EPIDEMIOLOGICAL STUDY OF AVIAN INFLUENZA IN BACKYARD CHICKENS AND OPEN FIELD-REARED DUCKS IN NORTHERN THAILAND

By

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ABSTRACT

EPIDEMIOLOGICAL STUDY OF AVIAN INFLUENZA IN BACKYARD CHICKENS AND OPEN FIELD-REARED DUCKS IN NORTHERN THAILAND

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Avian influenza (AI) is an infectious disease of birds, other animals, and humans caused by type A subtypes of the influenza virus. Highly pathogenic avian influenza (HPAI), caused by the virus subtype H5N1, currently occurs worldwide with the greatest burden in Southeast Asia where the disease was first reported. Even though the major outbreaks of the disease in this region have declined, the disease remains a major threat to the poultry industry and human health. It is generally hypothesized that the main reason for the disease to persist in this region is the existence of traditional backyard chicken and open field-reared duck raising systems. These traditional poultry raising systems are particularly strong in Thailand, but limited research has been conducted to determine their role in maintaining and spreading the AI virus.

This study was designed to test the hypothesis that backyard chickens and open field-reared ducks harbor the avian influenza virus. To test the stated hypothesis, three objectives were designed to address: 1) to determine the prevalence of the avian influenza by a combination of virus isolation and antibody testing; 2) to identify the risk factors associated with the laboratory-confirmed avian influenza by Logistic Regression Analyses; and 3) to generate the Geographic Information System (GIS) mapping of the laboratory-confirmed AI in Northern Thailand.

One thousand oropharygeal swabs of backyard chickens, one thousand cloacal swabs of open field-reared ducks, and two thousand serum specimens from the same individual birds (as well as data via questionnaire) were collected at the time of visit in 87 dusticts of the 6 provinces in the Nothern region of Thailand in 2009-2010.

Avian influenza virus isolation (egg incoculation; confirmed by hemagglutination test) and Agar Gel Immunodiffusion (AGID) test were conducted at Chiang Mai University (CMU) in Thailand; using the guidelines provided by the World Organization for Animal Health (OIE). No active AI virus infection was detected by egg inoculation, and no influenza A antibody was tested positive to AGID (in fact AGID test is fairly insensitive).

To confirm the primary test results, further serological tesing and virus subtyping were carried out at the USDA National Veterinary Services Laboratories (NVSL) in Ames, Iowa. Among 2,000 serum samples of both poultry species, 1.5% (15/1000) (of sera) from backyard chickens and 2.1% (21/1000) of sera from open field-reared ducks tested positive for antibodies against type A influenza virus; using the IDEXX MultiS-Screen ELISA Test Kit (Sensitivity and Specificity of the test is 95.4% and 99.7%). Out of 13 sera that were positive to ELISA and had adequate quantity for AGID test, only 1 chicken serum was tested positive. Thus, AGID is not a recommended test for screening of AI antibody. By way of logistic regression modeling and GIS mapping, the AI antibody positivity in the backyard chickens appeared to be significantly (p-value < 0.05) associated with the large flock size (>100 birds/flock) and farming proximity to the other farms. Because of a confounding effect found in the final model, multivariable analyses-risk factors for AI antibody positivity in the open field-reared ducks could not be identified.

AI virus sub-typing was conducted on the 21 adequate sera that were positive for ELISA. Three chicken sera (obtained from the same flock of birds tested positive to AGID) had been definitely identified positive to antibody against H5; using Hemaglutination-inhibition test. The fact that influenza A virus (H5 subtype) remains entrenched in Northern Thailand, it may put the country at risk of disease re-emerging. Therefore, the sustaining ongoing surveillance for early disease detection and preparedness for rapid disease response are still strongly recommended.

To the Two Heroes in My Life

My Father; Mr. Siddhinan D. Chantong and My PhD Advisor; Professor Dr. John B. Kaneene

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LIST OF SYMBOLS AND ABBREVIATIONS

&	=	and
-	=	(dash); minus; to (punctuation mark)
$	=	Dollar(s)
°C	=	degree(s) Celsius
>	=	greater than
<	=	less than
x	=	multiply by
# or No.	=	number
%	=	percent
® or TM	=	Trademark
/	=	(slash); by; divided by
AAF	=	amnioallantoic fluid
AI	=	Avian Influenza
AGID	=	Agar Gel Immunodiffusion (Test)
cc (cm^3)	=	cubic centimeter(s)
DLD	=	Department of Livestock Development (Thailand)
DOI	=	Digital Object Identifier (System)
e.g.	=	*exempli gratia* (Latin); for example
ELISA	=	Enzyme-Linked Immunoabsorbent Assay
et al.	=	*et alii* (Latin); and others
etc	=	*et cetera* (Latin); and other things, and so on

EU	=	European Union
FAO	=	Food and Agriculture Organization of the United Nations
g	=	gram(s)
GIS	=	Geographic Information System
HA	=	Hemagglutination (Test or Assay)
HI	=	Hemagglutination-inhibition (Test or Assay)
HPAI	=	Highly Pathogenic Avian Influenza
i.e.	=	*id est* (Latin); that is; in other words
µl	=	microliter(s)
ml	=	milliliter(s)
mm	=	millimeter(s)
µm	=	micrometer(s)
LPAI	=	Low Pathogenic Avian Influenza
nm	=	nanometer(s)
NVSL	=	National Veterinary Services Laboratories
OIE	=	*Office International des Epizooties* (French); (World Organization for Animal Health)
RNA	=	Ribonucleic Acid
RT-PCR	=	Reverse-Transcription Polymerase Chain Reaction
URL	=	Uniform Resource Locator
U.S.A.	=	United States of America
USDA	=	United States Department of Agriculture
WHO	=	World Health Organization

INTRODUCTION

RATIONALE

Avian influenza (AI) is an infectious disease of birds caused by the subtype of type A influenza virus. In addition to birds, the disease affects a wide range of avian and mammalian species, including humans, pigs, horses, and aquatic animals (Heinen, 2003; WHO, 2006).

Influenza A viruses infecting poultry can be subdivided into two groups; the very virulent highly pathogenic avian influenza (HPAI), and low pathogenic avian influenza (LPAI) viruses. The infection of HPAI characterized by the mild form to severe illness with the high mortality rate, up to 100% deaths in the infected animals (Swayne & Halvorson, 2008).

