3. MATERIALS AND METHODS

3.1 Location of the study area and data collection

This study as an experimental study using samples collected from pre-slaughter chicken proved to be free from avian influenza virus by isolation in embryonated chicken eggs and harvesting allantoic to detected haemaglutination test, then indirect test with Newcastle disease virus serum which free from disease at Animal Health and Technical Service in Bangkok. Cloacal swab and lung samples were collected from 100 chickens.

3.2 Highly pathogenic avian influenza virus (H5N1)

The avian influenza virus strain H5N1 (A/chicken-2 /NP/Thailand//2004) was used.

3.3 Sample size determination

30 cloacal swabs and 20 lungs were collected from 10 apparently healthy chickens per group. In this experiment, there were 10 set of 10 chicken each groups were examined. The factorial experimental design was used with 100 samples in each block of analysis method and each sample type (Table 1)

Table 1. Total number of experiment

Virus conc.	Cloacal swabs			Lung organs		
	Embryonated eggs.	MDCK cell line	RT-PCR	Embryonated eggs	MDCK cell line	RT-PCR
Control group	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{0}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{-1}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{-2}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{-3}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{-4}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{-5}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{-6}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{-7}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{-8}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
HA×10^{-9}	10 samples	10 samples	10 samples	10 samples	10 samples	10 samples
Total	100	100	100	100	100	100

3.4 Collection of the samples

3.4.1 Cloacal swabs

The cloacal swab of live pre-slaughter chickens were collected: using a dry cotton or polyester swab, it is inserted into the cloacal opening of live chickens and swabbed by inserting a swab deeply into the vent and vigorously swabbing the wall in place for a few seconds. Then it is slowly withdrawn with a rotating motion down the inside of the cloacal. The tip of the swab is put into a vial containing 30 ml of transport medium (isotonic PBS, pH 7.2, penicillin 2000 IU/ml, streptomycin 2mg/ml and fungizone 0. 025mg/ml). Repeat as the first swab, 3 cloacal swab per one chicken. Each group had 10 chickens and then pool 30 cloacal swabs. After that, divided into three groups. First group for positive control. Second group for negative control and the last group for test by differential concentration of virus suspension.

3.4.2 Lung samples

The lung samples were collected from live pre-slaughter chicken which were sent to an Animal Health and Technical Service laboratory in Bangkok. where the carcasses were opened, then 20 lungs were collected from 10 chickens per sample and then the organs were pool as one sample and homogenized . After that, divided into three groups. First group for positive control. Second group for negative control and the last group for test by differential concentration of virus suspension.

The specimens for virus isolation were chilled in the ice pack immediately after collection. The cloacal swabs and tissue samples must be kept at 4ºC for inoculated of differential concentration of virus suspension.

3.5 Titrating Avian influenza H5N1 virus for infectivity

The aim of titration is to measure the concentration of infectious avian influenza disease virus in a suspension. The unit of measurement of infectivity of virulent avian influenza disease virus is the 50 percent Embryo Infectious Dose Or EID50. To determine the infectivity titer of suspension of avian influenza disease virus, a series of ten-fold dilutions is carried out on the suspension. This data is used to calculate the inoculum.

Materials

Use specific pathogen free embryonated eggs, 9-11 days old. Candle the eggs and mark the inoculation site. 70% ethanol. needle 22 gauge, 1½ inch. syringe 1ml. egg hole punch. glue or varnish. 15 ml tubes & rack. 10 ml pipettes, forceps (sterile).

Methods

Step 1. Carry out ten-fold serial dilutions of the test suspension of virus.
The range of dilutions required will be determined by the estimated infectivity titre of suspension in each 0.2 ml of inoculum.

Step 2. Estimate the infectivity titre in the suspension by considering previous titrations and storage conditions. dilute from 10^{-1} to 10^{-9} .

Step 3. Inoculate via allantoic sac 5 eggs with each dilution. Use separate needle and syringe for each dilution.

Step 4. Incubate eggs for 4 days at 38ºC.

Step 5. After 4 days incubation, harvest allantoic fluid from each egg and test for hemagglutination to determine the presence or absence of Avian Influenza virus .

Step 6. Tabulate the results in the daybook.

Step 7. Perform the result of application of the Spearman-Karber formula to calculate the infectivity titre of the original suspension;

Log_{10} Median Dose = $(X_0 - (d/2) + d(\sum r_i/n_i)$

X_0 = log_{10} of the reciprocal of the lowest dilution at which all test inocula are positive.

d = log_{10} of the dilution factor (i.e. the difference between the log dilution intervals)

n_i = number of test inoculated used at each individual dilution (after discounting accidental losses)

$\sum(r_i/n_i) = \sum(P)$ = sum of the proportion of positive tests beginning at the lowest dilution showing 100% positive result.

$$EID_{50} = 8.0+1/2(1.0) - \frac{1.0(1+4)}{5}$$

$= 8.0+0.5-1.0$

$= 7.5$

$= 10^{7.5}$ per 0.2 ml.

The total amount of virus $10^{7.5\ EID50/\ 0.2ml}$

3.6 Preparation of serial ten fold dilution of virus inoculums

The stock virus concentration of avian influenza virus antigen (EID 50) was $10^{7.5\ EID50/0.2ml}$ Making ten-fold serial dilutions in PBS. The serial dilution of virus concentration were prepared for inoculated into preparation of sample.

Step 1. Set up the sterilized glass test tubes in a rack. Label each tube clearly to indicate the dilution of its content after the ten-fold serial dilution has been carried out.

Step 2. Use a micropipette to dispense 2.7 ml of the diluent (PBS 1x, pH 7.2) to all the labeled sterile tube.

Step 3. Use a micropipette to transfer 0.3 ml of the suspension of virus to the first tube and mix. This is the first tube of the ten-fold dilution.

Step 4. Use a micropipette with new sterile tip to carry out a second ten fold dilution.
Step 5. Continue the series of ten-fold dilutions until the last tube.

