacteria in supernatant can also be removed by other methods such as filtration through the 0.22 µm membrane filter (This technique may result in reduction of number of virus particles).

(4) Collect supernatant and store at 4°C until inoculation. In case supernatant is to be held for longer than 24 h before inoculation, it shall be stored at –70°C.

3.3.1.2 Egg inoculation

(1) Use 10–11 day-old embryonated chicken eggs. One sample shall be inoculated into 3-4 eggs for isolation of virus.

(2) Inoculate 0.1–0.2 ml of inoculum into the amniotic and allantoic cavity (Figure K.5).
(3) Incubate eggs at 35–37°C for 3–4 days and candle every 24 h. Eggs with embryos that have died within 24 h of inoculation are discarded.

(4) Refrigerate eggs with embryos which died later than 24 h after inoculation or at the end of incubation period at 4°C for 12 h or frozen at -20°C for 1 h.

(5) Harvest amniotic and allantoic fluids from eggs and centrifuge at 1500–1900 g for 10–20 minutes at 4°C. Fluids are evaluated for the presence of SIV with the HA test (Appendix E, Section E.2).

(6) If HA test is positive, other confirmation techniques, such as RT-PCR shall be performed (Appendix F).

(7) If HA test is negative, the fluid is re-inoculated into chicken embryonic eggs (second passage) and steps (1)-(6) above shall be repeated.

3.3.1.3 Cell culture virus isolation

Madin Darby Canine Kidney (MDCK) is the preferable cell line for SIV culture. The other cell lines can also be used such as mink lung cell line and primary swine kidney. Trypsin is required in cell culture medium for promoting the separation of haemagglutinin to be HA1 and HA2 before the HA1 will bind the cell surface receptors.

(1) Inoculate MDCK cell line in a 24-well culture plate. Final concentration of at least $10^6$ cell/ml shall be obtained by using 1 ml culture medium (minimal essential medium; MEM), supplemented with 5% fetal bovine serum (FBS) (Appendix D, Section D.4).

(2) Place the plate in an incubator at 37°C, 5% CO₂ for approximately 48 h, until the cells are formed in the manner of confluent monolayer covering 80-100% of the well’s area.

(3) Rinse confluent cell monolayer 3 times with 0.5 ml PBS or MEM and completely remove the washing solution.

(4) Add 0.2-0.3 ml of viral suspension. Dilute viral suspension with PBS to obtain 1:1, 1:2 and 1:3 dilutions. Each concentration should be duplicated. Place the plate in an incubator at 37°C, 5% CO₂ for 45-60 minutes.

(5) Remove viral suspension from confluent cell monolayer.

(6) Add 1 ml of culture medium that contains MEM and 1 µg/ml of trypsin into each well and place the plate in an incubator at 37°C, 5% CO₂.

(7) Observe cytopathic effect (CPE) every day for 5-7 days.

(8) If CPE is not detected, harvest monolayer cells and culture medium and freeze at -70°C and then thaw cell suspension at 0-4°C. Repeat freeze and thaw process for 2-3 times and perform the second passage by inoculating cell suspension into cell culture as described in
steps (4)-(7). To confirm test result, other techniques such as HA test and RT-PCR may be performed.

In case quantitative result is required, virus titration (Appendix H) and immunoperoxidase monolayer assay (IPMA) (Appendix I) may be performed.

(9) For interpretation of result, evaluation of CPE in cell culture shall be used in conjunction with other confirmation techniques.

3.3.2 Serological tests

Serological test is the detection of haemagglutinin antibodies specific to each subtype of influenza A virus. The serological test can differentiate SIV as H1 and H3 subtypes. To detect SIV infection, paired sera collected 10-21 days apart shall be carried out. Four-fold increasing in titre indicates SIV infection. If vaccine is not applied, the primary test for SIV is HI test.
Appendix A

Epidemiology, pathogenesis and clinical signs of swine influenza

(Section 3)

A.1 Epidemiology

Swine influenza is caused by type A influenza virus of family Orthomyxoviridae. The virus is a round shape, single-stranded RNA virus with a diameter of 80 – 120 nm. After several passages in cell culture, the shape of virus will change. The viral structure consists of lipid envelope and two types of glycoprotein protruding out of its surface i.e. the rod shape known as haemagglutinin and the mushroom-like structure called neuraminidase. Under the viral envelope is matrix protein (M), M1 and M2 where M2 is a fine structure containing 20-60 molecules of protein per virion and inner M1 layer covers the ribonucleoprotein complexes (RNP complexes). The RNP complexes consist of ribonucleic acid (RNA) which contains eight segments of viral genome. Each genome segment contains ribonucleic acid, nucleoprotein and acidic polymerase (PA) including the genome segments that play a role of transcription and replication of ribonucleic acids which are basic polymerase 1 (PB1) and basic polymerase 2 (PB2). In addition, non-structural proteins (NS) is also observed. Non-structural proteins 2 (NS2) is found in all virion, while non-structural proteins 1 (NS1) is specifically found only in cells infected with influenza virus. All genome segments are negative single-stranded RNA. Segregation of viral genome into segments allows genomic exchange during replication in host cells and may lead to the formation of a new virus strain.