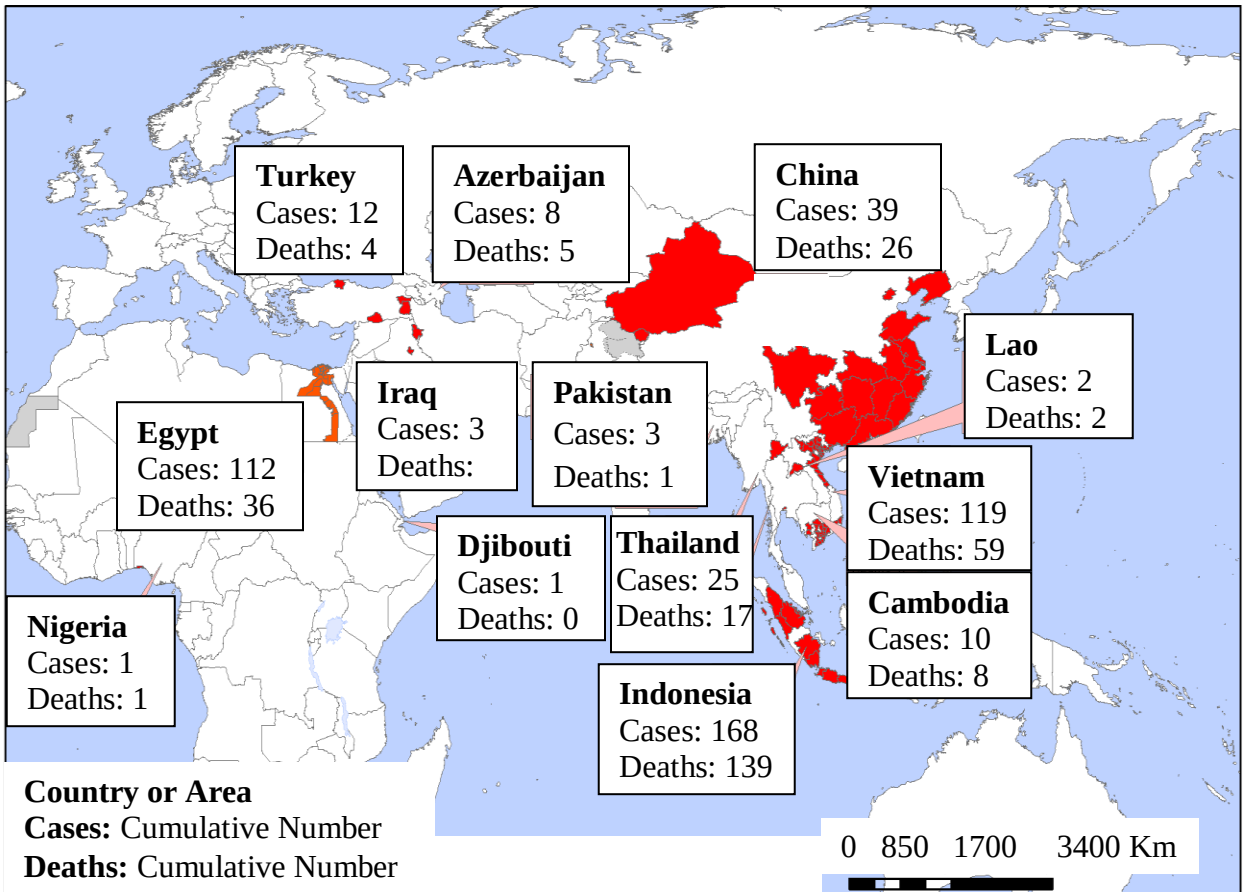
The recent emergence and spreading of the HPAI A/H5N1 in Thailand and other countries in Southeast Asia has brought avian influenza to the forefront of important animal diseases, and, at the same time, to overall public health concerns. Not only have the numbers of birds involved increased over the past several years, but also the impacts in terms of economic losses and social consequences have considerably escalated (Alexander, 2007).

Beyond the countries in Southeast Asia, currently the disease occurs worldwide with a total of over 60 countries having reported H5N1 in domestic poultry, and wildlife from 2003 - 2010 to the World Organization for Animal Health (OIE, 2010a) (**Appendix A**).

According to the World Health Organization (WHO, 2010a), there are 512 confirmed human cases affected from the disease. Of them, 304 deaths were reported in 15 countries distributed around the world (as of December 29, 2010) (**Appendix B**). The geographical distribution of the confirmed human cases since 2003 is presented in Figure I-1.

Figure I-1: Areas with confirmed human cases of H5N1 avian influenza, 2003-2010*

* For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

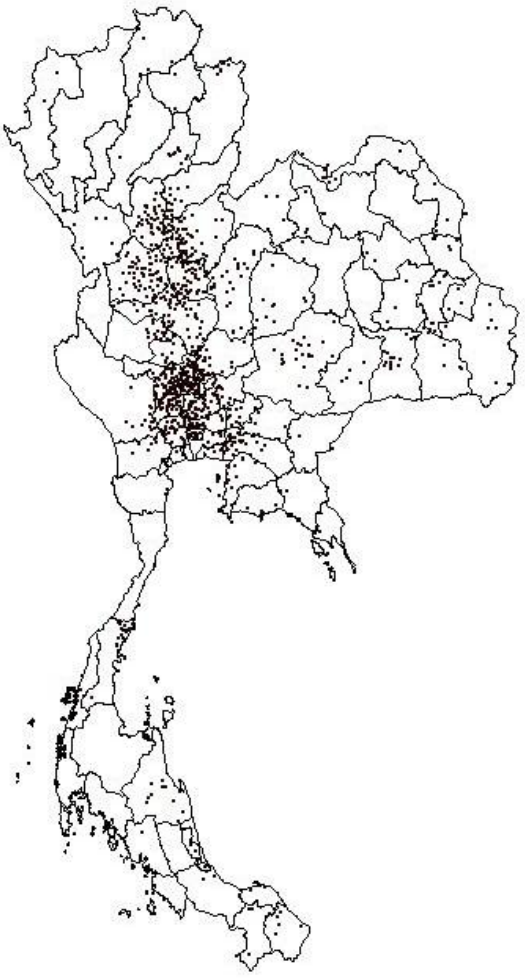


Source: WHO, 2010b

In Thailand where outbreaks of HPAI was first confirmed by Thailand Department of Livestock Development (DLD) in January 2004, four epidemics were reported between January 2004 and December 2006 (DLD, 2004; NaRanong, 2008; Thanapongtham, Hongchumpol, & Phungjiem, 2007). According to the OIE (2010b), several outbreaks were reported in 2007-2008. To date, there were 25 Thai human cases (17 deaths) reported to the World Health Organization (WHO, 2010a).

During 2004-2006 when the waves of outbreaks were remarkably reported in Thailand, more than 65 million birds died, or had to be destroyed, by the government's stamping-out policy (DLD, 2007; NaRanong, 2008; Tiensin et al., 2005). During the outbreak period, the disease was widely distributed in all regions of the country, which were mostly found in the Central and lower-Northern region of Thailand. The distribution of AI outbreaks in Thailand is illustrated in Figure I-2.

Figure I-2: The distribution of highly pathogenic avian influenza in Thailand during the 2004-2006 outbreaks



Source: Thanapongtham et al., 2007

PROBLEM STATEMENT

Backyard poultry, including open flock-reared ducks, were recently incriminated as having been the source of AI outbreaks in Thailand (Gilbert et al., 2006, 2007; Songserm et al., 2006a; Tiensin et al., 2005, 2007). These traditional systems of raising poultry are widely practiced in Thailand and in other countries in Southeast Asia. Since the birds can freely roam around the gardens/property, they are more likely to come in close proximity to humans and other animals. However, no systematic studies have been conducted in this avian species of poultry to assess the magnitude of the problem.