The virus concentration from ten-fold dilution into each tube and all dilution of virus inoculums were keep at -70 °C. (Prepare for inoculation into the experimental samples)

3.7 Laboratory diagnosis methods

Laboratory procedures: The study was be conducted following the procedure by the Animal Health and Technical Service Laboratory in Bangkok for avian influenza virus isolation and identification using specific pathogen free embryonated egg (Bangkok Agro. Co, Ltd.) and MDCK cell line (CMU. Lab.) for virus diagnosis. This procedure allows virus identification by Haemagglutination test, Haemagglutination inhibition test by using Newcastle disease virus serum and RT-PCR .

3.7.1 Virus isolation and identification

3.7.1.1 Processing materials for virus isolation

3.7.1.1.1 Cloacal swab

30 cloacal swabs collected will be pooled as one sample was suspended in buffer containing antibiotics 30ml (isotonic PBS, pH 7.2, penicillin 2000 IU/ml, streptomycin 2mg/ml and fungizone 0. 025mg/ml). Take the cloacal swab out before inoculation. After that, the positive control in first group inoculated 200 µl avian influenza virus HA $10^{7.5}$. Second group inoculated 200 µl PBS 1x for negative control. Last group inoculated 200 µl for initial suspension of virus concentration. The

suspension form 3 group were centrifuged at 3000 for 30 min in refrigerated centrifuge (4ºC) to sediment tissue debris and most bacteria, the topper clear liquid was collected, then the liquid was filtrated with filter membrane (0.45µm). For positive control group take the inoculated material 3ml into the microcentrifuge tube, The second group was negative control take the inoculated material 3ml into microcentrifuge tube, and the last group for initial suspension of virus concentration split into 10 duplicate.

For the next dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}) same procedure as mention above was used.

3.7.1.1.2 Tissue samples (lung organs)

20 lung samples collected from 10 chickens will be pooled as one sample for virus isolation. Grind tissue in a sterile mortar and pestle with crushed glass from a pasteur pipette, making a 10% suspension with transport medium was suspended in buffer containing antibiotics 30 ml (isotonic PBS, pH 7.2, penicillin 2000 IU/ml, streptomycin 2mg/ml and fungizone 0. 025mg/ml), then transfer homogenize sample with transport medium into centrifuge tube. For the positive control in first group inoculated 200 µl avian influenza virus HA $10^{7.5}$. Second group inoculated 200 µl PBS 1x for negative control. Last group inoculated 200 µl for initial suspension of virus concentration. The suspension form 3 group were centrifuge at 3000 for 30 min in refrigerated centrifuge (4ºC) to sediment tissue debris and most bacteria, the topper clear liquid was collected, then the liquid was filtrated with filter membrane (0.45 µm), After that the positive control group take the inoculated material 3ml into the microcentrifuge tube, The second group was negative control take the inoculated material 3ml into microcentrifuge tube, and the last group for initial suspension of virus concentration split into 10 duplicate.

For the next dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}) the same procedure as mention above was used.

3.8 Virus isolation by inoculation in embryonated eggs

Materials

Use specific pathogen free embryonated eggs (SPF), 9-11 days old. egg candler. 70% ethanol. needle, 22 gauge, Syringe 1 ml. 1½ inch. egg hole punch. glue or varnish. inoculum. discard tray.

3.8.1 Candling of eggs

Examine eggs with an egg candler and place with blunt end up into egg trays. discard any eggs that are infertile, have cracks, are underdeveloped, or that appear to have a porous shell.

3.8.2 Inoculation of eggs

Place eggs with blunt end up into holding the egg up to the candler, locate the embryo. egg trays and label each egg with a specific identification number. Wipe the tops of the eggs with 70% ethanol and punch a small hole in the shell over the air sac. Three eggs per specimen are usually inoculated in the allantoic sac. Aspirate 200 µl of processed sample into a tuberculin syringe with a 22 gauge, 1½ inch needle. Insert the needle into the hole of the egg. Using a short stabbing motion, inoculate 200 µl of the process sample which is prepare from cloacal swab and lung organs in each dilution for virus concentration into allantoic cavity, 10 samples per dilution. Remove the needle. Inoculate the two other eggs in the same manner with the same syringe and needle for a total of three eggs inoculated per specimen. Discard syringe into a proper safety container. Seal the holes punched in the eggs with a drop of glue. Incubate the eggs at 38ºC to 39 ºC and humidity of 60%-70% for 5 days.

3.8.3 Observation of inoculated eggs

Post- inoculation egg were candled to 5 days. Record deaths and note the time it takes for the embryo to die. Deaths within the first day are regarded as non-specific and discounted. Discard these embryos. Deaths on subsequent days are likely to be due to avian influenza disease virus. (Figure 2)

