CHAPTER 3

Materials and Methods

3.1 Sample size calculation

To meet the study objectives, especially the comparison of antibody titres pre- and post-vaccination and at different farms, before the study commenced, sample size was calculated using the Power Analysis and Sample Size software (PASS) for one-way ANOVA repeated measures (NCSS statistical software, 2008, Kaysville, UT, USA), to define the number of ducks required in each farm for each time of sample collection. Expected H5-specific antibody levels were based on experts' opinions and results of previous studies on antibody response against H5N1 vaccination in ducks conducted in several provinces in the Mekong Delta. With 80% power of test, it was determined that the sample should consist of 15 ducks per flock to cover potential lost during the observation period. In addition, at each of the selected farms, 5 control ducks were chosen to receive no vaccination.

3.2 Time, sampling sites and design of the study

This field study was conducted within 5 months, from July to early December 2017. Two districts (i.e Ba Tri and Giong Trom) of Ben Tre province which is located in the Mekong Delta of Viet Nam (see Figure 3.1), were selected by veterinary officers from the provincial SDAH because of their large population and high density of ducks. Experienced veterinarians and veterinary technicians were available to collect samples in these areas.

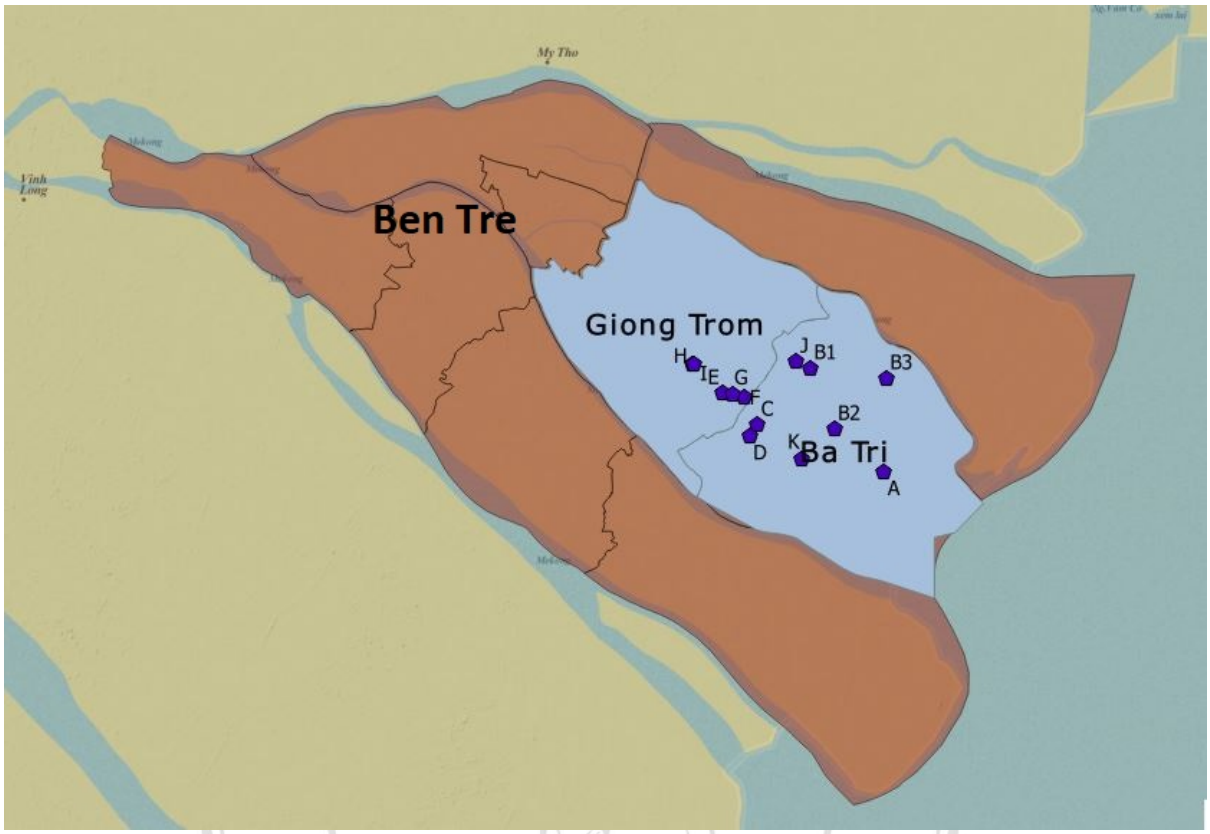

Figure 3.1 Location of the 11 farms sampled in the study.
Farms are coded A to K. B1 to B3 are 3 the locations of fields of 3 other households in the same district to which the duck flock of farm B were moved for grazing.

