

### 3. MATERIALS AND METHODS

#### 3.1 Place of sample collection

This study was performed at 2 live bird markets (LBM) in Harbin, the capital city of Heilongjiang Province, China.

##### 3.1.1 Background information of Harbin city pigeons

It is reported that the total population of pigeons in Harbin city is around 200,000 in 2005. According to the background investigation before the epidemiological study, a lot of Harbin citizens are pigeon fanciers and it is common for the citizens to hold the pigeons in their balcony. The pigeons for sightseeing in the public gatherings are also present with a big amount, for example in Sofya Church Square, which is located in the busy business centre of Harbin city.

The Harbin Racing Pigeon Union was build up in 1986, with the history of more than 20 years and approximately 2000 current members. The famous exhibition site of racing pigeons is located in ZhaoLin Park, where more than 170 different species of pigeons around the world were displayed here.

At the end of this study, one industrialized pigeon farm was found to be located in Shuangcheng District, 30km southwest from Harbin city. 124000 pairs of parent pigeons were fed on this 40000 m<sup>2</sup> farm and 300000 meat pigeons were produced every month.

### 3.1.2 Sample sites

There are mainly 2 live birds markets in Harbin City presently: JiangBian LBM and ChangChun Street LBM.

JiangBian LBM is a fixed market. Pigeon retailers in this market have their own fixed stores. Usually other species were also available in these retailers, such as parrots, quails and so on. The 5 retailers at JiangBian LBM handled approximately 150 pigeons per day from farms located around the Harbin city in the north-eastern part of China (around 30 pigeons per retailer). Sometimes they may also collect the pigeons from the city pigeon fanciers. So the pigeons resources are mainly the city pigeons distributed in Harbin City.

ChangChun Street LBM is some kind of fresh market. In addition to pigeons selling, some other kinds of goods are also there, such as dog, cat, vegetable, fruits, tabacoo and so on. The retailers in while the ChangChun Street LBM themselves are city pigeon fanciers. They have not their own fixed shop and the flexible transportation vehicles are used to hold their pigeons. As referred before, these fanciers keep the pigeons in their home building, which retain many public health risks especially in the densely populated communities. Around 10 pigeon fanciers in the Harbin city handled about 25-40 pigeons each. There was no report about the accurate data of pigeon population in both LBM until the study was done.

### 3.2 Sample size

When sampling for highly pathogenic H5N1 avian influenza virus it is critical that an appropriate sample size for each species or species group in each designated sample population is obtained.

Equation 1 provides a method for calculating the recommended sample size:

$$n = \lg(1-c) / \lg(1-p) \quad (\text{eq. 1})$$

Where  $n$  is the sample size,  $c$  is the desired level of confidence, and  $p$  is the prevalence of positive samples in the population. An adequate sample size should allow for >95% confidence that AI is detected at < 1.5% prevalence based on an assumed prevalence of 1.5% of highly pathogenic H5N1 avian influenza. A sufficient number of samples should be collected to give a 95% probability of detecting infection if the virus is present in 1.5% of samples. These criteria result in an estimated sample size of 200:

$$n = \lg(1-0.95) / \lg(1-0.015) = 200 \quad (\text{eq. 2})$$

### 3.3 Collection of the pigeons from the retailers in the LBM

A total of 5 pigeons were collected from 5 retailers out of the 15 retailers distributed in JiangBian LBM and ChangChun Street LBM each week and the collection duration was 8 weeks which designed to be December and March to verify the repeatability of the sampling design and the diagnostic methodology.

Each of the 15 retailer were coded with a number from 1 to 15, among which No.1-No.5 retailers were from JiangBian LBM and No.6 –No.15 from ChangChun Street (ChangChun St.) LBM (as described in Table 3.1).