Subtyping of influenza virus is performed by characterizing antigenic properties of glycoproteins of haemagglutinin and neuraminidase. Currently, not less than 17 subtypes of haemagglutinin glycoproteins and 9 subtypes of neuraminidase glycoproteins have been reported. All subtypes of SIV have been isolated from waterfowl which is a natural reservoir for influenza outbreak. Influenza subtypes have been reported in mammals such as human H1N1, H2N2 and H3N2, equine H3N8 and H7N7 and swine H1N1, H3N2 and H1N2. Outbreak of swine influenza was first reported in 1918 in the United States. Large number of pigs showed symptoms similar to human infected with influenza virus. Moreover, severe outbreak of human influenza occurred during the same period. Influenza virus subtype H1N1 (classical H1N1) was first isolated in 1930. Sporadic outbreaks of swine influenza subtype H1N1 and H3N2 have been reported worldwide. The occurrence of swine influenza has increased dramatically in many parts of the world since 1975, including Asia.
In Thailand, three SIV subtypes (H1N1, H1N2 and H3N2) have been reported. The first occurrence was in 1978 caused by H3N2 subtype, followed by the identification of H1N1 in 1988 and H1N2 in 2005. The investigation of SIV pathogenesis of H1N1 and H3N2 isolated from pigs in Thailand in 2005 showed that the virus can cause pathological lesion in respiratory system. Infected pigs showed symptoms similar to human e.g. coughing, sneezing, fever and anorexia. In addition, shedding of virus was reported for only 1-3 days after infection. Analysis of the 8 genome segments of SIV occurring in Thailand indicated that the virus was derived from the recombination of strains from Europe and America which resulted in genetic heterogeneity of SIV in Thailand.

In 2009, worldwide influenza outbreaks in human were reported. Type A influenza virus was identified, which was then named as human pandemic H1N1 2009 (pH1N1). The virus was a combination of influenza virus gene derived from swine, avian and human. In addition, distribution of pH1N1 in pigs was reported in many countries, including Thailand. The virus could be isolated from both farm pigs and pigs from the Pork expo in the United States. In Thailand, there was a report on pH1N1 that persisted for 4 months in a farm located in western region and it exchanged genomes with the endemic strain. An outbreak of the virus in other parts of Thailand has not yet been reported. The new virus recombinant only caused mild respiratory signs in pigs, which did not cause mortality. However, this shows that influenza virus occurs in Thailand and there is a risk of the emerging of a new influenza virus. Therefore, reassortment of the virus genome is of concern. Disease survey, diagnosis, and surveillance of swine influenza in Thailand are very important. The issue is concordance with the World Health Organisation’s recommendations on the importance of survey of type A influenza virus in human and other animals, especially in swine in order to prevent emerging and re-emerging diseases from animals to human as pH1N1.

A.2 Pathogenesis

Pathogenesis of SIV in an infected pig is initially associated with respiratory system. The virus attaches itself to cilia and invades through epithelial cells of nasal cavity, trachea, and bronchi. Replication of virus occurs in epithelial cells within 16 h post infection, which results in partial necrosis of bronchial epithelial lining, collapse of the alveoli and passive congestion of the lung. Epithelium cells necrosis are progressive due to the spreading of the virus to such cells. Tracheal exudate and wider area of collapsing alveoli are then observed. Within 24 h post infection, apical and cardiac lobes of the lung will become dark red. These lesions will become less visible and eventually lung will turn back to normal within 72 h post infection. In general, infection of SIV in swine occurs specifically in respiratory system, however, viremia may also occur. The virus is usually isolated from nasal cavity of the infected pig at the first day of infection. Infected pigs may not show any sign of illness or may exhibit different degrees of clinical signs depending on several factors such as route of infection, maternal antibody, virus subtype, management of farm environment, and secondary bacterial infection.
The three influenza subtypes, H1N1, H3N2, and H1N2 cause similar histopathological lesions or infection of the respiratory system as follows. Destruction and necrosis of bronchi and bronchiole epithelium were shown by flattening of the alveoli, exudative bronchitis, and interstitial pneumonia. Desquamated cells are found with the accumulation of neutrophil in bronchi and alveoli, followed by infiltration monocytes into lung tissue. Hyperemia and vasodilation of lung vessels are then observed with infiltration of lymphocytes, histiocytes and plasma cells into alveolar membrane, accordingly.