Travel fee and the costs of laboratory analyzing limited the number of farms involved in the study. Eleven household farms in the 2 districts with ducks 18 to 20 days old were selected randomly by veterinary officers of the provincial SDAH, representing a variety of breeds, flock sizes (from 100 to nearly 1,500 ducks per farm), and purpose of production (meat-type ducks and layer ducks). Categories of selected farms can be seen from Appendix B. Access to farms was possible only through the SDAH. Veterinarians, technicians of the District Veterinary Stations (DVS) and para-veterinarians working in the villages helped in farm contact, farm visit and sample collection.

The farming practice (e.g., housing, diet and feeding type, hygiene, stockpersonship etc.) of selected farms which are village smallholders having < 1500 birds, is quite similar from farm to farm. Ducks, which are mixed breed (F4 is most common), were bought from local hatcheries by the farmers. In most farms, ducks were kept in confinement or grazed around the household and returned overnight. In only one of the selected farms,

ducks were moved for grazing onto fields of some other households in the same district. In this later case, flock movement was recorded for sequential scavenging locations. At early visits, latitude and longitude coordinates of duck farms and flocks were obtained using a global positioning system (GPS) device. The geographical coordinates of all farm/flock locations were plotted using Quantum Geographic Information Systems (QGIS) software version 2.18.14 (QGIS Development Team, 2017).

3.3 Vaccine

The Inactivated Reassortant Avian Influenza Virus Vaccine subtype H5N1, Re-6 Strain (Zhaoqing Dahuanong Biology Medicine Co., Ltd., Guangdong, P. R. China) was used for vaccination. The vaccine contains the HA gene of A/duck/Guangdong/ 1332/2010 H5N1 clade 2.3.2 with the HA titre ≥ 1:256 (i.e., 8 $\log_2$ in the binary logarithm system) before deactivation and appears as a milky white oil emulsion. This is the only vaccine against H5N1 HPAI licensed for mass vaccination in the province where this study was conducted. Vaccine bottles (500 mL/bottle) were provided free of charge by the provincial SDAH but also were commercially available. The vaccine was used according to manufacturer's recommendations. Vaccine was stored at 2℃ ~ 8℃, recovered to normal room temperature, and evenly mixed by shaking the bottle well before each use. Dose and administration: 0.5 mL for ducks aged 2-4 weeks and 1 mL for those over 5 weeks, intramuscularly in the breast.

3.4 Vaccination

Ducks were vaccinated and sampled according to the schedule in Figure 3.2. Vaccinations were performed using automatic syringes either by competent farm workers or by local veterinarians following the guidelines of the national mass vaccination program against H5N1 HPAI for poultry regarding vaccine strain and schedule of immunization. Dose and administration were based on the manufacturer's recommendations. Each duck received a dose of 0.5 mL for the prime vaccination and 1 mL for the booster vaccination intramuscularly in the breast.

Among the group of selected ducks, five control ducks received saline instead of the H5N1 vaccine. The other 15 identified ducks and the rest of the flock were vaccinated twice with a 21-day interval between vaccinations (see in Figure 3.2). All non-vaccinated

and vaccinated ducks selected for sampling were followed until the end of the study (in layer ducks) or the end of production cycle (in meat-type ducks).