A few studies were performed in the free-grazing ducks in the Central part of Thailand (Amonsin et al., 2008; Gilbert et al., 2006, 2007; Songserm et al., 2006a; Tiensin et al., 2005, 2007). However, no studies have been conducted to determine the prevalence and risk factors associated with AI outbreaks in backyard chickens and open field-reared ducks in the Northern region of Thailand. The Northern region have a fast growing commercial and semi-commercial poultry industry, but at the same time the traditional systems of raising backyard chickens and open field-reared ducks are still widely practiced.

HYPOTHESES

Even though the major outbreaks of the disease in Thailand have declined, the disease remains a major threat to the poultry industry and human health. It is generally hypothesized that the main reason for the disease to persist in this region, despite major control efforts, is the existence of traditional backyard flocks and free- range ducks raising systems.

The following hypotheses were formulated:

1. Avian influenza viruses are still circulating in backyard chickens and open field-reared ducks in Northern Thailand. The study would be able to demonstrate this, using a combination of virus isolation and antibody detection methods.
2. Major risk factors associated with finding a virus or AI antibody bird(s) include: close proximity of the farm to wild birds and other animals, history of previous outbreaks, flock size, presence of live poultry markets, and human activities (i.e., land use, fighting-cock arenas, and poultry movement).

OBJECTIVES

In order to test the stated hypotheses, three objectives were designed:

1. Determine the prevalence of avian influenza infection by a combination of virus isolation and antibody tests in six provinces of Northern Thailand (Chiang Mai, Chiang Rai, Lampang, Phrae, Nan, and Phayao).
2. Determine the risk factors associated with laboratory-confirmed AI virus infection by Logistic Regression Analysis.
3. Generate the Geographic Information System (GIS) - mapping of the AI distribution, using the ArcGIS® program (Environmental Systems Research Institute [ESRI], Inc., Redlands, CA, USA).

SIGNIFICANCE AND IMPACT OF THE STUDY

The baseline information obtained in this study could generate the up-to-date information on disease prevalence and its associated risk factors. Moreover, the study could define disease patterns in respect to spatial distribution (place and time) of AI in Northern Thailand. This particular information would be useful in formulating hypotheses and in designing long-term studies aimed at establishing monitoring, and setting up strategic measures for the future AI prevention, control, and eradication in the Northern region and in Thailand as a whole.

OVERVIEW OF THE DISSERTATION

This PhD dissertation is divided into 6 chapters based on the variety of topics related to epidemiological study of avian influenza in general. The study of avian influenza in backyard chickens and open field-reared ducks in Northern Thailand in particular is mainly specified, and then elucidated in this dissertation. Moreover, the National Strategic Plans for Avian Influenza Control and the effectiveness of the plans and other related issues are consecutively reviewed.

Chapters 1 to 3 consist of the literature reviews of AI viruses, their characteristics and outbreaks in Thailand, including the outbreak consequences during 2004-2008. Poultry production systems and the roles of backyard chickens and open field-reared ducks as the significant sources of H5N1 outbreaks in the country were consequentially reviewed. Chapter 4 presents the epidemiological study of avian influenza in backyard chickens and the finding results. Chapter 5 is dedicated for the epidemiological study of avian influenza in the open field-reared ducks. Effectiveness of AI surveillance systems in Thailand is evaluated and discussed in the Chapter 6. Further discussion, conclusions, and recommendations are also presented in Chapter 6.

REFERENCES

REFERENCES

Alexander, D.J. (2007, July 26). An overview of the epidemiology of avian influenza. *Vaccine Journal, 25(30),* 5637-44.

Amonsin, A., Choatrakol, C., Lapkuntod, J., Tantilertcharoen, R., Thanawongnuwech, R., Suradhat, S., et al. (2008, November): Influenza virus (H5N1) in live bird markets and food markets, Thailand. *Emerging Infectious Diseases Journal, 14(11),* 1739-42.

Department of Livestock Development (DLD, 2004). Avian influenza situations and control measures: Available from URL: http://www.dld.go.th/home/bird_flu/history_47.html

Department of Livestock Development (DLD), Thailand. (2007). Avian influenza situations and control measures: Available from URL: http://www.dld.go.th/home/bird_flu/birdflu.html

Gilbert, M., Chaitaweesup, P., Parakamawongsa, S., T., Premashthira, Tiensin, T., Kalpravitdh, W., Wagner, H., et al. (2006, February). Free-grazing ducks and highly pathogenic avian influenza, Thailand. *Emerging Infectious Diseases Journal, 12(2),* 227-34.

Gilbert, M., Xio, X., Chaitaweesup, P., Kalpravitdh, W., Premashthira, S., and Boles, S., et al. (2007). Avian influenza, domestic ducks and rice agriculture in Thailand. *Agriculture, Ecosystems, and Environment Journal, 119*, 409–415.

Heinen, P. (2003). Swine influenza: a zoonosis. *Veterinary Science for Tomorrow.* Available from URL: http://www.vetscite.org/publish/articles/000041/print.html

NaRanong, V. (2008). Structural changes in Thailand's poultry sector: avian influenza and its aftermath. *TDRI Quarterly Review* 2008; 23: 3-10.

Songserm ,T., Jam-on, R., Sae-Heng, N., Meemak, N., Hulse-Post, D.J., Sturm-Ramirez, K.M., et al. (2006a, April). Domestic ducks and H5N1 influenza epidemic, Thailand. *Emerging Infectious Diseases Journal, 12(4),* 575-81.

Swayne, D.E. & Halvorson, D.A. (2008). Influenza. In Y.M. Saif (Ed.), *Disease of poultry* (12th ed.) (pp. 153-84). Ames, IA: Blackwell.

Thanapongtham, W., Hongchumpol, N., & Phungjiem, P. (2007). The outbreaks of avian influenza in Thailand, 2004-2006 [Thai version]. *The Department of Livestock Development, Thailand*. Available from URL: www.dld.go.th/home/bird_flu/16-10-49KAN/file/3-1-50.doc

Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., et. al. (2005, November). Highly pathogenic avian influenza H5N1, Thailand, 2004. *Emerging Infectious Diseases Journal, 11(11),* 1664-72.

Tiensin, T., Nielen, M., Songserm, T., Kalpravidh, W., Chaitaweesub, P., Amonsin, A., et al. (2007). Geographic and temporal distribution of highly pathogenic avian influenza A virus (H5N1) in Thailand, 2004-2005: an overview. *Avian Diseases Journal, 51*, 182-8.