A.3 Clinical signs

Clinical observation of sick pig and pattern of disease outbreak in pig farms often show that infected animals have acute respiratory signs like human influenza. The outbreak of SIV occurs rapidly and morbidity rate may reach 100% while mortality rate is low (1-4%).

**Signs:** Clinical presentations of swine influenza include fever, uveitis and swelling of ocular area, ocular discharge, coughing, sneezing, clear nasal discharge (Figure K.1), depressed, and anorexia causing reduction of weight gain. Diarrhea may occasionally be present. Piling of weaning pig can be observed. Sow may have a miscarriage from high fever.

**Incubation period:** 1-3 days

**Clinical presentation period:** 3 – 7 days, the average of clinical presentation period does not exceed 5 days. Without secondary infection, pig can be self recovery. Lengthening of clinical presentations up to 15 days due to weather condition and environmental factors has been reported in some countries.

**Transmission period in farm:** wide spread of SIV in pig house can occur within 7-14 days

**Shedding of SIV:** Sick pig sheds virus via respiratory secretion such as nasal discharge and phlegm. In Thailand, the experiment shows that pig can shed virus up to 4 days but in some countries the shedding period can last up to 15 days.
Appendix B

Sampling
(Section 3.1)

B.1 Sampling or sample collection by nasal swab

Sampling shall take into account the health status of herd:

- If the animal initially shows respiratory signs, at least 20 nasal swabs shall be collected from the sick animals.
- If farm has an SIV outbreak within 30 days but respiratory sign is absent at the day of sample collection, a total number of 20 nasal swabs shall be collected from suspected animals.
- In order to confirm SIV-free status in a farm that the latest outbreak occurred more than 30 days ago and there is no pig with signs of respiratory disease on the day of sample collection, nasal swab shall be performed on normal individual and the number sample collected shall adhere to Table B.1.

**Table B.1** Sample collection by nasal swabbing technique for confirmation of SIV-free status in farm

<table>
<thead>
<tr>
<th>Total number of animals in farm (N)</th>
<th>Total number of sample collected based on suspected level of SIV shedding (N)</th>
<th>Detection 1% of shedding (99% confidence)</th>
<th>Detection 1% of shedding (95% confidence)</th>
<th>Detection 5% of shedding (99% confidence)</th>
<th>Detection 5% of shedding (95% confidence)</th>
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Appendix C

Sample collection method by nasal swabs

(Section 3.2.1)

C.1 Materials and methods

C.1.1 Materials for nasal swabbing technique

- 3-5 ml screw cap test tube
- Sterile dacron or rayon or polyester swab should be used, however, non-fluorescence, bleach-free cotton swab with 15 cm long stalk may also be used. The stalk should be immediately excluded after used because it may interfere with the diagnosis.

C.1.2 Collection method

C.1.2.1 Hold pig’s face upward and lift up the snort. In piglet, the animal may be restrained by keeping the pig in standing posture facing the collector. Snare may also be used in restraining the animal (Figure K.2).

C.1.2.2 Clean outside of the nostrils with towel to prevent contamination

C.1.2.3 Insert the cotton tip of the swab into the nostril, and move swab in the dorsal-median direction. Then, carefully circle the swab to obtain nasal epithelium and secretion as much as possible. The same swab should be applied for both nostrils. The depth of insertion depends on pig’s ages as follows:

- 0-4 weeks old piglet, 1 cm;
- 4-7 weeks old piglet, 2 cm;
- >7 weeks old fattening pig, 3-4 cm;
- Breeder, at least 4 cm, however, the depth may be adjusted as appropriate.

C.1.2.4 Put the cotton tip in transport medium immediately after used. Remove or break the stick below the opening of the test tube to allow the cap to be closed and close tightly.

C.1.2.5 Label the test tube, details should include animal’s identification code, ages, signs, date of collection and farm location. Samples shall be immediately kept at 2-8°C until arriving at the laboratory. Samples should arrive at the laboratory within 24 h after collection.
Appendix D

Preparation of antibodies and chemicals for ELISA technique

(Sections 3.2.1 and 3.3.1.3)

D.1 PBS preparation for transport medium

<table>
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<th>Ingredient</th>
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<tr>
<td>Na₂HPO₄</td>
<td>1.15 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
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(1) Add deionized water until 1 L of volume is obtained.
(2) Autoclave the solution.