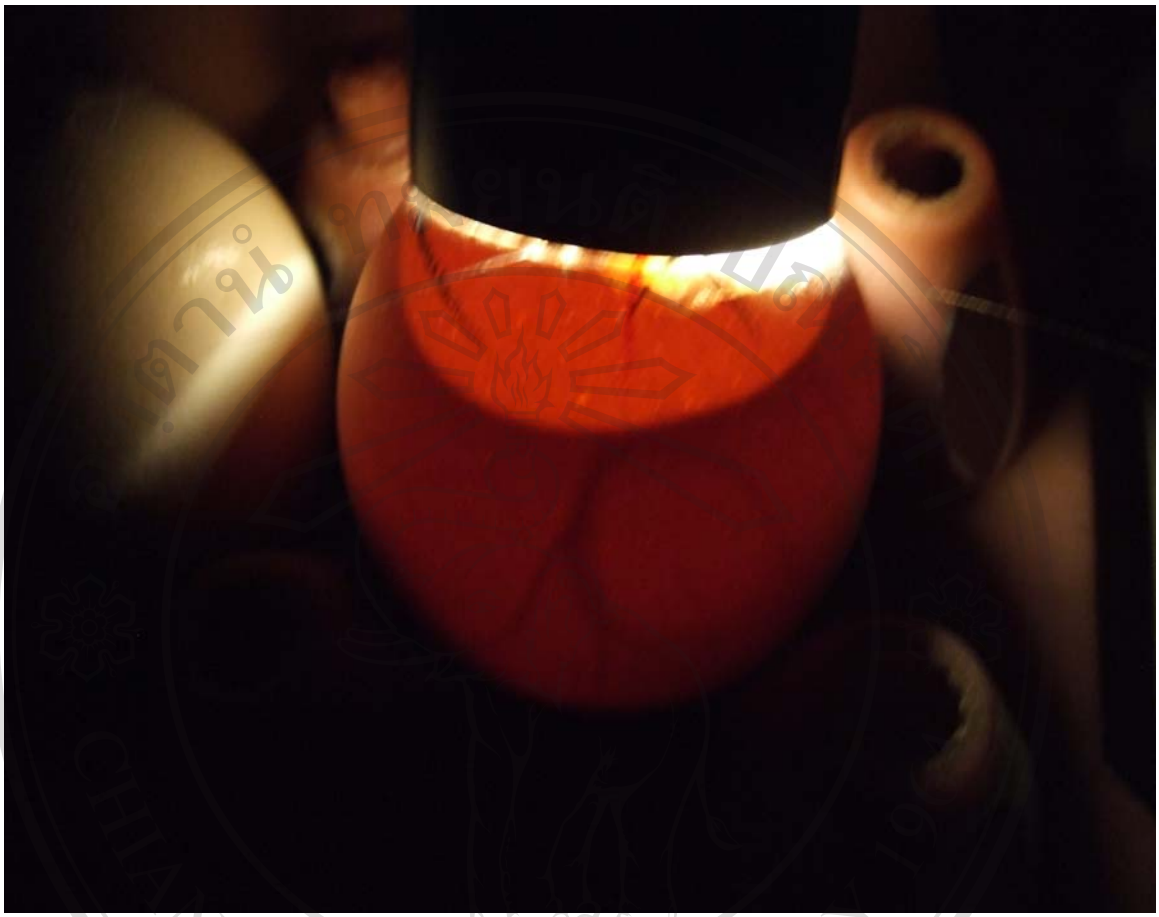


Figure 2: Observation of inoculated eggs

3.8.4 Harvesting of inoculated chicken eggs

Eggs are chilled at +4ºC overnight or for 4 hours before harvesting. Label one plastic tube (15ml) for each egg with the specimen number. Clean off the top of each egg with 70% ethanol. With sterile forceps, break the shell over the air sac and push aside the allantoic membrane with the forceps. Then using a syringe and needle, pierce the amniotic sac and remove as much amniotic fluid as possible. Place harvest in a separate tube, combine the allantoic fluid from the three eggs inoculated per specimen. Centrifuge harvested fluids and perform a hemagglutination test and incubate at 4ºC/30 mins.

3.8.5 Haemagglutination test

Material

Use 96 wells microtiter plate with V-bottom shape. multichannel micropipette 5-50 µl. , single micropipette 5-50 µl. microtip 1-200 µl. timer. phosphate buffer saline (PBS) pH 7.2. 1% chicken red blood cell. positive and New castle disease virus serum.

Method

Using the V-bottom disposable tray. Dispense 50 µl of allantoic fluid from each sample into a well of the microwell plate. Use a separate tip for each sample. Include negative and positive control allantoic fluid samples on one of the plates. Dispense 50 µl of PBS into the wells. Add 25 µl of 1 percent red blood cells to each well. Gently tap sides of the plate to mix. Place a cover on the plate. Allow the plate to stand for 45 minutes at room temperature. Observe and record the results.

Read results.

Interpretation of the result

HA negative: A sharp button of red blood cells at the bottom of the V-bottom well.
HA positive: A hazy film of red blood cells, no button or a very a small button of red blood cells at the bottom of the V- bottom well. This will be show complete haemagglutination and contains one haemagglutinating unit.

The positive result from hemagglutination test occur in the first passage, so harvest allantoic fluid and store at -70ºC. For the negative result from haemaglutination test in the first passage and continuing inoculate allantoic fluid from first passage in second passage.

The positive sample from Haemagglutination test continuing to confirmed Haemagglutination inhibition test by using Newcastle disease virus (NDV) antiserum.

3.8.6 Haemagglutination Inhibition test. (HI identification)

Using V-bottom disposable tray. Dispense 25 µl of allantoic fluid from each sample into a well of the microwell plate. Use a separate tip for each sample. Include negative and positive control allantoic fluid samples on one of the plates. Add 25 µl of Newcastle disease virus (NDV) antiserum. Add 25 µl of 1% chicken red blood cell suspension to each well. Gently tap sides of the plate to mix. Place a cover on the plate. Allow the plate to stand for 45 minutes at room temperature. Observe and record the results.

Read results.

Interpretation of the result

HI negative: A sharp button of red blood cells at the bottom of the V-bottom well.

HI positive: A hazy film of red blood cells, no button or a very a small button of red blood cells at the bottom of the V- bottom well.
This will be show complete haemagglutination inhibition and contains one haemagglutinating inhibition unit.

If the Newcastle disease virus (NDV) Haemagglutination test is negative. then, confirmed by subtyping with direct rapid test by using vet smart® test .

3.8.7 Rapid test by using vet smart® test

The commercial test kit vet smart test ®. Rockeby Avian Influenza Virus Antigen was used.

The kit is a Avian Influenza virus antigen detection (Test Type A) for invitrogen diagnostic use, Rockeby biomed. Singapore. Pte. Ltd).

This assay is designed to measure the quality of antigen. The 200 µl of the samples were taken from each tube which suspected avian influenza result from hemagglutination inbition test and pool as one sample. Take a portion of the sample (atlantoic fluid) from microcentrifuge tube with the sample collection swab. Insert the swab into the specimen tube containing assay diluent. Mix the swab until the sample has been dissolved into the assay diluent. Leave the test tube until the large particles have settled down to the bottom of the tube. Remove the test device from the foil pouch, and place it on a flat and dry surface. Using the disposable dropper provided, take the supernatant from extracted sample in the tube. Add eight drops into the sample hole with disposable droper.
(Figure 3). Interprete test results after 10 minutes.