The weekly selection of the retailers in the 2 markets was a random lot process. 3-6 numbers were selected randomly every time out of the 15 numbers before the sampling started. These 3-6 selected No. represented the retailers to be sampled every week sampling at different duration. The retailers sampled in this study using the random lot method were recorded from December, 2006 to the beginning of April, 2007 as Table 3.2.

**Table 3.1 The background information and coding system of the pigeon retailers**

Code No. of the retailer in this study	Market name	Retailer type	Total No. of the pigeons held
1	JiangBian	fixed	30
2	JiangBian	fixed	30
3	JiangBian	fixed	30-40
4	JiangBian	fixed	40
5	JiangBian	fixed	40
6	ChangChun St.	flexible	25-30
7	ChangChun St.	flexible	50
8	ChangChun St.	flexible	40
9	ChangChun St.	flexible	25-30
10	ChangChun St.	flexible	25-30
11	ChangChun St.	flexible	25-30
12	ChangChun St.	flexible	35-40
13	ChangChun St.	flexible	35-40
14	ChangChun St.	flexible	35-40
15	ChangChun St.	flexible	25-30

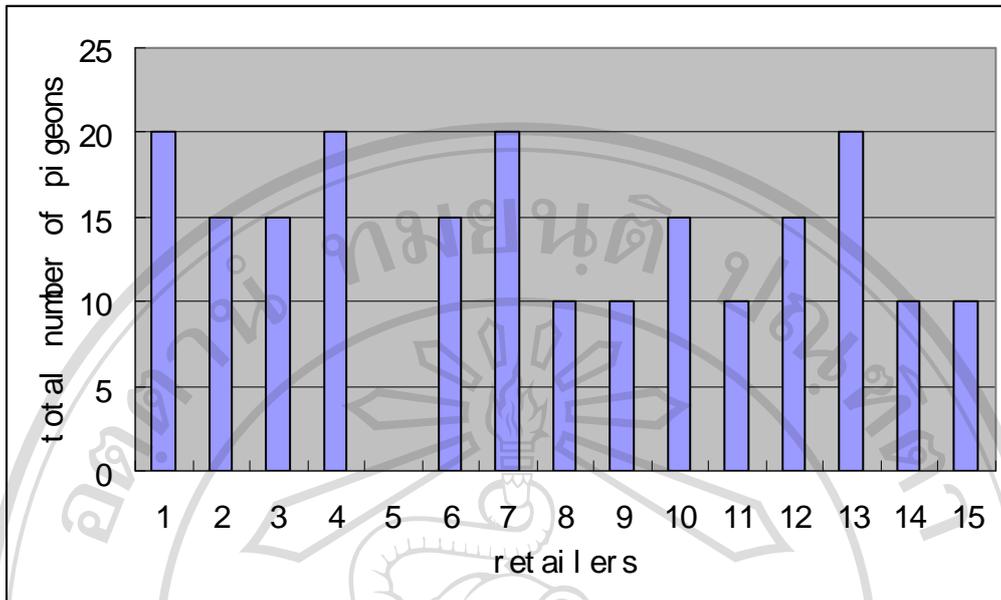
**Table 3.2 The lot schedule of the weekly sampling in different durations from Dec, 2006 till the beginning of April, 2007**

Sampling	Week	Duration	Retailer* sampled
	1st week	Dec 1st ,2006- Dec 5th,2006	13, 6, 4, 8
1st sampling	2nd week	Dec 6th,2006- Dec 9th,2006	7, 1, 14, 10
	1st week	Dec 16th,2006-Dec 21th,2006	4, 10, 7, 3, 11, 6
2nd sampling	2nd week	Dec 22th,2006-Dec 25th,2006	9, 14, 13, 12, 2, 3
	1st week	Feb 22nd,2007-Feb 28th,2007	1, 3, 4
3rd sampling	2nd week	Mar 2nd,2007-Mar 8th,2007	7, 13, 2
	1st week	Mar 18th,2007- Mar 23rd, 2007	15, 9, 4, 13, 1
	2nd week	Mar 25th,2007- Mar 30th,2007	7, 6, 12, 8
4th sampling	1st week	April 2nd,2007- April 6th,2007	11, 2, 1, 12, 10, 15

Note: Here the coding number represents the corresponding retailers described in Table 3.1 when getting the lot of different retailers to be sampled.