D.2 Transport medium preparation

(1) pH 7.4 PBS, or Eagle's minimal essential medium (MEM)
(2) 1% bovine serum albumin (BSA)
(3) Antibiotic such as penicillin G (2x10⁶ U/l), streptomycin (200 mg/l), gentamicin (250 mg/l)
(4) Divide the medium to 1-2 ml/tube.

D.3 Important equipment and chemicals for virus isolation by cell culture technique

- class II biological safety cabinet
- T-75 or T-25 tissue culture flasks
- 24 well tissue culture plates
- Eagle's minimal essential medium (MEM)
- bovine serum albumin fraction V, 7.5% solution
- fetal bovine serum (FBS)
- trypsin-EDTA
- trypsin, tosyl phenylalanyl chloromethyl ketone (TPCK) treated (type XIII from bovine pancreas)
- gentamicin reagent solution
- penicillin-streptomycin

D.4 Preparation of MDCK culture medium

(1) Prepare from 500 ml working MEM (preparation of working MEM is depended on its manufacturer protocol).
(2) Add 25 ml of FBS (5% FBS in MEM is obtained).
(3) Add 5 ml penicillin-streptomycin.

D.5 Preparation of cell culture medium MDCK

(1) Prepare from 500 ml working MEM (preparation of working MEM is depended on its manufacturer's protocol).
(2) Add 25 - 50 ml BSA (5-10% BSA in MEM is obtained).
(3) Add 5 ml penicillin-streptomycin.
(4) Add 0.5 ml TPCK-trypsin stock (to obtain final concentration of 2 µg/ml).

D.6 Preparation of 2 µg/ml TCPK-trypsin stock

(1) Dilute 20 mg TPCK-trypsin with 10 ml sterile deionized water or sterile saline water (depending on manufacturer’s recommendation).
(2) Filtrate through 0.22 µg/ml filter paper.
(3) Divide MEM into 1.5 ml/tube and store at -20°C.

D.7 Essential equipment and chemicals for HI test

- centrifuge and centrifuge tubes
- incubator
- V-bottom microtitration plates
- deep well microtitration plates
- 56°C water bath
- single channel and multichannel pipette
- pipette tips
- biological safety cabinet
- reagent reservoirs

D.8 Preparation of 10x PBS, pH 7.4 for HA and HI test

<table>
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<tr>
<th>Chemical</th>
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<tr>
<td>Na₂HPO₄</td>
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<td>NaH₂PO₄</td>
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<tr>
<td>NaCl</td>
<td>85.00 g</td>
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(1) Add weighed chemical to 1000 ml sterile deionized water and mix well.
(2) Dilute 10 X PBS with sterile deionized water at the ratio of 1: 9 before use.
(3) Adjust pH to 7.4 ± 0.1.
Appendix E

Diagnosis of Swine Influenza by serological tests

(Sections 3.3, 3.3.1.2 and 3.3.2)

E.1 Preparation of 0.5% chicken red blood cells suspension

(1) Obtain 3 ml of whole blood from fully mature chicken. Mix the blood with Alsever’s solution at the ratio of 1:1 and slowly flip the tube up and down to prevent blood clot.
(2) Centrifuge at 800 g for 10 minutes and remove supernatant.
(3) Wash the cells 3 times with PBS (10 times volume to the blood), slowly flip the tube up and down and centrifuge at 800 g for 10 minutes.
(4) Remove supernatant.
(5) Add PBS, 10 times the volume of the blood and mix by slowly flip the tube up and down.
(6) Repeat steps (3) to (5) for two more times.
(7) Centrifuge at 800 g for 10 minutes.
(8) Remove supernatant. Packed red cells can be kept at 4°C for 1 week.
(9) Add 10 ml PBS to the 500 µl packed red cells from (8) to obtain 0.5% red cell concentration before use and shake lightly. The 0.5% chicken red blood cells can be kept at 4°C for no longer than 1 week. If the breakdown of the red blood cells is observed, prepare a new batch. Mix the cells well before use.