Interpretation of the result ;

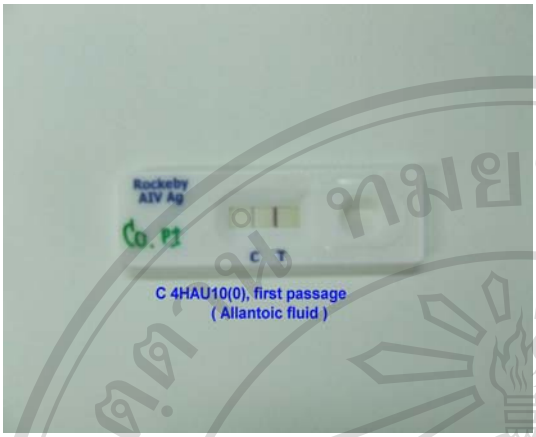
C : control band is found only this band it is the negative result

T : test group is test the sample

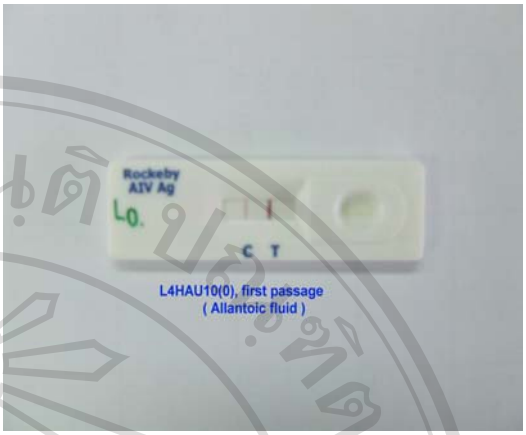
Negative result from the test found C band

Positive result from the test found both of C and T band

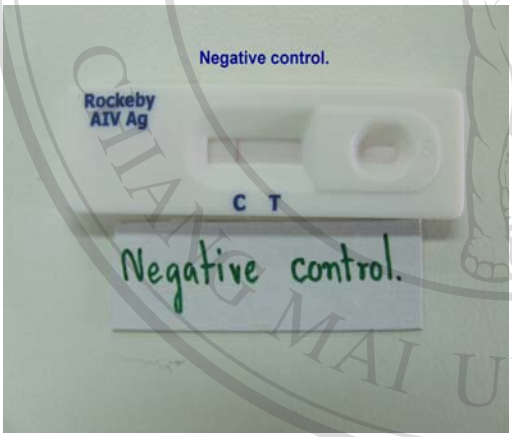
The direct rapid test by using vet smart® test for the last step to detect antigen in the suspension of experimental study in each dilution of virus concentration. The positive result in rapid test use to performable record the result. (For virus isolation and detection by using embryonated eggs)



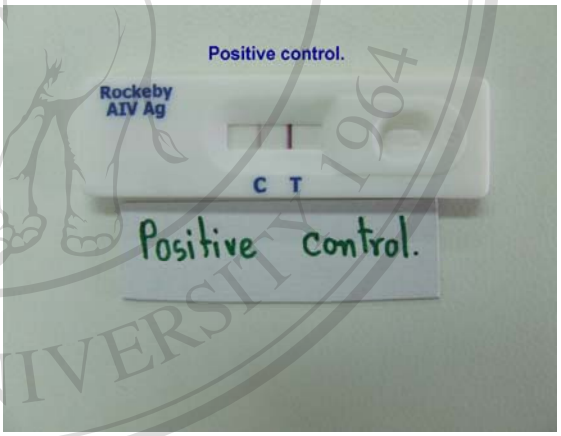
CHA10^0, First passage
(Allantoic fluid)



LHA10^0, First passage
(Allantoic fluid)



Negative control



Positive control

Figure 3: Interpretation of the result form allantoic fluid by using direct rapid vet smart® test

3.9 Virus isolation by inoculation on Madin-Darby Canine Kidney cells (MDCK)

Materials

Use Madin-Darby Canine Kidney cells (MDCK), American Type Culture Collection (CMU, Lab). T-75 and T-25 tissue culture flasks, canted neck. Corning Cat. 430720. Dulbecco's Modified Eagle Medium (D-MEM), Bovine serum albumin fraction V, 7.5% solution, Fetal bovine serum, 40 nm filtered, Trypsin-EDTA.

3.9.1 Preparation of MDCK cells in tissue culture flasks

The procedure for preparing an MDCK cell suspension is described for confluent T-75 flasks. If cell culture flasks of other sizes are used, the volumes have to be adjusted accordingly. One T-75 flask with a confluent monolayer of MDCK cells contains approximately 10^7 cells. Decant medium and add 5 ml of trypsin-EDTA pre-warmed to 37°C. Distribute trypsin-EDTA over entire cell sheet by gently rocking the flask for 1 min. Remove trypsin-EDTA with pipette. Add another 5 ml of trypsin-EDTA solution and rock flask as described above for 1 min. Remove trypsin-EDTA with pipette. Add 1 ml of trypsin-EDTA solution. Distribute trypsin-EDTA over entire cell sheet and incubate flask at 37ºC until all cells detach from plastic surface (5 - 10 min). The flasks may need shaking or tapping to detach cells. Add 1 ml of FBS to inactivate remaining trypsin. Add 8 ml of complete D-MEM. Pipette up and down gently to break up cell clumps. Transfer the 10 ml mixture to 90 ml of complete D-MEM containing 10% FBS for a final concentration of 10% FBS. (This cell suspension contains approximately 10^5 cells per ml.). The remaining cell suspension can be used to seed T-75 flasks for cell passage.

3.9.2 Inoculation of cell culture

Add 6 ml (600,000 cells) of this cell suspension to an appropriate number of T-25 flasks. Incubate flasks at 37ºC in 5%CO_2 incubator 2 to 3 days.

3.9.3 Preparation of flasks

Check the cells with microscope at 40X magnification. Replace growth medium with medium for virus growth. Be sure to use proper media as indicated. Decant growth medium into a beaker and wash three times with 6 ml of D-MEM containing 2 µg/ml of TPCK-trypsin.