And the total number of the pigeons investigated was 205 pigeons out of 15 retailers. The Pigeon collecting distribution among 15 different holders was referred as in Figure 3.1.

The pigeons bought from one retailer each time were put in different cages and transported back to lab. Being bought from Harbin and holding at least 20 pigeons at the same time, the cages were treated by the 75% alcohol spraying and following 6-hour ultra-violet disinfection. Before reaching and being put into the isolation feeder, the pigeons were given coding on the feet or the wing by the metal circle. The number information and the pigeons retailers No. were recorded correspondingly.



**Figure 3.1 The Pigeon collecting distribution among 15 different holders**

After being transported from the LBM at every sampling duration, pigeons investigated in this study and were held in the BioSafety Level-2 (BSL-2) isolation feeder with negative-pressure ventilation, which are facilitated in the Poultry Infection Laboratory belonging to Lab Animal Centre of Harbin Veterinary Research Institute. To avoid the possible horizontal transmission between pigeons from different retailers, the sampling of swabs and blood was done immediately after the pigeons mixed together in the same isolation feeder.

As to the animal welfare issues, the pigeons were released to the nature if the pigeons were healthy after being held in the isolation feeder until the end of the diagnosis of avian influenza infection and also the result of the diagnostic tests of avian influenza was negative. Some diseased pigeons but verified with negative results of avian influenza infection were not diagnosed to be infected with other pathogens and destroyed by the Animal Rendering Apartment in Harbin Veterinary Research Institute. To be specified, the pigeons with positive results against the serological diagnosis of Newcastle Disease were also killed and rendered.

### 3.4 Types of Samples

The sampling work of 3 kinds samples were done with every pigeon bought from LBMs immediately after the pigeons being put into the isolation feeder, including oropharyngeal swabs, cloacal swabs and venous blood.

#### 3.4.1 Oropharyngeal swabs

The oropharyngeal swab of live pigeons was obtained from the oropharynx by inserting a dry cotton fiber-tipped swab into the posterior wall of the oropharynx and gently swabbing the wall, and the swab was placed in 1 ml Sterilized PBS with the end concentration of Penicillin G ( $2 \times 10^3$ /ml) and polymyxin B ( $2 \times 10^3$ /ml) in a 1.5 ml sterilized centrifuge tube and marked the no. of pigeon and the type of the swab. The samples were put on the ice immediately and brought back to the laboratory and kept in  $-70^\circ\text{C}$  until being tested.

#### 3.4.2 Cloacal swabs

The cloacal swab from live pigeons was swabbed by inserting a dry cotton swab deeply into the vent and vigorously swabbing the wall. The swab was softly and deeply stained with the fecal material or excretions and is then placed in 1ml sterilized PBS as a described in 3.4.2. The samples were put on the ice immediately and brought back to the lab and kept in  $-70^\circ\text{C}$  until being tested.