E.2 Haemagglutination test (HA test) for testing haemagglutination property of the red blood cells

Haemagglutinin proteins on a surface of the virus have ability to attach to the specific receptor on red blood cell surface which results in red blood cell agglutination. This ability is then used to detect the presence of SIV. HA test is usually performed after the method of viral isolation because HA test requires high amount of virus. In addition, false positive is likely to occur because there are other viruses and bacteria that may interfere with the reaction. The result shall be read within 30-45 minutes. Otherwise, red blood cell will spontaneously agglutinate resulting in false positive. HA test methods are described as follows:

(1) Use micropipette for adding 50 µl of PBS to each well of a 96-well microtitre plate.
(2) Add 50 µl of the solution per well from embryonated fluid or the virus from cell culture to the first row of the plate, 2-4 wells per sample. Thoroughly mix by pipetting the solution up and down.
(3) Perform 2 fold dilutions by adding 50 µl of the solution from the first row to the second row. Serially repeat the step until the solution reaches the row before last. The last row is used for a negative control.

(4) Add 50 µl of 0.5% chicken red blood cells (Section E.1) into each well.

(5) Mix well by tapping the plate(s) lightly and place the plate(s) at room temperature for 45 minutes.

(6) Read test result. The positive result is shown by the complete agglutination of red blood cells at the bottom of the wells (Figure K.6).

The well(s) which has the most dilute antigen is recorded as 1 HA unit (HAU).

E.3 Haemagglutination inhibition test (HI test) for testing inhibitory property to prevent erythrocyte agglutination of the antibody in serum

HI test is a method for detecting antibody that possesses ability to prevent viral haemagglutinin protein to attach itself to the specific receptor on red blood cell to form virus-erythrocyte complexes which result in red blood cell agglutination. Pig begins to develop antibody to the virus within 5-7 days of infection. The highest level of antibody titre is observed during 3-6 weeks of infection. Level of maternal antibody detected in pig usually prolongs for 4-8 weeks. However, it depends on the initial level of the antibody in colostrum received during nursing period. The benefits of HI test are the ability to perform in live pig and convenience in collection of blood. The method is simple to perform in general laboratories. Pair serum should be collected at 10-21 days apart in order to detect the increase in antibody level. In addition, HI test can be used to differentiate SIV subtype because antibodies to haemagglutinin protein of SIV are specific to each SIV subtype. Hence, this technique requires all of the SIV subtypes to be used as references. HI test methods are described as follow:

(1) Dilute viral antigen to the concentration of 4 HAU or 8 HAU per 25 µl in 0.01 M PBS, at pH 7.2-7.4. Back titration should be performed to confirm the correct antigen concentration

(2) Add 25 µl PBS into each well of a 96-well V- or U-bottom microtitre plate.

(3) Add 25 µl of serum sample into the first well of each row. Three replicates per sample are recommended.

(4) Perform a two-fold serial dilution.

(5) Add 25 µl of reference antigen (1) (positive control antigen) into each well.

(6) Add 50 µl PBS to wells to test a precipitation of red blood cells.

(7) Cover microtitre plate(s) and incubate at room temperature for 10-30 minutes.

(8) Add 50 µl of 0.5% red blood cell suspension to each well and shake the plate(s) to mix thoroughly.

(9) Cover microtitre plate(s) and incubate at room temperature for 10-30 minutes or until erythrocytes in control well are settling at the bottom of the plate.
(10) For reading of test result, HI test is positive when there is an evidence of erythrocyte agglutination inhibition. Erythrocytes are settled down at the bottom of the well which have a button-like appearance or tilt the plate and observe flow rate of erythrocyte, the rate should be the same as that of erythrocyte in positive control well(s).

(11) For interpretation of result, antibody to any SIV subtype is presented if the level HI titre is read at 1:40 or higher, when the viral antigen of that specific SIV subtype are used at the concentration of 4 HAU/25 µl or 8 HAU /25 µl.

Pair serum collected at 10-21 days interval should show 4 times increase in HI titre. If the level of HI titre is less than 1:40, the tested animal is not infected with SIV.

Remarks
1. Positive control serum and negative control serum are required in order to confirm a test result.

2. Serum normally contains non-specific inhibitor, which prevent blood clotting and aggregation of red blood cells. This non-specific inhibitor may contribute to false positive. Consequently, receptor destroying enzyme should be added to inhibit the non-specific inhibitor. Moreover, WHO recommends that positive result is accepted when HI titre is 1:40 or higher. Eradication of non-specific inhibitor is described as follows:

(1) Prepare 100 units/ml of receptor destroying enzyme (RDE) by making 1/10 RDE dilution in calcium saline solution or as recommended by the manufacture’s protocol.

(2) Mix 50 µl of reference serum (reference serum for H1N1, H1N2 and H3N2 isolates) with 200 µl RDE and incubate in water bath at 37°C for 12 - 18 h.