World Health Organization (WHO) (2006). Avian influenza ("bird flu"). *WHO | Avian Influenza Fact sheet*. Available from URL: http://www.who.int/mediacentre/factsheets/avian_influenza/en/index.html

World Health Organization (WHO) (2010a). Cumulative number of confirmed human cases of avian influenza A/(H5N1) Reported to WHO: 29 December 2010. *WHO | Avian Influenza Archives*. Available from URL: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_12_29/en/index.html

World Health Organization (WHO) (2010b). Areas with confirmed human cases of H5N1 Avian Influenza since 2003. *WHO | Avian Influenza Maps*. Available from URL: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_H5N1inHumanCUMULATIVE_FIMS_20100831.png

World Organization for Animal Health (OIE) (2010a). 63 countries report H5N1 avian influenza in domestic poultry/wildlife, 2003-2010. *Avian Influenza*. Available from URL: http://www.oie.int/eng/info_ev/en_AI_factoids_2.htm[Cited on 2010 December 09]

World Organization for Animal Health (OIE, 2010b). Update on highly pathogenic avian influenza in animals (Type H5 and H7) 2007 and 2008. Available from URL: http://www.oie.int/downld/AVIAN%20INFLUENZA/A2008_AI.php [Cited on 2010 December 09]

CHAPTER 1

AVIAN INFLUENZA VIRUSES; CHARACTERISTICS AND IDENTIFICATION: A REVIEW

INTRODUCTION

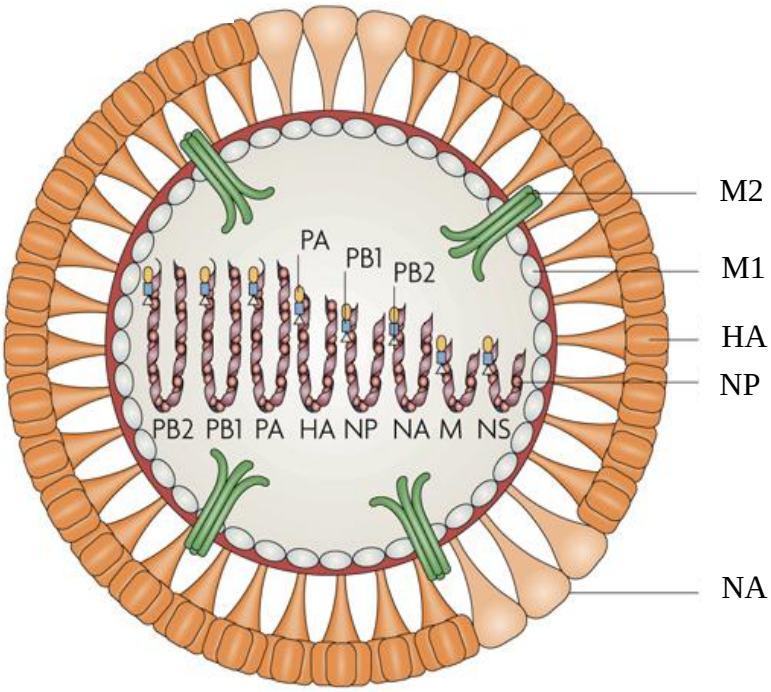
Avian influenza (AI) viruses are classified as members of the family *Orthomyxoviridae* (from the Greek *orthos,* 'standard or correct' and *myxo,* 'mucus'; meaning their ability to bind to mucus). The influenza viruses that constitute this family are classified into types A, B or C based on differences between their nucleoprotein (NP) and matrix (M) protein antigens. AI viruses that typically infect a wide range of avian and mammalian species (including humans, pigs, horses and aquatic mammals), belong to type A. Influenza B and C viruses are almost exclusively isolated from humans, although influenza C virus has also been isolated from pigs and influenza B has recently been isolated from seals (Heinen, 2003).

Influenza A viruses are further categorized into subtypes according to the antigens of the hemagglutinin (HA) and neuraminidase (NA) projections on their surfaces (Figure 1-1a). The hemagglutinin allows the virus to attach to the surface of a host cell, while the neuraminidase allows the virus to be released (Gauthier-Clerc, Lebarbenchon, & Thomas, 2007). There are 16 haemagglutinin subtypes and 9 neuraminidase subtypes of influenza A viruses, and avian influenza viruses have representatives in all of these subtypes (WHO, 2008).

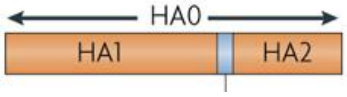
Highly pathogenic avian influenza (HPAI) viruses infecting poultry have been restricted to subtypes H5 and H7, although not all H5 and H7 viruses caused HPAI. All other viruses, i.e. low pathogenic avian influenza (LPAI), cause a milder primarily respiratory disease, unless exacerbated (Alexander, 2007). This initial chapter of the dissertation therefore reviewed only general characteristics, epidemiology, and identification of influenza viruses classified in type A.

Figure 1-1: The schematic representation of influenza A virus **(A)** and cleavage site **(B)**

A. Influenza A Virus



B. Hemagglutinin



Cleavage Site

Source: Subbarao & Joseph, 2007

MORPHOLOGY AND CHARACTERISTICS

Influenza A viruses have a spherical or filamentous morphology, and are medium-sized with a diameter of 80 to 120 nm. The virus is segmented, negative sense, stranded RNA, and is enveloped. The lipid membrane of the virion is derived from the host cell in which the virus replicated. From the surface of the envelope extend the two transmembrane glycoproteins; rod-shaped hemagglutinin (HA) and mushroom-shaped neuraminidase (NA) that are commonly called ‘spike’ (Swayne & Halvorson, 2008). The morphology of influenza A virus is presented in Figure 1-1a.

A third transmembrane protein, matrix protein M2, also exists on the surface of virion, but only 20-60 molecules per virion are present. The matrix protein M1 forms a layer beneath the envelope, and so gives structure to the virus and binds to the ribonucleoprotein (RNP) to form RNP complex. The complexes consist of ribonucleic acid (RNA) associated with nucleoprotein (NP) as well as the polymerase PA, PB1, and PB2 that are responsible for RNA replication and transcription. Two non-structural proteins (NS) are also associated with the virus: NS2 is found in the virion, while NS1 is found only in the infected cells.

The influenza virus genome consists of eight unique segments of single-stranded RNA, which have negative polarity. Each RNA strand encodes only one protein, except for strands 7 and 8 which encoded two (Heinen, 2003).

The replication cycle of influenza virus starts with the cleavage of HA0 into HA1 and HA2 (Figure 1-1b) by enzymes present in the respiratory and intestinal tract of the host, i.e. the trypsin-like enzymes. The enzymes are mostly produced by the host, but may also be derived from bacteria, which therefore can promote the influenza infection.

After HA cleavage, the receptor-binding site of HA1 can attach to a terminal sialic acid residue of a cell surface receptor; once attached to the host cell the virus is digested (receptor-mediated endocytosis). NA functions as a receptor-destroying enzyme by cleaving terminal sialic acid residues from the receptor. Therefore, NA releases progeny virions from the host cell in which they arose and facilitates virus spread. The progeny virions can infect other cells or can be transmitted to another individual where the cycle of transmission begins. The type A influenza viruses can be shed out and transmitted to a wide range of their natural hosts.