#### 3.4.3 Sera collection

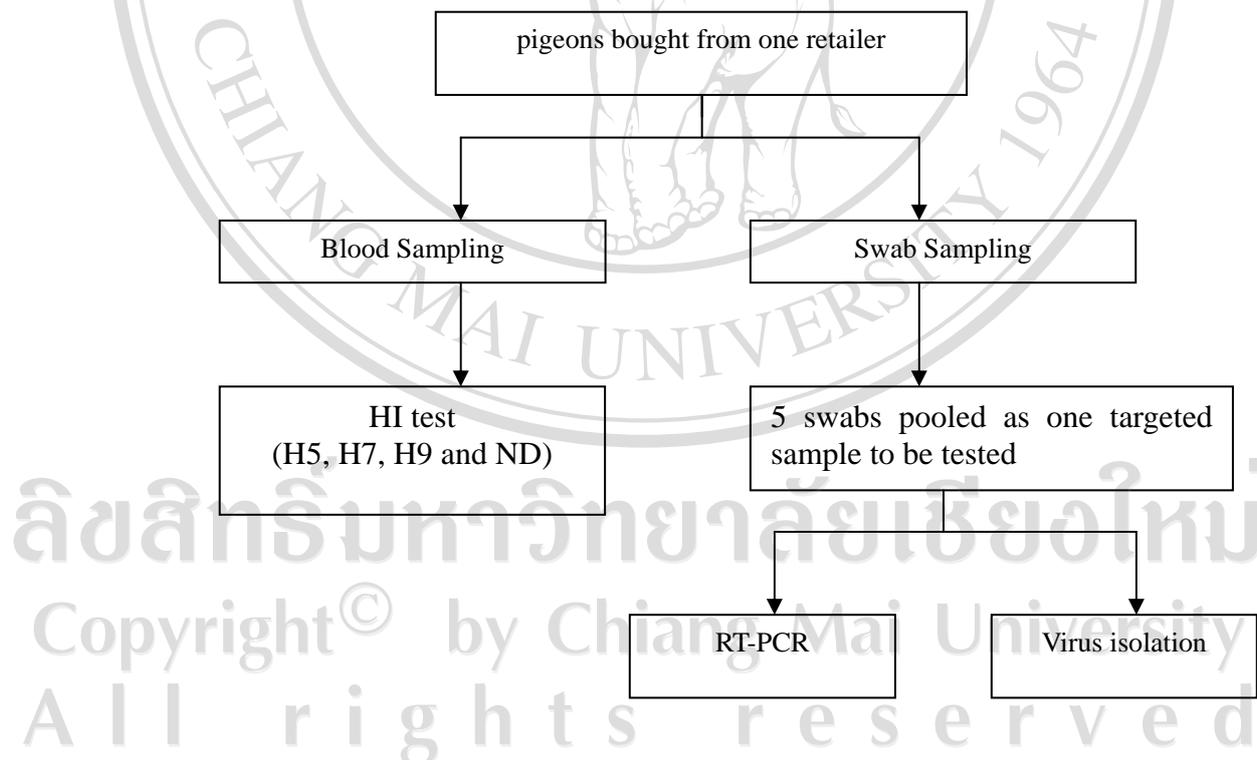
In serological surveillance studies, 2-3 ml of whole blood was taken from the wing vein of every pigeon and only a single sample of serum was available. Every blood sample was marked clearly with the isolator No. and the Pigeon code.

The blood was put horizontally in  $37^\circ\text{C}$  incubator for 2-3 hours and kept in the  $4^\circ\text{C}$  for overnight. The clotting of blood happened and the sera can be poured off into

1.5ml sterilized centrifuge tubes, which were centrifuged at 2500 rpm/15 mins to separate the remained red blood cells (RBCs) and serum. The yellow and transparent sera were pipetted off into 1.5ml sterilized centrifuge tubes, and the RBCs were discarded. Serum samples from pigeons were coded well and stored at -20°C until being tested.

### 3.5 Lab diagnostic methods

Three tests were run at a parallel level Fig 3.1. All the procedures were adopted from the recommendation of national standard on the serological and virological tests of avian influenza virus and Newcastle Disease virus.



**Figure 3.2 The flow chart of the diagnostics methods**

### 3.5.1 Virus isolation

#### 3.5.1.1 Oropharyngeal and cloacal swabs samples pooled

The frozen swabs samples stored in  $-70^{\circ}\text{C}$  were thawed in  $4^{\circ}\text{C}$  refrigerator for overnight. After the samples thawed, centrifuge with 11000 rpm under  $4^{\circ}\text{C}$  for 15 minutes and take 0.2 ml out of each oropharyngeal or cloacal swab sample.