3.9.4 Inoculation of flasks

Remove D-MEM from flask with sterile pipette. Inoculate 200µl of processed sample of each specimen into a T-25 flask using sterile pipettes including positive and negative control. Allow inoculum to adsorb for 30 minutes at 37ºC in 5%CO_2 incubator. .After that add 6 ml of complete media (D-MEM) containing 2 µg/ml of TPCK trypsin without calf serum to T-25 flasks. Observe daily for cytopathogenic effect (CPE) to 5 days.

3.9.5 Harvesting of flasks

Harvest the cell culture if CPE occurred on cells and observed by collecting supernatant fluid and adding stabilizer such as bovine serum albumin to a final concentration of 0.5%. Harvest by day 6 or 7, even if no CPE is observed. Label one plastic tube (15ml) for each flask with the specimen number. Centrifuge harvested

fluids at 3000 for 5 min. in refrigerated centrifuge (4ºC) to remove cells and perform a haemagglutination test and incubate at 4ºC/30 min.

Interpretation by observation on MDCK cells

If there is CPE on the cell line occur post inoculation in the first passage. Then harvested the media to detect Haemagglutination test. For the negative result in the first passage and continuing inoculated in second passage.

The Cytopathogenic effect was manifested by cell rounding, cell shrinkage and foci of cell destruction. Floating into the supernatant.

3.9.6 Haemagglutination test

(See 3.8.5)

3.9.7 Haemagglutination Inhibition test (HI identification)

(See 3.8.6)

3.9.8 Rapid test by using vet smart® test

(See 3.8.7)

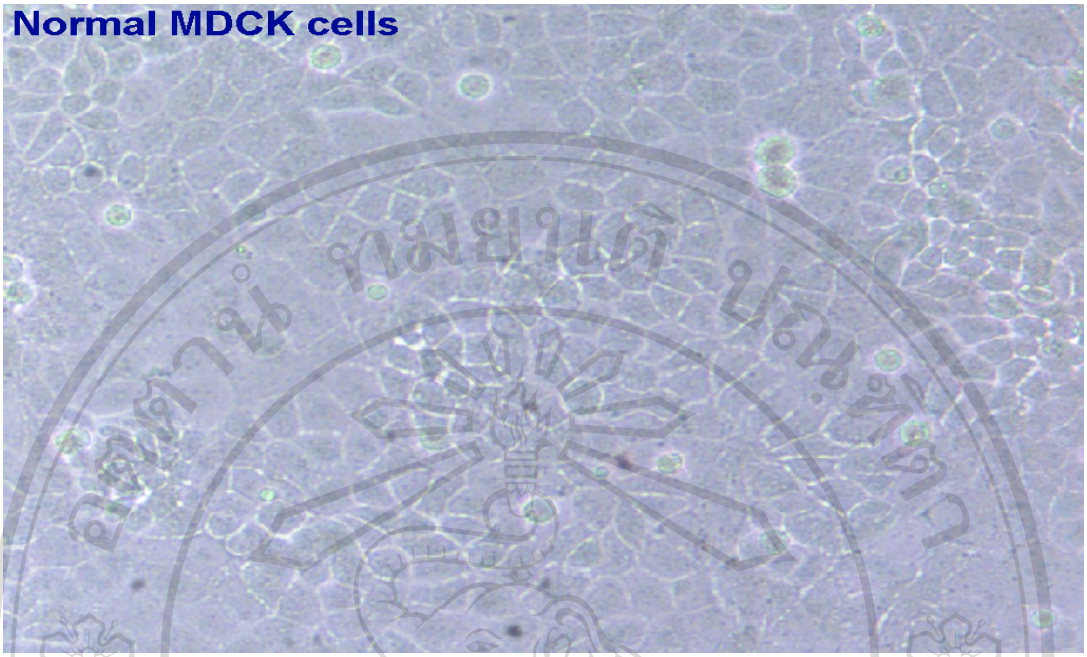
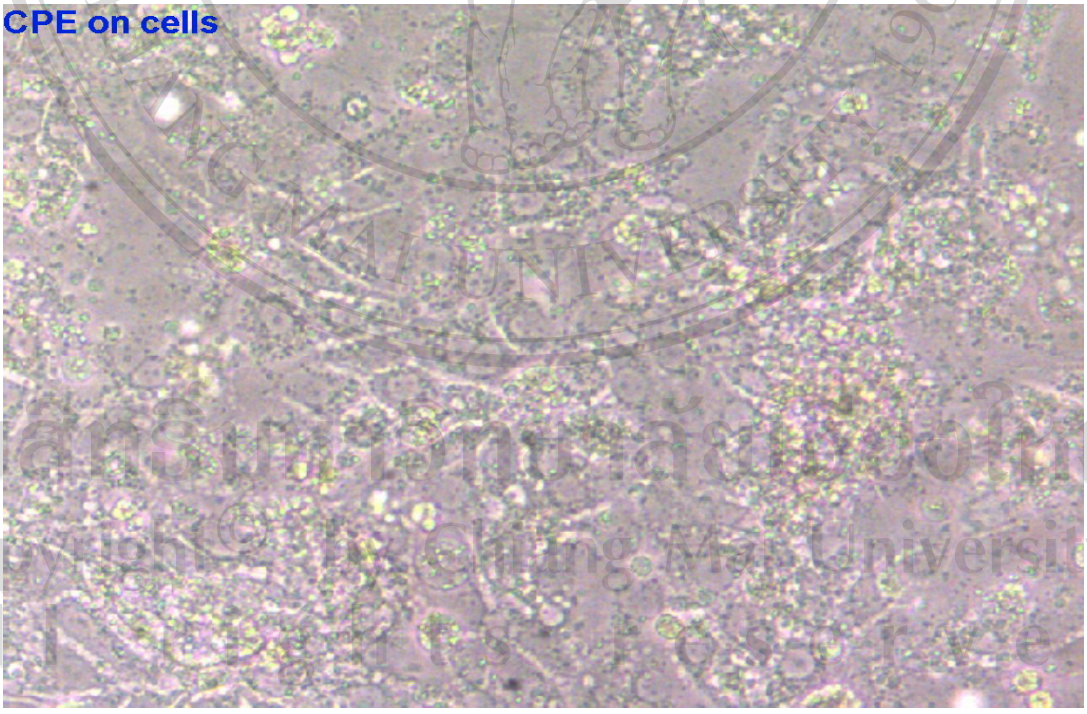
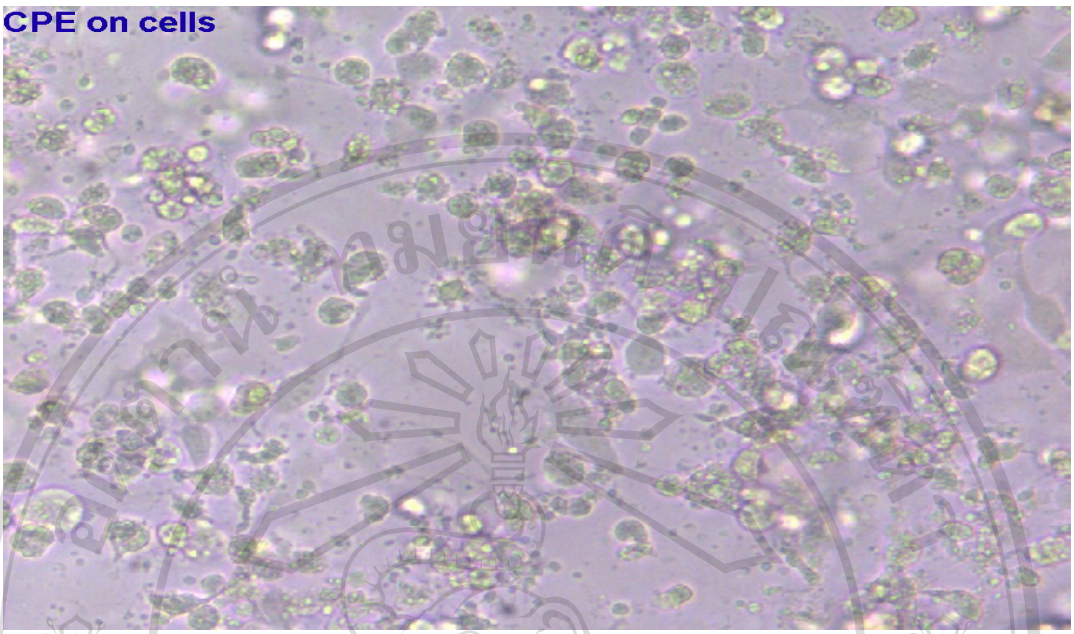