(3) Add 150 µl of 2.5% sodium citrate and incubate at 56°C for 30 minutes.

(4) Mix 200 µl of the serum in (2) with 25 µl PBS.

(5) Add 50 µl of 50% concentration of red blood cell suspension, shake/agitate to mix thoroughly and incubate at room temperature for 30 minutes or incubate at 4°C, overnight.

(6) Centrifuge at 800 g at 4°C for 10 minutes.
Appendix F

Diagnosis of Swine Influenza by Molecular Biological Techniques
(Sections 3.3, 3.3.1.2 and 3.3.1.3)

Diagnosis of Swine influenza by molecular biological technique comprises of 2 steps as follows:

1. RNA extraction by conventional method or by any commercial RNA extraction kits, which have been approved that the sensitivity and specificity are similar to the conventional method.

2. Detection of SIV antigen by RT-PCR, which includes cDNA synthesis, followed by replication of target DNA. RT-PCR can be performed either by a one-step RT-PCR method or by primary constructing cDNA (Appendix J) and then perform a conventional PCR on the constructed cDNA.

F.1 RNA extraction

1. Add 750 µl Trizol LS to 250 µl of processed sample of nasal swab or lung tissue (as indicated in Section 3.3.1.1) and mix thoroughly by pipetting the solution up and down.

2. Incubate the sample in (1) at room temperature for 5 minutes

3. Add 200 µl Chloroform and shake/agitate to mix thoroughly for 15-20 seconds.

4. Incubate at room temperature for 15 minutes.

5. Centrifuge at 10,000 rpm at 4°C for 15 minutes.

6. Add 500 µl isopropanol and mix thoroughly.

7. Incubate at room temperature for at least 10 minutes.

8. Centrifuge at 10,000 rpm at 4°C for 15 minutes and remove supernatant.

9. Wash the precipitate with 75% ethanol, at the ratio of 1 µl : 500 µl.

10. Centrifuge at 10,000 rpm at 4°C for 15 minutes and remove supernatant.

11. Place at room temperature for approximately 20 minutes or until the precipitate is dry.

12. Redissolve the precipitate in 40 µl in diethylpyrocarbonate (DEPC)-treated water for cDNA synthesis. If RNA is not used immediately, store the RNA in -70°C until used.

**Remark:** Commercial RNA extraction kit may also be used. The methods should adhere to the manufacturer instruction.

F.2 cDNA synthesis and a one-step RT-PCR method

Use a one step RT-PCR commercial kit: Access Quick™ RT-PCR Kit (Promega, USA). Primers sequences from 5´– 3´, used in the reaction are as follow:

1. Prepare 22 µl Master mix in 0.5 ml test tube as indicated in Table F.1
**Table F.1 PCR Master mix**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAse free water</td>
<td></td>
<td>8.0 μl</td>
</tr>
<tr>
<td>Master mix 2X</td>
<td></td>
<td>12.5 μl</td>
</tr>
<tr>
<td>Primer MF</td>
<td>25 pmol/μl</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Primer MR</td>
<td>25 pmol/μl</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>AMV Reverse Transcriptase</td>
<td></td>
<td>0.5 μl</td>
</tr>
<tr>
<td><strong>Total (μl)</strong></td>
<td></td>
<td>22.0 μl</td>
</tr>
</tbody>
</table>

**Table F.2 Examples of primer used in the RT-PCR of swine influenza virus**

<table>
<thead>
<tr>
<th>Direction</th>
<th>Base sequence</th>
<th>PCR-product size (base pair:bp)</th>
<th>Specificity level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward (MF)² / Reverse (MR)²</td>
<td>5’-TGATCTTCTTGAAAATTTTGAG-3’ / 5’-TGTTGACAAAATGACCATCG-3’</td>
<td>276 bp</td>
<td>M gene (Influenza A)</td>
</tr>
</tbody>
</table>

**Remark:** Primers were designed from a part of viral M gene. The primers are for the detection of type A influenza virus. Additional primers are required in order to test the specific HA and NA genes.

2 Add 3 μl of viral RNA, close the test tube and

(3) Place the test tube in thermocycler and set PCR conditions as follows:

- Step 1 Reverse transcription 48°C 45 seconds
- Step 2 Initial PCR activation 94°C 3 seconds
- Step 3 Denaturation 94°C 20 seconds
- Step 4 Annealing 55°C 20 seconds 35 cycles
- Step 5 Extension 72°C 30 seconds
- Step 6 Final extension 72°C 10 minutes

(4) While the PCR thermocycler is working, prepare 1.5% agarose gel in TBE buffer. Heat the undissolved gel using microwave; apply medium heat or approximately 300 – 600 watt, for 3 - 5 minutes. Pour dissolve gel into horizontal gel box with comb that provides sufficient channel for the test samples. Wait until the gel is dry and then place the gel in electrophoresis chamber containing TBE buffer. Level of TBE buffer must be completely above the gel.