Five oropharyngeal and 5 cloacal swabs samples taken from 5 pigeons from one retailer at every sampling point will be pooled respectively. Pooled samples (1ml) were put into 1.5 sterilized centrifuge tubes and kept on the ice all the time and ready for the SPF (Specific Pathogen Free) embryonated chicken eggs.

#### 3.5.1.2 Inoculation of 9-11 days old SPF Embryonated Chicken Eggs

SPF eggs used in this study were provided by the Laboratory Animal Centre of Harbin Veterinary Research Institute.

Examine eggs with an egg candler and place with blunt end up into egg trays. any eggs were discarded if infertile, having cracks, underdeveloped, or appearing to have a porous shell. Place eggs with blunt end up into egg trays and label each egg with a specific identification number (3 eggs per specimen). Wipe the tops of the eggs with 70% ethanol and punch a small hole in the shell over the air sac.

Three eggs per samples were inoculated with  $100\mu\text{l}$  into the allantoic cavity. Inoculate the specimen and store the remainder at  $-70^{\circ}\text{C}$ . Seal the holes and incubate the eggs at  $37^{\circ}\text{C}$  for 72 hours and observe the eggs living status every 24hours after inoculation.

After 72-hour incubation Eggs were chilled at  $4^{\circ}\text{C}$  overnight 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. Using a 10ml pipette, the allantoic fluid was aspirated and placed in a labeled plastic tube. Then using a syringe and needle, pierce the amniotic sac and remove as much amniotic fluid as possible. Place harvest in a separate tube, but because of the low volume of amniotic fluid obtained from each egg, it is necessary to combine the allantoic fluid from the three eggs inoculated per specimen. Centrifuge harvested fluids at 3000rpm/5minutes to remove blood and cells and perform a haemagglutination test (HA test) and incubate at 4°C about 30 mins.

No HA was present, the specimen were passaged by 2 times before reporting inability to recover virus from the specimen. Centrifuge the tubes at 3000 rpm/5minutes to remove excess blood and tissues. The isolate were stored at -70°C.

#### 3.5.1.3 Haemagglutination Inhibition test (HI test)

Once the positive results of HA test in 3.5.1.2 presented, the harvested fluid should be done with HA titration and then the following HI test (discussed in detail in 3.5.3).

To be briefly, after HA titration, v-shape micro titer plates were labelled. Add 25µl of PBS to wells B through H (B1- H12) of each numbered column. Add 25 µl of the H5N1 reference serum (provided by Chinese National Reference Lab for Avian Influenza) to the first well (B1-A12) of the numbered column. Prepare serial dilutions of the positive reference sera by transferring 25µl from the first well of the numbered columns 1-12 to successive wells. Discard the final 25µl after row H.

Add 25µl of titrated antigen to all wells (B1-H12) in H5 reference sera. Add 25µl of PBS instead of antigen to the set of treated sera for antigen controls (A1-H12). Mix the contents of the plates by shaking on a mechanical vibrator for 10 sec. Cover the plates and incubate at room temperature (22°C to 25°C) for 15 min. Add 25 µl of standardized RBCs to all wells. Mix as before. Cover the plates and allow the RBCs to settle at room temperature (22°C to 25°C) for the appropriate time according to RBCs being used. Record the HI titers.

The same procedures were applied with the reference serum against H7 and H9 avian influenza viruses and Newcastle Disease virus to exclude the presence of these antigens.

### 3.5.2 Virus detection: RT-PCR

#### 3.5.2.1 RNA Extraction from the swabs

Samples for avian influenza virus detection were pooled in the same way as the 3.5.1.1. Mix together the following materials: 350  $\mu$ l Lysis Buffer RLT, 3.5  $\mu$ l  $\beta$  – mercaptoethanol, 200  $\mu$ l virus suspension and 550  $\mu$ l 70% ethanol. Positive and negative control 200 $\mu$ l was mixed respectively with the above RNA extraction agents.