Figure 4: normal MDCK cells

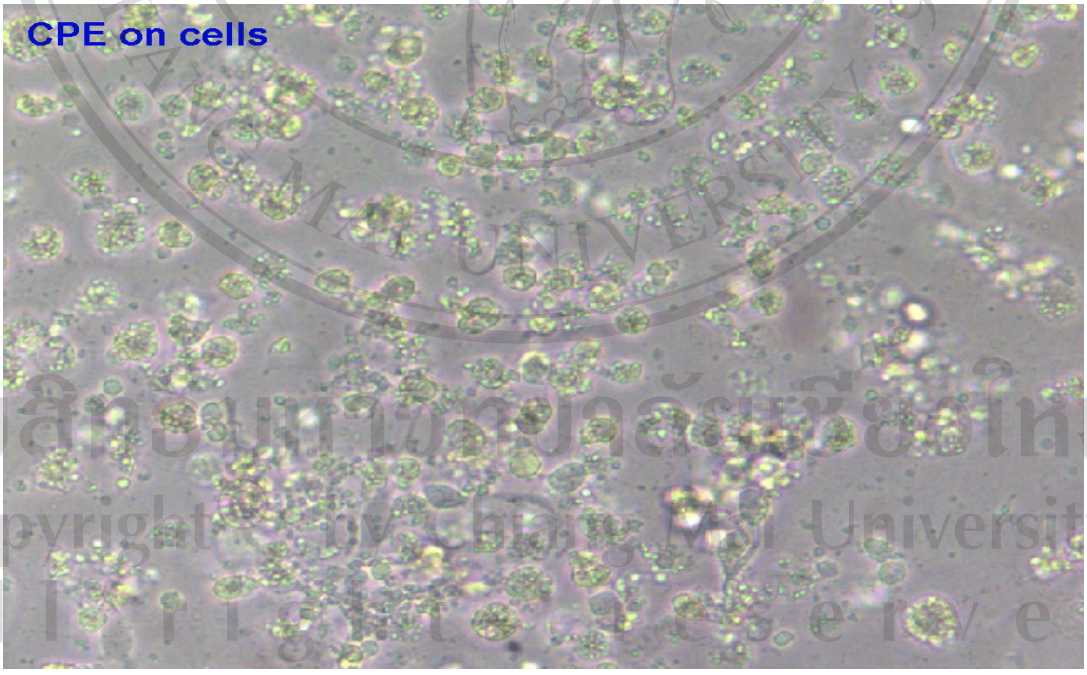


Cytopathogenic effect on cells; cell shrinkage and manifested by cell rounding

Figure 5: Cytopathogenic effect on cells



Cytopathogenic effect on cells; foci of cell destruction and floating into the supernatant



Cytopathogenic effect on cells; foci of cell destruction and floating into the supernatant

Figure 5: Cytopathogenic effect on cells

3.10 Virus identification by RT-PCR

3.10.1 Ribonucleic acid Extraction (RNA Extraction)

The commercial test kit Viral Nucleic Acid Extraction kit® (RBC Real Biotech Corporation, Taiwan), was used.

Viral Nucleic acid Extraction kit is specially designed for purification of viral RNA/DNA from cell-free samples such as serum, plasma, body fluids and the supernatant of viral infected cell culture.