(5) Mix 10 μl of PCR product with 2 μl of 6X loading dye and load into the gel. The first column is preserved for DNA marker (100 bp).

(6) Discriminate the PCR product with gel electrophoresis, at 100 voltages for 45 - 60 minutes.

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(7) Stain agarose gel with 2 µl/ml ethidium bromide in distill water for 15 minutes. Florescence dye may also be used to stain DNA.

(8) Read result with visible-UV gel transmission to observe size of PCR product.

(9) Interpretation of result, size of DNA band of type A Swine influenza virus (Figure F.1), is adhered to primers used in the reaction as described in Table F.2.

Figure F.1 276 bp PCR product obtained by RT-PCR technique, using specific primers targeting M gene of Swine influenza virus.
Appendix G
Immunohistochemistry (IHC)
(Section 3.3)

G.1 Principle

IHC is a method for detecting SIV antigens in formalin-fixed, paraffin-embedded tissues. This method is suitable for retrospective study because tissues can be preserved for a long period of time in a paraffin block. Specificity of detection is limited to the type of antibody used. For instance, monoclonal anti-influenza A (nuclidean) is able to detect all type of influenza A virus, however, it cannot differentiate between influenza subtypes. In order to identify each subtype, other techniques such as PCR may be required. Sensitivity of the tests is depended on tissue quality due to tissue preservation by formalin, which may obscure viral antigens present in a tissue.

G.2 Influenza virus detection by Immunohistochemistry technique

1. Chop tissue into 0.5-1 cm pieces, and bath in 10% buffered formalin for less than 24 h and then embed in a paraffin block.
2. Deparaffinise by applying heat to the sliced formalin-fixed, paraffin-embedded tissue (tissue thickness should be less than 4 µm), and rehydrate through immersion in 100%, 95%, 80% and 70% alcohol, respectively. Wash the samples with tap water and distilled water.
3. Retrieve antigen by adding 0.1% proteinase K, and incubate at 37°C for 10 minutes.
4. Rinse 3 times for 5 minutes in PBS and block non-specific endogenous peroxidase with 3% H2O2 in absolute ethanol, at room temperature.
5. Rinse 3 times for 5 minutes in PBS.
6. Block non-specific binding with 1% bovine serum albumin in PBS, at 37°C for 30 minutes.
7. Rinse 3 times for 5 minutes in PBS.
8. React with monoclonal anti-NP influenza A antibody at appropriate concentration and time.
9. Rinse 3 times for 5 minutes in PBS.
10. Apply 1:400 biotinylate rabbit anti-mouse IgG, at 37°C for 30 minutes.
11. Rinse 3 times for 5 minutes in PBS.
12. Apply envision polymer (Envision Polymer DAKO, Denmark), at 37°C for 30 minutes.
13. Rinse 3 times for 5 minutes in PBS.
14. Stain with 0.05% DAB (3, 3´-diaminobenzidine tetrahydrochloride 0.01 M Tris-HCL, pH 7.6) (Sigma, USA) for 30-60 minutes.
15. Rinse with distilled water for 1 minute.
(16) Counterstain with Mayer’s hematoxylin for 45 minutes.

(17) Rinse thoroughly with tap water for 5 minutes.

(18) Rehydrate tissues rapidly by immersion in 70%, 80%, 95% and 100% alcohol, respectively, and cover with glass slide.

(19) Read result, positive result is indicated by the presence of brown staining in cell’s nucleus. Negative result is indicated by the absence of brown staining in the nucleus.

(20) Interpretation of result, reddish-brown staining of antigens in tissue indicates that there is an evidence of SIV infection in this particular tissue.

**Recommendation:**

Sufficient humidity should be applied at all time by keep the sample in moist chamber.
Appendix H

Quantitative determination of SIV by virus titration

(Section 3.3.1.3)

(1) Culture MDCK cells in 24-well cell culture plate, and obtain a concentration of more than $10^6$ cell/ml. Add 200 µl of minimal essential medium (MEM), containing 5% fetal bovine serum (FBS) into each well.

(2) Place in 37°C, 5% CO₂ incubator for approximately 48 h, until the cells are formed in the manner of confluent monolayer (80-100% of the well’s area).