A/Turkey/England/N28/73(H5N2), A/African starling/983/79(H7N1) and A/Turkey/ Wisconsin/1/66(H9N2) which was originated from Veterinary Reference Lab of United Kingdom and kept by Harbin Veterinary Research Institute, were used as amplified target to be positive controls (Figure 4.1- 4.5). The allantoic fluid harvested from non-inoculated SPF egg was used as negative control.

Pipette the mixture onto the pink RNease mini spin columns (Qiagen RNeasy™ Total RNA Isolation Kit) and centrifuge for 15 seconds at 10,000 rpm. Remove the column and empty the liquid from the bottom collection tube. Replace the column in the collection tube. Add 700  $\mu$ l Wash Buffer RW1 to the column and centrifuge for 15 seconds at 10,000 rpm. Transfer the column to a clean collection tube and add 500  $\mu$ l Wash Buffer RPE to the column. Centrifuge for 15 seconds at 10,000 rpm. Remove the column and empty the liquid from the collection tube. Add 500 $\mu$ l Wash Buffer RPE to the column and centrifuge for 2 minutes at 12,000 rpm. Transfer column to a 1.5 ml micro-centrifuge tube and pipette 20 $\mu$ l RNase free H2O directly onto the filter of the column. Wait 1 minute. Centrifuge for 1 minute at 10,000 rpm. Sample was ready for cDNA synthesis. RNA was stored at  $-20^{\circ}\text{C}$ .

### 3.5.2.2 Synthesis of cDNA

All reactions were carried out on ice. Label one 0.5 ml micro-centrifuge tube for each RNA used. The negative control is water. To 4  $\mu$ l of RNA and negative control (water blank) add 0.5  $\mu$ l of primer 'Uni-12' with the sequence of GGA GCA AAA GCA GG (E. Hoffmann1, et al. 2001). Incubate for 5 minutes at 72 °C. Make a cocktail of the following:

- 1.5  $\mu$ l of H<sub>2</sub>O
- 2.0  $\mu$ l of Reverse Transcriptase buffer
- 0.5  $\mu$ l of 10 mM dNTP mix
- 0.5  $\mu$ l of RNasin
- 1.0  $\mu$ l of Reverse Transcriptase

Add 5.5  $\mu$ l of the cocktail to each tube Incubate the RNA/Primer mix with the cocktail , total volume 10  $\mu$ l at 42°C for 1 hour. Then stop the RT-reaction by incubation for 5 minutes at 95 °C. Primer used (designed and provided by Dr. Xiurong Wang, Harbin Veterinary Research Institute):

**Table 3.4 Primers used in this experiment**

Name	Sequence	Length (bp)
Uni 12	GGA GCA AAA GCA GG	12
H5/591 forward	GGA ATG ATA GAT GGN TGG TAY GG	591
H5/591 backward	GTG TTT TTA AYT AMA ATC TGR ACT MA	
H7/525 forward	ACAAGGAGAGGGAACTGCTGCAGATT	525
H7/525 backward	CCCATTGCAATGGCCAGAAGTATGAA	
H9/808 forward	AGCAAAAGCAGGGGAAYWWC	808
H9/808 backward	CCATACCATGGGGCAATTAG	
M/1015forward	GAAGGTAGATATTGAAAGATG	1015
M/1015backward	GAAACAAGGTAGTTTTTACTC	

W = (AT); Y = (CT); N = (AGTC); M = (AC)

### 3.5.2.3 PCR Reaction

From the cDNA synthesized take only 1.5  $\mu\text{l}$  for each PCR reaction. This is added to 48.5  $\mu\text{l}$  of the master mix. Make a PCR reaction Master Mix as follows: (Run blank for each primer pair):