EPIDEMIOLOGY AND PATHOBIOLOGY

- **Host Range**

The natural reservoir of influenza viruses is birds, and consequently, many subtypes are known as avian influenza viruses. Avian influenza viruses normally do not infect species other than birds, but have been found infrequently in a range of other animal species, including marine mammals, other terrestrial mammals, and humans (Causey & Edwards, 2008).

The first reported isolation of an influenza virus from feral birds was in 1961 when the researchers isolated the virus strain from the common terns (*Sterna hirundo*) in South Africa (Becker, 1966), but it was not until the mid-1970s that any systematic investigation of influenza in feral birds was undertaken. These revealed the enormous pools of influenza viruses now known to be present in the wild bird population (Alexander, 2000, 2007).

Avian influenza viruses had been isolated recently in a variety of wild birds worldwide; underlining the importance of wild birds in viral epidemiology. The viruses have been found in at least 12 orders, 105 species from 26 different families of wild birds (Munster et al., 2007); including free-living birds, captive caged birds, waterfowls, gulls, passerines, waders, terns, pleasant, falcons, shorebirds, sea birds, and other domestic poultry (Alexander, 2000, 2007; Gauthier-Clerc, et al., 2007; Olsen et al., 2006; Stallknecht & Shane, 1998).

Wild birds, especially waterfowl and shorebirds, have long been a focus for concern by the poultry industry and considered to be the potential major source of influenza viruses for epidemic in domestic poultry (Alexander, 2000; Friend, Franson, & Ciganovich, 1999).

Domestic poultry susceptible to avian influenza viruses include chickens, turkeys, ducks, guinea fowl, domestic geese, quails, and pleasant (OIE, 2010c). Until the spread of highly pathogenic avian influenza (HPAI) A/H5N1 in Asia, HPAI viruses had been isolated rarely from wild birds. When they had been isolated, it was usually in the vicinity of outbreaks of HPAI in poultry or geographically close to known outbreaks in the domestic birds (Alexander, 2007).

HPAI A/H5N1 emerged after the end of 1996 in Hong Kong, and started to spread again in 2003 in populations of domestic poultry in Southeast Asia (Gauthier-Clerc et al., 2007). As early as the beginning of 2004 in Thailand, besides domestic poultry, cases of H5N1 infection were reported also in domestic dogs and cats (Tiensin et al., 2005; Songserm et al., 2006a, 2006b). The carcasses were taken to Kasetsart University for laboratory diagnosis. The authors found the evidence of H5N1 infection in both cat and dog carcasses from the necropsy's gross lesions, histopathology, and reverse transcription (RT-PCR) methods. A study conducted by Butler (2006) in the infected zone of Thailand revealed that 25% of dogs and 7% of cats carried antibodies to H5N1, indicating they had been infected with the disease. However, cats were more susceptible to the disease than dogs (Swayne, 2010).

In October 2004, H5N1 infection in captive tigers at Sriracha Tiger Zoo in the Eastern region of Thailand, apparently by ingestion of contaminated chicken meat; 147 tigers in the zoo died or were euthanized to prevent disease transmission (Tiensin et al., 2005).

Moreover, H5N1 can also infect the wild carnivores, e.g., the Owston's civet in Vietnam (Roberston et al., 2006), a marten in Germany, and a mink in Sweden (WHO, 2006). The virus had also been passed to wild animals in captivity, including ferret, tigers, and leopard (Lednicky et al., 2010; Thanawongnuwech et al., 2005; Tiensin et al., 2005; WHO, 2006).

- **Disease Transmission**

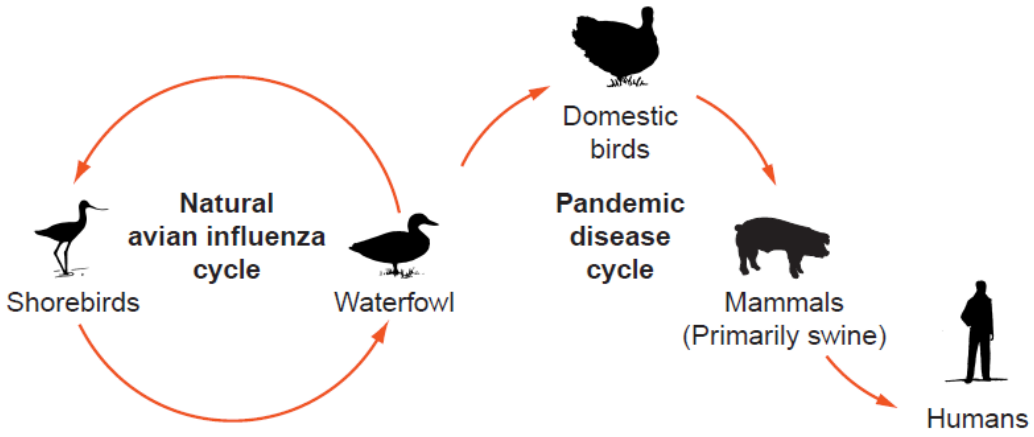
The mechanisms by which influenza viruses pass from one bird to another and bring about infection are poorly understood. According to Alexander (2000), bird to bird transmission is extremely complex and depends on strain of virus, the species of bird, and environmental factors. However, shorebirds and wild waterfowl (most notably ducks) are now recognized as the natural reservoir of influenza viruses. The virus multiplies in the gastrointestinal tract producing large amounts of virus usually without producing clinical signs, but they can excrete the viruses for long periods of time (Jacob, Butcher, Mather, & Miles, 2009).

The WHO (2006) stated that at least some migratory waterfowl are now carrying the H5N1 virus in its highly pathogenic form, sometimes over long distances, and introducing the virus to poultry flocks in areas that lie along their migratory routes.

An avian influenza virus can be introduced into the susceptible domestic poultry through the five different means; (1) direct exposure to infected birds; (2) exposure to equipment or materials that are contaminated with the virus in respiratory secretions or feces; (3) movement of people with the virus on shoes, clothing, or hands; (4) the virus contaminated water (waterborne, e.g. drinking water or water sources); and (5) the virus moved in the air (airborne, e.g. in dust or aerosol droplets) (WHO, 2006).

Avian influenza viruses are able to be transmitted from animals to humans in two main ways: (1) directly from the infected birds or from avian virus-contaminated environments to people, and (2) through an intermediate host, such as a pig. When the human-to-human transmission of influenza virus is efficient, the conditions can probably be set for pandemic spread of the disease (Baigent & McCauley, 2003). The transmission cycle of AI is shown in Figure 1-2.