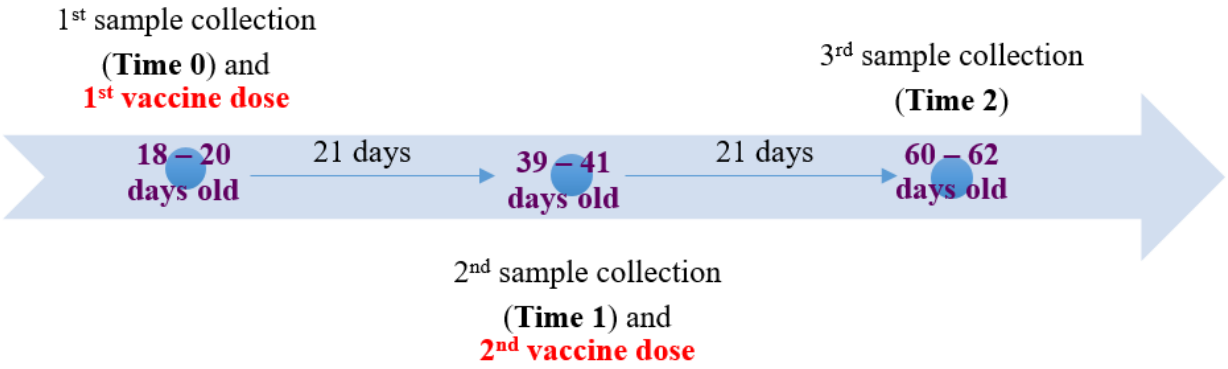

Figure 3.2 Immunization and sample collection schedule used in the study. All ducks in the selected farms received two intramuscular injections in the breast at 21-day intervals. Time 0, 1 and 2 represent samplings at 1day pre-vaccination, 21 days after the prime vaccination (21 dpv) and 21 days after the booster vaccination (21 dpbv), respectively. "Time 0" of blood sampling on day 18 to 20 was carried out to detect H5-specific antibodies potentially derived from maternal immunity, natural infection, or other unknown factors.

3.5 Sample collection and sample treatment

All the techniques of sample collection, storage, and shipment for this study was conducted under the relevant national technical regulation on animal diseases guidelines (QCVN 01-83:2011/ BNN PTNT). Sample collection was approved by the provincial SDAH and was performed by experienced veterinarians and technicians to minimize suffering of ducks during the sampling process. Blood sampling was performed right before each time of vaccination. In each farm, with the help of duck owner, ducks were chased into a corner of a pen and caught until obtaining the required sample size. In each flock, 20 ducks were selected to be identified individually with leg bands and were blood sampled. All of the identified ducks were individually blood sampled 3 times at 21-day intervals: pre-vaccination, 21 days after a prime vaccination, and 21 days after a booster vaccination (see in Figure 3.2).

Approximately 1 to 2 mL of blood was taken from each individual duck via the medial metatarsal vein and allowed to clot for 30-60 minutes at room temperature before

being transported to the laboratory the same day. Serum was separated from the blood by centrifugation and stored at either +4°C or -20°C until testing.

In addition to blood sampling, pooled tracheal swabs from 5 vaccinated ducks in each of the selected farms were also collected during the last blood sample collection and sent to the Regional Animal Health Office VI (RAHO VI) in Ho Chi Minh city where they were tested using real-time reverse transcriptase–polymerase chain reaction (RRT-PCR) to confirm that there was no H5 HPAI virus circulating in the farms during the observation period.

3.6 Antigen

Based on the commercial availability of H5N1 vaccines and antigen in Vietnam, the H5N1 virus strain A\Ck\Scot\59 (product code RAA 7002 – APHA Scientific, Surrey, UK) was used as antigen for serological assays described below as it is the only strain licensed for H5-specific antibody detection by laboratory diagnosis in the country. The reagent is 1 mL lyophilized in glass ampoules which are reconstituted with 1 mL sterile distilled water before use. One batch of HA antigen was used for all sera in serological tests to minimize variability of the HI testing.

3.7 Collection and preparation of chicken's erythrocytes

Erythrocytes used for the serological assays are collected from healthy roosters that were previously tested to be negative to H5-specific antibodies (specific-pathogen free (SPF) chickens). Fresh red blood cells (RBCs) were prepared every time right before the tests according to the following steps:

(1) Collect whole blood from roosters' brachial veins in an equal volume of Alsever's solution for preserving RBCs (referred to as solution 1).

(2) Dispense 10 - 20 mL of solution 1 into a 50-mL centrifuge tube and fill with PBS solution. Invert the tube gently several times to wash the RBCs. Centrifuge at 3000 rpm for 5 min (C-28A centrifuge, Boeco, Germany). Discard the supernatant and surface layer of white cells from the tube. Refill the tube with fresh PBS solution. Repeat the washing and centrifugation cycle 2 more times. Washed RBCs can be stored at

4°C for up to 1 week. The suspension should be discarded if the RBCs show hemolysis.

(3) Prepare a 10% suspension for treatment of duck serum samples and a 0.5% suspension of RBCs with PBS for use in the HI assay. Concentration of the RBC suspension was measured by Microhematocrit Tube method using standard capillary microhaematocrit tubes and centrifuging at 10,000 rpm for 5 min (HAEMATOKRIT 200 centrifuge, Hettich GmbH & Co.KG, Germany).

The procedure above was describe by Killian M.L. in 2008 (E. Spackman, 2008) with some modification.