- 5  $\mu\text{l}$  PCR buffer
- 38.65  $\mu\text{l}$  H<sub>2</sub>O
- 1  $\mu\text{l}$  10 mM dNTP mix
- 3  $\mu\text{l}$  25 mM MgCl<sub>2</sub>
- 0.25  $\mu\text{l}$  Taq DNA polymerase
- 0.3  $\mu\text{l}$  Forward primer (1 $\mu\text{g}/\mu\text{l}$ )
- 0.3  $\mu\text{l}$  Reverse Primer (1 $\mu\text{g}/\mu\text{l}$ ) (primers setting as above)

Spin briefly to collect. Add a drop of mineral oil to the top of the tube. Place tube in thermocycler. Program for amplification:

- (1) 94 °C for 2 minutes
- (2) 94 °C for 1 minute (denature)
- (3) 50 °C for 1 minutes (anneal)
- (4) 72 °C for 3 minutes (extend)
- (5) Repeat from step 2, 30 times
- (6) 72 °C for 8 minutes
- (7) 4 °C until usage

### 3.5.2.4 Agarose Gel Electrophoresis of the PCR Products

Preparation of Agarose Gels: Weigh out the desired amount of agarose and place in an Erlenmeyer flask with a measured amount of electrophoresis buffer (for a 2% gel, add 2 gm of agarose and 100ml of TBE Buffer (1X)) to flask and boil it over. Dissolve the agarose in a boiling in a revolving-plate microwave oven. All the grains of agarose should be dissolved and the solution clear. Cool the solution to 70°C for concentrations 2%, add 0.5 mg/ml ethidium bromide into the cooled agarose gel, mix and pour immediately. Allow the gel to set for one-half hour before using. Make sure to use the same electrophoresis buffer in the gel as for the running buffer.

Remove tape from gel frame and place the gel into the electrophoresis chamber; cover the gel with 1x TBE. Label the 0.5 ml micro-centrifuge tubes separately. Remove 4 µl of the PCR product from each reaction tube to a corresponding 0.5 ml micro-centrifuge tube (remove PCR product from underneath oil); mix with 3 µl gel loading buffer. Load 4 µl molecular weight marker (2000) to the first well of the 1% 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 at 120V for 30-40 minutes. Visualize presence of marker and PCR product bands with a hand-held UV light. It is desirable to have an ultraviolet light source emitting light at 302 nm wavelength. Document gel with a photograph and compare the size of the PCR-fragments with the marker.

The expected size of PCR products for influenza A/H5 is 591 bp, for A/H7 is 525 bp, and for A/H9 is 808 bp. But the absence of the correct PCR products (i.e. a negative result) cannot rule out the presence of influenza virus.

### 3.5.3 Serological test

In serological diagnosis procedure, the 205 serum specimens from 205 pigeons were titrated antibodies to 4 reference antigens: against the H5, H7, H9 avian influenza virus and Newcastle Disease virus. All the 4 reference antigens were provided by Harbin Veterinary Research Institute, which are the commercialized diagnosis kits for serological tests of avian influenza and Newcastle Disease.

Red blood cell (RBC) suspension (1%) was prepared from SPF chickens provided by Laboratory Animal Centre of Harbin Veterinary Research Institute.

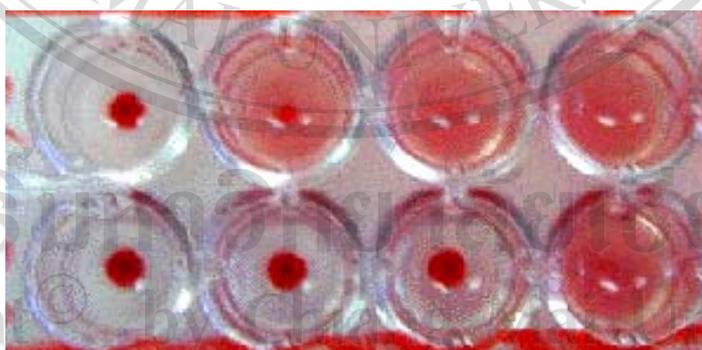
#### 3.5.3.1 Haemagglutination test (HA test)

HA test was used for the titration of reference antigen before the HI test. Every package of the 4 lyophilized antigens were dissolved by 1ml sterilized PBS (pH 7.0-7.2) as recommended in the user guide from the 4 commercial diagnosis kits. The dissolved antigens were kept in 4°C.