Figure 1-2: Global cycle of avian influenza viruses in animals and humans



Source: Friend et al., 1999

- **Pathotypes and Pathogenicity**

According to Alexander (1982); Heeney (2006); Swayne & Suarez, 2000; and Suarez (2008), avian influenza viruses can be divided into 2 pathotypes based on their pathogenicity, i.e., the ability to produce disease. The World Organization for Animal Health (OIE-Terrestrial Animal Health Code, 2010d) defines the pathotypes of AI viruses as follows:

1. High pathogenicity notifiable avian influenza (HPNAI) viruses have an intravenous pathogenicity index (IVPI) in the 6-week-old chickens greater than 1.2 or, as an alternative, cause at least 75% mortality (6 deaths out of 8) in 4- to 8-week-old chickens infected intravenously. H5 and H7 viruses, which do not have an IVPI of greater than 1.2 or cause less than 75% mortality in an intravenous lethality test, should be sequenced to determine whether multiple basic amino acids are present at the cleavage site of the hemagglutinin molecule (HA0). If the amino acid motif is similar to that observed for other HPNAI isolates, the isolate

being tested should be considered as HPNAI. In general terms, HPNAI are synonymous with HPAI viruses by definition.

2. Low pathogenicity notifiable avian influenza (LPNAI) viruses are all influenza A viruses of H5 and H7 subtypes that are not HPNAI viruses. The LPAI pathotype can include AI viruses from any of the 16 HA (H1- H16) and 9 NA (N1-N9) subtypes, while LPNAI viruses are a subset of LPAI viruses.

The ability of LPAI viruses to mutate into HPAI viruses, particularly in poultry, and the diversity of viruses circulating in wild bird populations emphasize the potential importance of wild birds as a primary source of the zoonotic introduction of influenza into human populations (Baigent & McCauley, 2003).

- **Incubation Period**

Incubation period is defined as the period of time between exposure to the pathogen and the onset of clinical signs (Toma, Vaillancourt, Dufour, et al. (Eds.) (1999). Incubation period of AI ranges from as short as a few hours in intravenously inoculated birds to 3 days in naturally-infected individual birds, and up to 14 days in flock and depending on the dose of viruses, the route of exposure, the species exposed, and the ability to detect clinical signs. According to the OIE (2009), incubation period of AI can range 2 – 3 days in humans and 1 – 7 days in poultry. Incubation period in mammals is even shorter.

CLINICAL SIGNS AND PATHOLOGICAL FINDINGS

Clinical signs of disease are extremely variable and depend on other factors including host species, age, sex, concurrent infections, acquired immunity, and environmental factors (Swayne & Halvorson, 2008).

- **Low Pathogenicity Avian Influenza (LPAI) Viruses**

  - **LPAI: Clinical Signs and Signalment**

For the LPAI viruses, high morbidity (>50%) and low mortality rates (<5%) are typical, but mortality rate can be quite high if accompanied by secondary pathogens or if the disease occurs in young birds. Most infections by LPAI in wild birds produce no clinical signs. However, in experimental studies in ducks, LPAI virus infections suppressed T-cell functions and produced a one-week depression in egg production (Swayne & Halvorson, 2008).

In domestic poultry (chickens and turkeys), clinical signs reflect abnormalities in the respiratory, digestive, urinary, and reproductive organs. The most frequent signs represent infection of the respiratory tract and include mild to severe respiratory signs, such as, coughing, sneezing, rales (crackles), rattles, and excessive lacrimation. In layers and breeders, hens may exhibit increased broodiness and decrease egg production. In addition, domestic poultry will exhibit generalized clinical signs including huddling, ruffled feathers, depression, listlessness, decrease activity, lethargy, decreased feed and water consumption, and occasionally diarrhea. Emaciation has been reported but is infrequent because AI is acute not a chronic disease.

  - **LPAI: Gross Lesions**

Gross lesions are variable depending on the virus strain, length of the time from infection to death, the host species, and the presence of secondary pathogens. The infraorbital sinuses may be swollen, especially in turkeys, and have accompanying mucoid-to-mucopurulent nasal discharge. The most frequent lesions are rhinitis and sinusitis, whose character varies between catarrhal, fibrinous, serofibrinous, mucopurulent, and fibrinopurulent. The fibrinopurulent inflammation usually is accompanied by secondary bacterial infections.

The tracheal mucosa may be edematous with congestion, occasionally hemorrhages, and serous to caseous luminal exudates. The tracheal exudates occasional occlude airways with resulting asphyxiation. Fibrinopurulent bronchopneumonia may be present, and is usually accompanied by secondary bacterial pathogens such as *Pasteurella multocida* or *Escherichia coli* (Swayne & Halvorson, 2008).

- **High Pathogenicity Avian Influenza (HPAI) Viruses**

  - **HPAI: Clinical Signs and Signalment**

By definition, HPAI viruses express high mortality in chickens both in filed cases and experimentally in the intravenous pathogenicity test and intranasal infectivity and pathogenesis studies. Typically, HPAI viruses also express high lethality in other gallinaceous birds, but the mean death times (MDTs) are usually lengthened compared with those in chickens and vary with individual HPAI virus strains. In wild birds and domestic birds, most HPAI viruses either do not replicate or replicate to a limited degree, and produce few clinical signs because of poor adaptation to non-gallinaceous species.

In domestic birds, turkeys, and related galliformes, clinical signs reflect virus replication and damage to multiple visceral organs and cardiovascular, nervous systems and integument. Specific clinical manifestations depend on the level of damage and which organs or organ systems are affected. In the per-acute stage, birds may be found dead prior to the appearance of any clinical signs or with few clinical signs other than listlessness, recumbency, and a comatose state. Closer observation of remaining birds has revealed reduced activity; decrease sensitivity to stimuli, reduction in “in-house” noise, dehydration, and decreased feed and water intake that rapidly progressed to severe listlessness and death.

In breeders and layers, egg production will drop precipitously with typical decline leading to total cessations of egg production within 6 days. Diarrhea may be evident as bile- or urine-stained loose dropping with variable amounts of intermixed mucus.

If the clinical course was less per-acute and birds survive for 3 to 7 days, individual birds may exhibit nervous disorders such as tremors of head and neck, inability to stand, torticollis, opisthotonus, nysagmus, paresis, paralysis, excitation, convulsions, rolling or circling movements, tremors and incoordination (lack of organization), shaking of head, abnormal fait, paralysis of wings, and unusual position of head and appendages. Neurological signs are not specific for HPAI, and may be identical but less frequent than neurological signs of velogenic Newcastle disease (ND) and other noninfectious and infectious diseases.

Respiratory signs are less prominent than with LPAI virus infection, but, if present, have included rales (crackles), sneezing, and coughing. Other galliforme birds (e.g. quails) may have less per-acute disease than chickens and turkeys, although clinical signs and the duration of morbidity may be similar.