(3) Prepare viral suspension using MEM and 1 µg/ml trypsin in test tube to obtain the concentrations of 1:10, 1:10², 1:10³, 1:10⁴, 1:10⁵ or until a prefer concentration is achieved. Store at 4°C or place on ice while waiting for the cultured cell washing procedures.

(4) Remove cell culture medium (1) and wash the rinse 3 times with 100 µl PBS/well.

(5) Add 100 µl of viral suspension (3) to the confluent monolayer. Three-four replicates should be applied. Add MEM and 1 µg/ml trypsin instead of viral suspension to the wells used as negative control.

(6) Place the plate in 37°C, 5% CO₂ incubator, and observe the forming of cytopathic effect (CPE) every day for 5 days.

(7) Apply the immunoperoxidase monolayer assay (IPMA) to the samples and calculate viral titre by the methods described by Reed and Muench, 1938.

(8) Viral suspension may also be used for HA test or real time RT-PCR.
Appendix I

Immunoperoxidase monolayer assay (IPMA)

(Section 3.3.1.3)

(1) Remove cell culture medium from culture plate from article 3.3.1.3. Preserve cells with 100 µl/well of 4% formalin diluted with 0.5% Phosphate Buffered Saline Tween-20 (PBST). Leave the plate at room temperature for 30 minutes.

(2) Remove formalin and rinse 3 times with 100-200 µl of 0.5% PBST. During the third wash, submerge with PBST for 5 minutes.

(3) Add anti-influenza A nucleoprotein monoclonal antibody at ratio of 1:2000. Dilute with 50 µl 0.5% PBS containing 1% BSA (freshly prepare) and place at room temperature for 60 minutes.

(4) Remove solution and rinse with 0.5% PBST as described in (2).

(5) Add anti-mouse IgG conjugate in the ratio adhered to the manufacturer protocol. Dilute with 50 µl 0.5% PBS containing 1% BSA (freshly prepare) and place at room temperature for 60 minutes.

(6) Remove solution and rinse with 0.5% PBST as described in (2).

(7) Add 100 µl of substrate to each well. The substrate’s compositions are as follow: AEC: acetate buffer: 30% H₂O₂ at the ratio of 1 µl: 19 µl: 20 µl (this amount is sufficient for 2 plates), and place at room temperature for 60 minutes.

(8) Positive result is indicated by the presence of brown staining in cell’s nucleus when compare to positive control. Negative result is indicated by the absence of brown staining in the nucleus.

(9) Interpretation of result, SIV infection is confirmed by the presence of reddish-brown stain of the nucleus.
Methods for cDNA synthesis (reverse transcription) are as follows:

1. Use 1 µg RNA as RNA template
2. Synthesis the first strand cDNA. The optimal condition of PCR reactions requires 20 µl mixtures of:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexamer primer 144R</td>
<td>0.75 µM</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1 mM</td>
</tr>
<tr>
<td>M-MLV (Moloney murine leukemia virus reverse transcriptase)</td>
<td>2.5 Unit</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5 mM</td>
</tr>
<tr>
<td>PCR buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl)</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

3. Place the samples in thermocycler and increase temperature to initiate the reactions.
4. Set temperature at 42 °C for 15 minutes to activate reverse transcription reaction of RNA template to obtain the first strand cDNA. Then, increase temperature to 100 °C for 5 minutes to stop the reactions of reverse transcriptase enzyme, and adjust temperature to 5 °C before PCR is further applied.
Appendix K

Illustrations of Swine influenza diagnosis

Figure K.1 (a) The clinical signs showing acute respiratory disease
(b) Uveitis (ocular inflammation)
(c) Clear nasal discharge
Figure K.2 Sample collection for swine influenza virus diagnosis, collection of nasal secretion by nasal swab technique.

Figure K.3 Demonstration of blood collection in piglet.
**Figure K.4** (a) and (b) Tissue collection areas of the lungs (circles)

**Figure K.5** Inoculation locations used for virus isolation and propagation in chick-embryonated egg.
Source: http://wenliang.myweb.uga.edu
**Figure K.6** HA test (a) Red blood cell agglutination indicating positive result (b) button-like appearance result indicating negative result

**Figure K.7** HI titre at 1:1280, button-like appearance of red blood cell (Box No. 9)

**Figure K.8** Immunoperoxidase monolayer assay (IPMA) staining technique (a) Reddish-brown staining of nucleus indicating SIV infection (b) Normal cells without reddish-brown staining of nucleus