Using V-shaped 96-well micro titer plates, add 25  $\mu$ l of PBS (pH 7.0-7.2) to #2 through #12 (A1 - E12) wells of each lettered row. Add 25  $\mu$ l of each reference antigen to the first well (A1- D1) of the lettered rows except rows E. Prepare an RBC control well in row E (E1) by adding 25  $\mu$ l of PBS.

Make serial twofold dilutions by transferring 25  $\mu$ l from the first well of lettered rows to successive rows. Discard the final 25  $\mu$ l. Add 50  $\mu$ l of RBC suspension to each well on the plate. Mix by using a mechanical vibrator or by manually agitating the plates thoroughly. Incubate the plates at room temperature (22 °C to 25°C). Check cell control for complete settling of RBCs.

Haemagglutination occurs when the RBCs are in suspension after the RBC control has settled completely. This is recorded using a "+" symbol. When a portion of the RBCs is partially agglutinated or partially settled, a "+/-" symbol is used. In the absence of haemagglutination, chicken RBCs form a compact button on the bottom of the wells. A "-" symbol is used to record the absence of haemagglutination. Haemagglutination can be determined by tilting the plates and noting the absence of tear-shaped streaming of erythrocytes which flow at the same rate as RBC controls. The RBC control should be completely settled either as a compact button or "halo".



**Fig 3.3 the determination of haemagglutination**

Record HA activity of the 4 reference antigens. Dilute 4 reference antigens in sterilized PBS (pH7.0-7.2) based on the HA activity of the 4 antigens to yield 4 HA units in 25 $\mu$ l. The diluted antigens were kept on the ice and used for the following test within 2-3 hours.

### 3.5.3.2 Haemagglutination Inhibition test (HI test)

Label v-shape micro titer plates. One plate was used for 6 samples tested against one out of 4 reference antigen. The reference antigen and reference serum used here were all from Harbin Veterinary Research Institute.

The titration of serum samples to H5N1 avian influenza virus as following:

Add 25 $\mu$ l of PBS to wells from A1 to H12 of each column. For negative positive serum control, add 25 $\mu$ l PBS to the A1. For positive control, 25 $\mu$ l H5N1 reference serum was added to B1. Each serum ready to be tested was added to the first wells of the rested 7 rows (C1- H1) of the numbered column. Prepare serial twofold dilutions by transferring 25 $\mu$ l from the first well of the numbered columns 1-12 to successive wells. Discard the final 25 $\mu$ l after row H.

Add 25 $\mu$ l of standardized 4HA unit H5N1 antigen to all wells (A1- H12). Add 25  $\mu$ l of PBS instead of antigen to the set of treated sera for controls (A1- H1). Mix the contents of the plates by shaking on a mechanical vibrator for 10 sec or by agitating the plates manually. Cover the plates and incubate at room temperature (22°C to 25°C) for 15 min.

Add 25 $\mu$ l of RBCs to all wells. Mix as before. Cover the plates and allow the RBCs to settle at room temperature (22°C to 25°C) for the 45 min until the known-positive serum wells of row B exhibit a tight, well-circumscribed button of unagglutinated, sedimented erythrocytes. The reciprocal of the highest dilution of serum at which there was complete inhibition of haemagglutination. Record the HI titers. Here 1:8 was regarded as positive results. The titration of serum samples to H7 and H9 avian influenza virus and Newcastle Disease virus were done in the same procedure.