- **HPAI: Gross Lesions**

In gallinaceous poultry, HPAI virus infections produce a variety of edematous, hemorrhagic, and necrotic lesions in multiple visceral organs, cardiovascular and nervous systems, and the integument. With the per-acute phase (1 – 2 days post intranasal inoculation), no gross lesions are typically seen. In the acute phase (days 2 – 5), chickens have ruffled feathers and swelling of head, face, upper neck, leg shanks, and feet from subcutaneous edema and may have accompanying petechial-to-ecchymotic subcutaneous hemorrhages, especially of the nonfeathered skin (Swayne & Halvorson, 2008).

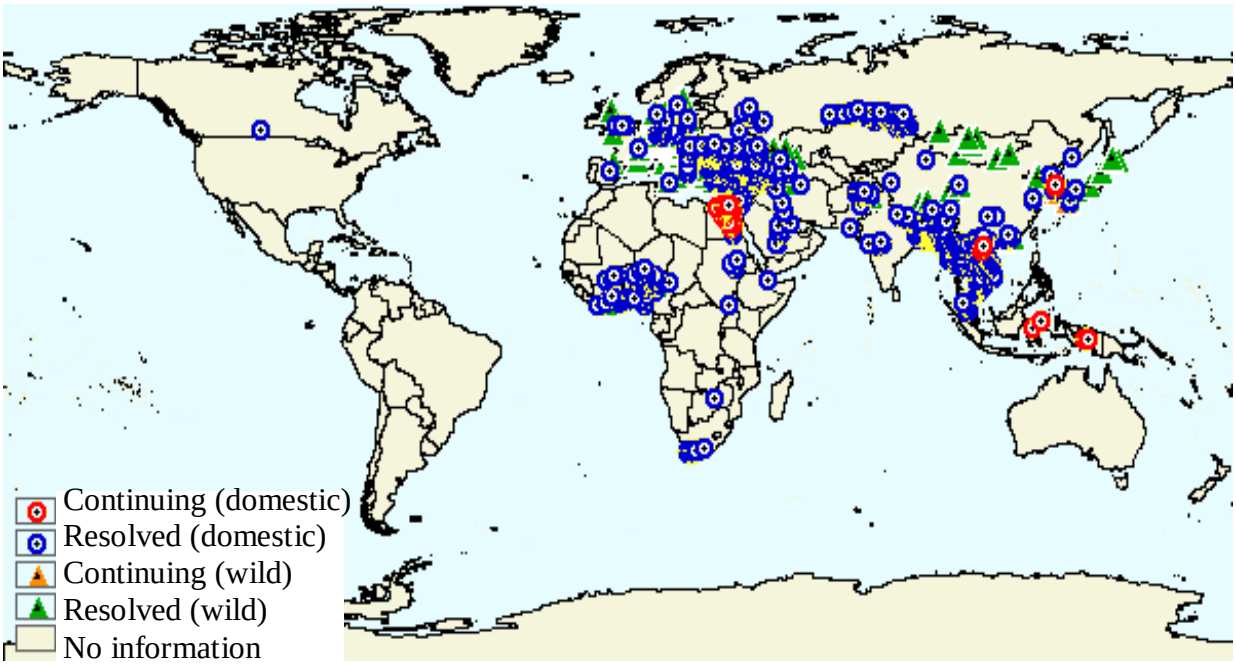
Some viruses produce hyperemia and edema of eyelids, conjuctiva, and trachea. Necrotic foci, peticheal-to-ecchymotic hemorrhage, and cyanosis of the wattles, combs, and snood may be common, and such lesions are used to identify suspect HPAI cases. The cyanosis results from ischemic necrosis following vascular infarction.

The gross lesions in visceral organs vary with virus strains, but most consistently include hemorrhages on serosal or mucosal surfaces and foci of necrosis within parenchyma of multiple visceral organs (Swayne & Halvorson, 2008). Especially prominent are hemorrhages in the coronary fat and on the epicardium, on the serosa and the mucosa of proventricus and within the pectoral muscles. Occasional hemorrhages are present on the inner surface of the sternum and in the caecal tonsils and Meckel's diverticulum. The pancreas may have light orange to brown mottling from necrosis. With the recent H5N1 HPAI viruses and classic fowl plague viruses, necrosis and hemorrhage in Peyer's patches of the small intestine have been common, as has been severe edema and hemorrhage in the lungs and with some virus gross edema of the brain.

GEOGRAPHICAL DISTRIBUTION

Low pathogenic avian influenza (LPAI) viruses commonly occur worldwide in wild birds and poultry. Highly pathogenic avian influenza (HPAI) viruses have been eradicated from domesticated poultry in most developed nations (OIE, 2009). The Asian lineage HPAI A/H5N1 outbreaks began among poultry in Southeast Asia in 2003. Since then, H5N1 viruses have been spreading into domesticated or wild birds in other regions of Asia as well as parts of Europe, the Pacific, the Middle East and Africa. Although some countries have eradicated the virus from their domesticated poultry, this epidemic has been ongoing until the present time (January, 2011). The global distribution of avian influenza from 2005 – 2010 is illustrated as Figure 1-3.

Figure 1-3: Globally geographic distribution of avian influenza from 2005-2010



Source: OIE, 2011a

DETECTION AND IDENTIFICATION

Successful detection of the avian influenza (AI) virus, viral antigen, nucleic acid, or antibody is dependent upon the collection of the appropriate sample type, the quality of the sample, and the proper storage and handling of the sample (Killian, 2008). Sera are acceptable samples for ELISA or agar gel precipitin tests, but not for real-time RT-PCR. Likewise, swabs and/or tissues are acceptable for real-time RT-PCR and virus isolation. The sample type will also depend on the type of birds that are being tested; oropharygeal swabs should be collected from poultry, and cloacal swabs should be collected from waterfowl.

The National Veterinary Services Laboratories (NVSL, USDA-APHIS, Ames, IA, USA) recommends the use of brain-heart infusion (BHI) broth without antibiotics for sample collection. However, a buffered balanced-salt solution with antibiotics may also be used for the collection of tracheal and cloacal swab samples (Spackman & Suarez, 2008; Woolcock, 2008).

The presence of protein in the viral transport media helps to prevent the degradation of live virus, while the antibiotics added into the medium help to reduce the bacterial contamination during handling and transport to the laboratory.

The avian influenza (AI) virus is usually isolated and propagated by inoculating either swab or tissue sample extracts from infected birds into the chorioallantoic sac of 9-11 day-old embryonating chicken eggs (Woolcok, 2008). After 4-7 days of incubation, amnioallantoic fluid (AAF) is taken from the eggs containing dead or dying embryos as they arise and tested for hemagglutination (HA) activity. Detection of HA activity indicates a high probability of the presence of an influenza A virus or of an avian paramyxovirus. Note that fluids that give a negative reaction should be passaged into at least one further batch of eggs (OIE, 2005).