3.8 Duck sera pre-treatment

Duck sera were heat-inactivated at 56°C for 30 min then treated with 10% roosters' RBC suspension to remove nonspecific inhibitors and prevent nonspecific HA reaction that might otherwise occur during HI assay in the sera of species other than chickens. The ratio of PBS:serum:RBCs was 2:1:1 (e.g. 0.1 mL of PBS + 0.05 mL of serum sample + 0.05 mL of 10% RBCs). The reagent plate was kept for 60 min at room temperature, waiting for the 10% RBCs suspension to settle. The supernatant was aspirated to be tested by the HI assay. The final concentration of a duck serum was 1:4 after pretreatment.

3.9 Serological assays

H5-specific antibodies in sera of ducks following vaccination were detected and quantitated by HI assay. Haemagglutination test (HA) was performed for antigen quantification to achieve 4 haemagglutination units (HAU) of the antigen used in the HI test. Both of the serological tests were performed in plastic V-bottom microtitre plates using two-fold dilutions and 0.5% specific-pathogen-free chickens' red blood cells (RBC).

The tests were conducted by the Veterinary Diagnostic Laboratory and Treatment Division of SDAH Ben Tre province (license number LAS-NN 59; ISO/IEC 17025: VILAT-0043) following the procedure described in the national technical standard of diagnostic procedure for animal diseases (TCVN8400-26:2014) and guidelines of the OIE Terrestrial Manual (OIE, 2009), with approved modification such as the routine HPAI

post-vaccination serological surveillance. The HA antigen solutions were back-titrated before each use to ensure that a correct amount of antigen had been used. Reference known titre positive sera and negative control sera were included in each test plate. The reference sera were provided by the RAHO VI which is a national reference laboratory for Avian Influenza. V-bottom microtitre plates were horizontally oriented so that reconstituted antigen was diluted 12 wells across. Rows and columns on each plate were numbered so that the contents of each plate cell are uniquely identified.

3.9.1 Haemagglutination (HA) test

The HA test was performed according to the following steps, similar to those described by Terregino and Capua (2009):

(1) Dispense 0.025 mL of PBS solution into each well of the first 3 rows of a V-bottomed microtitre plate.

(2) Give 0.025 mL of reconstituted antigen suspension into well A1 and B1.

(3) Make 2-fold dilutions from column 1 to column 12 (from 1:2 to 1:4096) of antigen suspension across the plate by using a multi-channel micropipette. Discard the last 0.025 mL.

(4) Dispense 0.025 mL of PBS solution into each well of row A, row B, and row C. Row C is for RBC control.

(5) Add 0.050 mL of 0.5% RBC suspension to each well, cover the plate and tap gently to mix and keep at 4°C for 40 – 45 min.

(6) Plates are read after 40 – 45 min (at 4°C) when the RBCs in control wells have settled and assumed a button shape. The samples are read by tilting the plate at approximately 45 degrees, using a white background and observing the appearance of tear-shaped streaming of the RBCs.

Result interpretation:

There will be no streaming in analyzed wells with HA reaction. The HA activity will result in a fine layer of RBCs lining the entire bottom of the well. In contrast, RBCs will settle at the center of the well in the shape of a button if HA activity is absent. When tilting the plate, RBCs in wells having no HA reaction should flow at the same speed as the RBCs in the control wells containing only PBS solution and RBCs.

The HA titre of the antigen suspension is the highest dilution that causes RBCs' agglutination (no streaming). This dilution is defined as containing 1 haemagglutinating unit (HAU).

To perform the HI test, a 4 HAU antigen solution will be used, i.e. containing 4 times that antigen concentration. For instance, if the HA titre obtained is 1:512 (1 HAU), 4 HAU will be obtained by dividing that titre by 4 (512:4 = 128). A dilution of 1:128 of the original undiluted antigen solution will be used to prepare the 4 HAU antigen solution for the HI test. This was explained previously by Calogero T. and Ilaria C. (Capua & Alexander, 2009).

Back titration:

To ensure that the correct amount of antigen will be used in the HI test, another plate is prepared for the back titration of the prepared 4 HAU antigen solution. HA reactions must be observed in the first 3 wells (well 1^{st} – 3^{rd}) of the 4 HAU control testing plate. In well 4^{th}, a partial agglutination (half of a tear-shaped drop) and in wells 5^{th} and 6^{th} no HA activity should be seen. Wells 1^{st} – 6^{th} correspond to 4 HAU, 2 HAU, 1 HAU, 0.5 HAU, 0.25 HAU and 0.125 HAU respectively.

3.9.2 Haemagglutination inhibition (HI) test

The HI test was performed to detect and quatitate H5-specific antibodies in sera of ducks following vaccination according to the following steps:

(1) Dispense 0.025 mL of PBS solution into all wells of a V-bottomed microtitre plate.