Viral RNAs were extracted using a Viral Nucleic Acid Extraction Kit from supernatant of lung organ and cloacal swab: Transfer 200µl sample (supernatant from lung organs and cloacal swabs) into a microcentrifuge tube (not provided) if sample volume is less than 200 µl with PBS (not provided). Add 400 µl of VB Buffer (carrier RNA added) to the sample, mix by vortexing. Incubate at room temperature for 10 minutes. Place a VB column in a 2ml collection tube. Add 400 µl of 70% ethanol to the sample lysate and mix immediately by vortexing. Apply 600 µl of ethanol-added mixture from previous step to the VB column. Centrifuge at 6,000 xg (8,000 rpm) for 1 minute. Discard the flow-through and apply any remaining mixture from step 1 to the same VB column. Centrifuge at 6,000 xg (8,000 rpm) for 1 minute. Discard the collection tube containing the flow-through and transfer the VB column to a new 2 ml Collection Tube. Add 400 µl of Wash 1 buffer into the VB column. Centrifuge at 6,000 xg for 30 seconds. Discard the flow–through and place the VB column back in the collection tube. Add 600 µl of Wash Buffer (ethanol added) into the column. Centrifuge at 6,000 xg for 30 seconds. Discard the flow-though and place the VB-column back in the collection tube. Centrifuge at full speed (14,000 rpm) for 3 minutes to dry the column matrix. Place dried VB column in a clean microcentrifuge tube. Apply 50 µl of RNase- free water into the center of the column matrix. Stand for 3 minutes until water is fully absorbed by the matrix. Centrifuge at full speed for 1

minute to elute purified nucleic acid. Eluted nucleic acid is free of inhibitors, nucleases, proteins and other impurities, and ready for direct application in PCR and RT-PCR or other molecular assays.

3.10.2 PCR reaction

The commercial test kit Superscript III One-step RT-PCR with Platinum Tag®, Invitrogen was used.

The system is designed for the convenience, and reproducible detection and analysis of RNA molecules by a RT-PCR. Both DNA synthesis and PCR are performed in a single tube using gene specific primers and target RNAs from either total RNA or mRNA.

This assay used for identification of avian influenza virus strain H5N1. Add the 50 µl per sample of the following components to a 0.25ml thin wall microcentrifuge tube. This is added to 50 µl of the master mix. Make a PCR reaction Master Mix as follows: (Run blank for each primer pair); 2 µl Nuclease-free Water. 25 µl 2X React Buffer mix. 10 µl 50 mM MgSO. 1 µl 10 pM Primer AIVFmix (MF, forward primer: 5'- TGA TCT TCT TGA AAA TTT GCA 3' 276 bp. H5F: 5' GAC TCA AAT GTA AAG AAC CTT TA 3' 189 bp. N1F: 5'GTT TGA GTC TGT TGC TTG GTC 3' 131 bp). 1 µl 10 pM Primer AIVR mix (MR, reverse primer 5' TGT TGA CAA AAT GAC CAT CG3' 276 bp. H5R : 5' CCA CTT ATT TCC TCT CTG TTT AG 3' 189 bp. N1R : 5' TGA TAG TGT CTG TTA TTA TGC C 3' 131 bp) were synthesized by (Yong, 2004). 10 µl RNA Template 200-800 ng. 1 µl Enzyme Mix. Mix the components, cap the tube and centrifuge briefly to the bottom of the tube. Place tube in thermocycler. was performed with a DNA Engine (PTC 200). Peltier Thermal Cycler (BIO-RAD, Hercules, lif.) as follow: a. 50ºC for 30min. 94ºC for 2min. 94ºC for 15 sec (denaturation). 55ºC for 30 sec. (annealing). 68ºC for 1 min (extension), and repeat from step 2 for 35 cycles. After 35 cycles, the DNA was given a final extension step at 68ºC for 5 minutes and 25ºC until usage.

3.10.3 Gel preparation

For gel preparation, dilute the Super Agarose 10% suspension to a required agarose concentration with an electrophoretic buffer. To prepare 50 ml of 2% gel, pour 45 ml of an electrophoresis buffer into a glass flask, and pipet 5 ml of the suspension into the buffer. Use repeated pipetting of the electrophoretic buffer to wash the residual agarose from the pipette. Mix the diluted suspension and heat it to boiling in a microwave or on a hot plate with stirring, until all the agarose is melted and dissolved to form a clear homogenous solution. Cool the solution to 60 – 70° C. and add dye if desired. Pour the molten super agarose into an electrophoretic tray, position the comb in the molten gel and allow it to harden by storing at room temperature for 1 - 1.5 hr.

3.10.4 Agarose Gel Electrophoresis of the PCR products

Remove tape from gel frame and place the gel into the electrophoresis chamber; cover the gel with 1x TBE. Label the 0.5 ml microcentrifuge tubes separately. Remove 5 µl of the PCR product from each reaction tube to a corresponding 0.5 ml microcentrifuge tube (remove PCR product from underneath oil); mix with 3 µl gel loading Dye buffer**.** Load 5 µl molecular weight marker (Gene ruler 100 bp DNA ladder plus) to the first well of the 2% agarose gel. Pipette 7 µl of PCR reaction, positive control and negative control to wells of the gel separately. Close lid on chamber and attach the electrodes. Run the gel and carry out electrophoresis at 300V approximately 35 minutes until the bromophenol blue marker dye has migrated 2/3 of the way down the gel.

The gels were stained in a 2 mg/L ethidium bromide staining bath for 5 -10 minutes and visualized by presence of marker and PCR product bands, and visualized by using a UV transilluminator. (Alpha Innotech, Alphalmager® HP.). It is desirable to have an ultraviolet light source emitting light at a 302 nm wavelength. Document gel with a photograph. Compare the size of the PCR-fragments with the marker.

Interpretation of results

In experiment A the expected sizes of the PCR-products for H5 is 189 bp, and N1 is 131 bp.