(2) Give 0.025 mL of each serum (previously diluted by 1:4) into wells from A1 to A9 of the first row of the plate. This row A is for sera control (later, without antigen).

(3) Give 0.025 mL of each diluted serum into wells from B1 to B9 of the second row of the plate.

(4) Give 0.025 mL of each negative reference serum into the well A10 and B10 and place 0.025 mL of each reference known titre positive serum in the well A11 and B11 of the plate.

(5) Perform 2-fold dilutions of all sera vertically across the plate, from row B to row H by using the multi-channel micropipette. Discard the last 0.025 mL from row H.

(6) Add 0.025 mL PBS into all wells of column 12. This column is for RBC control.

(7) Add 0.025 mL of diluted antigen containing 4 HAU in each well containing individual duck serum sample under examination or reference serum, start from the row with highest dilution (row H) to row B. Do not add diluted antigen to the sera control row (row A) or the RBC control column (column 12). Cover the plate and tap gently to mix and keep at room temperature (18 – 25°C) for 60 min.

(8) Add 0.050 mL 0.5% RBCs to all wells. Cover the plate, and gently tap the plate to mix and place at 4 -8°C. Plates can be read after 45 – 60 min, after the RBCs control has settled, by tilting the plate at approximately 45 degrees, using a white background and observing the tear-shaped streaming appears at the same speed as that occurring in the RBCs control wells.

Result interpretation:

The HI antibody titre was determined as the highest serum dilution causing complete inhibition (described above) of haemagglutination of RBCs using 4 HAU of antigen (Pedersen, 2008). The HI titre was reported as a base-2 logarithm value (i.e., $\log_2$). The starting dilution in the HI test was 1:8 (i.e., 3 $\log_2$ in the binary logarithm system) according to the fact that titres <1:16 (4 $\log_2$) are considered negative while samples with a titre ≥ 4 $\log_2$ were defined as seropositive using the official cut-off of 1:16 (4 $\log_2$) in compliance with Vietnam's national regulation on post-vaccination surveillance for AI which is based on the OIE's recommendation (MARD-DAH, 487/TY-DT, 2009; OIE, 2009). By regulation, a titre of 4 $\log_2$ is regarded as protection threshold as value is assumed to be the minimum protective antibody titre at the individual level.

3.10 Statistical analysis

For the overall sample data and for each farm data, the geometric mean titre (GMT), coefficient of variation (CV) and seroprotection rate (SPR) were calculated. Antibody

titres were transformed into $\log_2$ values as mentioned above before further analysis. Descriptive data on HI antibody titres were averaged both by individual farms and overall, by times of sample collection (hereinafter referred to as 'time'), and are presented as the mean ± SE.

GMTs of antibodies were calculated as the average $\log_2$ titres at each of the 3 times of sample collection. Titres < 3 $\log_2$ were regarded as 2 $\log_2$ for the calculation of GMT. Variations in antibody titre were presented as the coefficient of variation (CV) reported as a percentage (%) (CV = 100 x standard deviation/mean). The HI titre of 4 $\log_2$ is assumed to be the protection threshold as mentioned above, and thus the seroprotection rate (SPR) has been calculated as the proportion of subjects with seropositivity (i.e. HI titres ≥ 4 $\log_2$).

A general least-squares (GLS) was used to compare the differences in GMT by time. The GLS model was constructed by using R statistical software version 3.3.2 (R Development Core Team, 2016) with nonlinear mixed-effects (nlme) package version 3.1-128 (Pinheiro et al., 2016). The GMT of antibodies was the dependent variable, while independent variables, including 'farm' and 'time', were defined as fixed effects (independent variable). The individual duck was a random effect. In addition, the potential confounding farm effects were classified by location, purpose of duck production, farm scale and tested by the GLS model. The Akaike information criterion (AIC) for the GLS modeling was determined from models with different covariance structures; however, the AIC of the autoregressive process of order 1 (*ar1*) had the lowest value, indicating the best-fitting model. Tukey's test was used for multiple comparisons when mean differences were significant. Results with p-value < 0.05 were considered statistically significant.

3.11 Ethics statement

Although all sampling and laboratory analyzing activities of this study were carried out totally similar to the routine veterinary surveillance by the SDAH in the province, an animal ethics approval for this research was received from the Institutional Ethical Review Board of Hanoi University of Public Health (IRB-HUPH, approval number 308/2017/YTCC-HD3). The IRB was registered with the U.S. Department of Health and Human Services (IORG number 0003239, FWA number FWA00009326). Permission to

conduct the study was obtained from the SDAH Ben Tre province which was also a collaborator on the project.