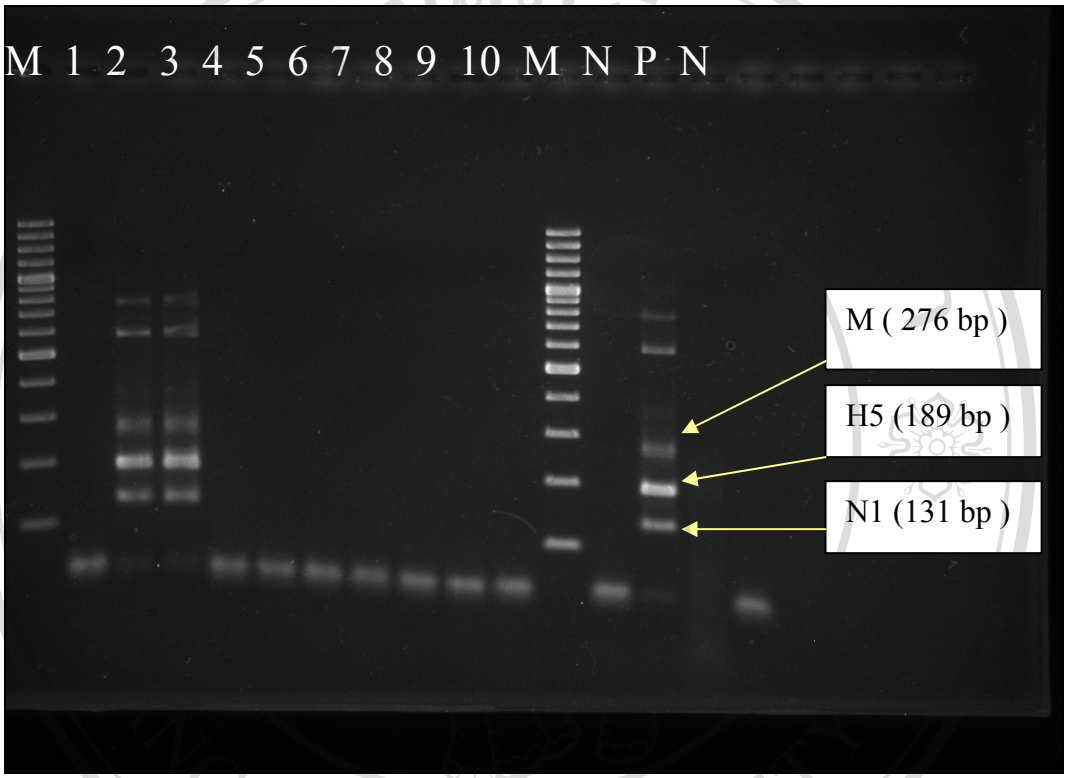


Figure 6: The result of suspension from cloacal swabs at concentration 4HAU10^{-3}

Figure 6: The size of PCR products of multiplex AIV is 276 bp, 189 bp and 131bp, corresponding to the influenza A virus Matrix gene, and N1 gene, respectively. Lane M, 100 bp standard maker ; lane2,3 are positive samples; lane 1,4,5,6,7,8,9,10 are negative result. Lane N is negative control and lane P is positive control.

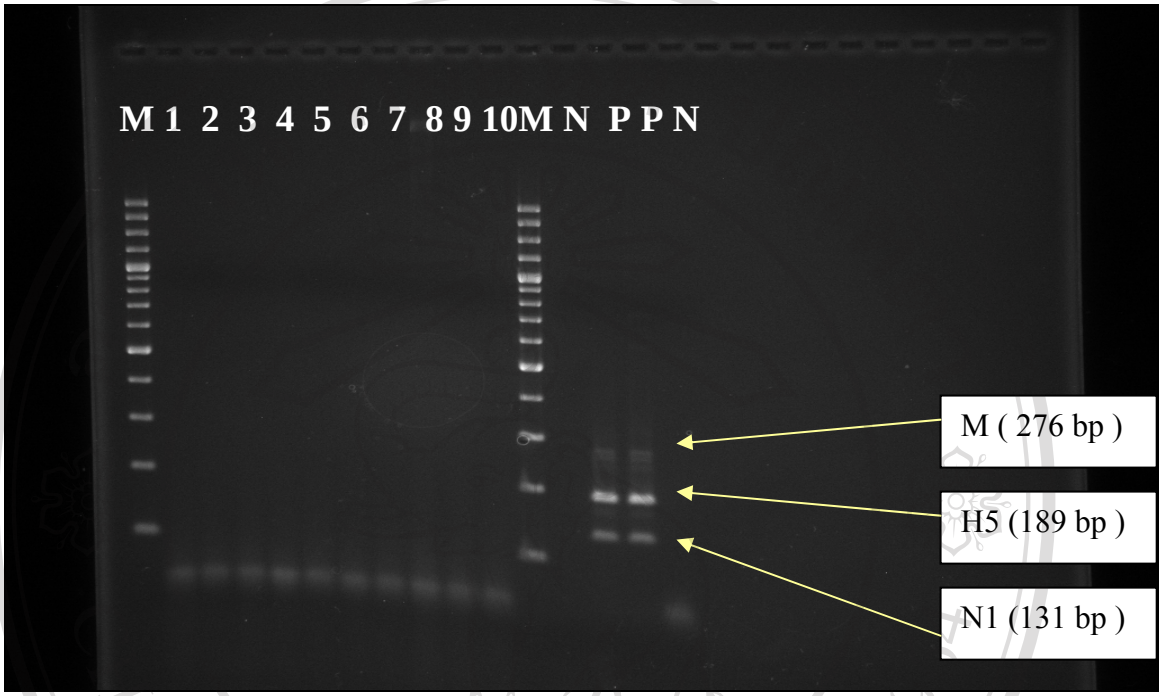


Figure 7: The result of suspension from lung organs at concentration $4HAU10^{-3}$

Figure 7: The size of PCR products of multiplex AIV is 276 bp, 189 bp and 131bp, corresponding to the influenza A virus Matrix gene, and N1 gene, respectively. Lane M, 100 bp standard maker; lane1,2,3,,4, 5,6,7,8,9 and 10 are negative result. Lane N is negative control and lane P is positive control.

3.11 Data management and statistical analysis

All the data were managed and analysed using STATA version 9. Survival analysis test was to be performed to compare viral isolation and identification between three diagnostic methods.

Log-rank test for equality of survivor functions is used to compare the difference between samples obtained from 2 different types of samples; between one by one of pair of methods; among 3 different methods (virus isolation and identification by using embryonated eggs, MDCK cells and RT-PCR). and a *P* value of ≤0.05 was considered significant.