Further confirmation that the virus isolated is indeed AI virus can be determined by Agar Gel Immunodiffusion (AGID) Assay. Alternatively, the presence of AI virus can also be confirmed by the use of reverse-transcription polymerase chain reaction (RT-PCR) or by the test of a commercially available immunoassay kit specific for type A influenza, e.g. Directigen™ Flu A (Becton, Dickson and Company, Franklin Lakes, NJ, USA) or *Flu* Detect® (Synbiotics Corporation, San Diego, CA, USA).

Antibody to influenza A virus can be detected by hemagglutination-inhibition (HI) test, where the quantity of antibody titers can be measured using inhibition activity. Use of the agar gel immunodiffusion (AGID) test is a satisfactory way to indicate antibody to virus in poultry serum, but various enzyme-linked immunosorbent assays (antibody detection ELISA tests) are now also available, e.g., IDEXX FlockCheck® MultiS-Screen (IDEXX Laboratories, Inc., Westbrook, ME, USA). Influenza A virus sub-typing (for 16 H- and 9 N subtypes) can be conducted using hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) assays.

REFERENCES

REFERENCES

Alexander, D.J. (1982). Ecological aspects of influenza viruses in animals and their relationship to human influenza: a review. *Journal of Royal Society of Medicine, 75,* 799-811.

Alexander, D.J. (2000, May 22). A review of avian influenza in different bird species. *Veterinary Microbiology: An International Journal, 74(1-2),* 3-13.

Alexander, D.J. (2007, July 26). An overview of the epidemiology of avian influenza. *Vaccine Journal, 25(30),* 5637-44.

Baigent, S.J. & McCauley, J.W. (2003). Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. *BioEssays Journal, 25,* 657-71.

Becker, W.B. (1966). The isolation and classification of tern virus: influenza virus A/tern/South Africa/1961. *Journal of Hygiene, 64,* 309–320.

Butler, D. (2006). Thai dogs carry bird-flu virus, but will they spread it? *Nature Journal, 439,* 773.

Causey, D. & Edwards, S.V. (2008). Ecology of avian influenza virus in birds. *Journal of Infectious Diseases, 197,* S29–33

Friend, M., Franson, J.C., & Ciganovich, E.A. (Eds.) (1999). Avian influenza. In *Field Manual of Wildlife Diseases: General Field Procedures and Diseases of Birds* (pp. 181-4). Washington, D.C.: Biological Resources Division, U.S. Geological Survey.

Gauthier-clerc, M., Lebarbenchon, C., & Thomas, F. (2007). Recent expansion of highly pathogenic avian influenza H5N1**:** a critical review. *Ibis: The International Journal of Avian Science, 149 (2),* 202–214.

Heeney, J.L. (2006). Zoonotic viral diseases and the frontier of early diagnosis, control and prevention. *Internal Medicine Journal, 260,* 399–408.

Heinen, P. (2003). Swine influenza: a zoonosis. *Veterinary Science for Tomorrow.* Available from URL: http://www.vetscite.org/publish/articles/000041/print.html

Jacob, J.P., Butcher, G.D., Mather, F.B., & Miles, R.D. (2009). Avian influenza in poultry [Electronic version]. University of Florida, PS38, 1-4. Available from URL: http://doacs.state.fl.us/ai/aiindex.htm

Killian, M.L. (2008). Avian influenza virus sample types, collection, and handling. In E. Spackman (Ed.), *Avian Influenza Virus Methods in Molecular Biology, 436,* (pp. 7-18). Totowa, NJ: Humana Press.

Lednicky, J.A., Hamilton, S.B., Tuttle, R.S., Sosna, W.A., Daniels, D.E., & Swayne, D.E. (2010): Ferrets develop fatal influenza after inhaling small particle aerosols of highly pathogenic avian influenza virus A/Vietnam/1203/2004 (H5N1) [Electronic version]. *Virology Journal, 7, 231*, 1-15. Available from URL: http://www.virologyj.com/content/7/1/231

Munster, V.J., Baas, C., Lexmond, P., Waldenstrom, J., Wallensten, A., Fransson, T., et al. (2007, May). Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog 3(5): e61. doi:10.1371/journal.ppat.0030061

Olsen, B., Munster, V.J., Wallensten, A., Waldenstrom, J., Osterhaus, A.D.M.E., & Fouchier, R.A.M. (2006, April 21). Global pattern of avian influenza A virus in wild birds. *Science, 312 (5772),* 384-8. doi: 10.1126/science.1122438

Roberston, S.I., Bell, D.J., Smith, G.J., Nicholls, J.M., Chan, K.H., Nguyen, D.T., et al. (2006): Avian influenza H5N1 in viverrids: implications for wildlife health and conservation. *Proceedings of Royal Society Biological Sciences 273*, 1729–32. doi:10.1098/rspb.2006.3549

Saurez, D.L. (2008). Influenza A virus. In D.E. Swayne (Ed.), *Avian Influenza* (pp. 3-22). Ames, IA: Blackwell.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Meemak, N., Pariyothorn, N., et al. (2006b, April). Avian influenza H5N1 in naturally infected domestic cat. *Emerging Infectious Diseases Journal. 12 (4),* 681-3.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Pariyothorn, N., Payungporn, S., et al. (2006c, November). Fatal avian influenza A H5N1 in a dog. *Emerging Infectious Diseases Journal. 12 (11),* 1744-7.

Spackman, E. & Suarez, D.L. (2008). Detection and identification of the H5 hemagglutinin subtype by real-time PCR. In: E. Spackman (Ed.), *Methods in Molecular Biology, Avian Influenza Virus, 436,* (pp. 27 – 33). Totowa, NJ: Humana Press.

Stallknecht, D.E. & Shane, S.M. (1988). Host range of avian influenza virus in free-living birds. *Veterinary Research Communications, 12,* 125-141.

Subbarao, K. & Joseph, T. (2007, April). Scientific barriers to developing vaccines against avian influenza viruses. *Nature Reviews Immunology 7,* 267-78. doi:10.1038/nri2054

Swayne, D.E. & Suarez, D.L. (2000). Highly pathogenic avian influenza. *Revue scientifique et technique, 19*, 463-82.

Swayne, D.E. (2010). Avian Influenza. In C.M. Kahn (Ed.), *The Merck Veterinary Manual* (10th ed.) (pp. 2498-2500). Whitehouse Station, N.J.: Merck & CO.

Thanawongnuwech, R., Amonsin, A., Tantilertcharoen, R., Damrongwatanapokin, S., Theamboonlers, A., Payungporn, S., et al. (2005). Probable tiger-to-tiger transmission of avian influenza H5N1. *Emerging Infectious Diseases Journal, 11,* 699-701.

Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., et. al. (2005, November). Highly pathogenic avian influenza H5N1, Thailand, 2004. *Emerging Infectious Diseases Journal, 11(11),* 1664-72.

Toma, B., Vaillancourt, J.P., Dufour, B., Eloit, M., Moutou, F., March, W., et al. (Eds) (1999). *Dictionary of Veterinary Epidemiology*. Ames, IA: Iowa State Press, 284 pages.

United States Department of Agriculture, National Veterinary Services Laboratories Testing Protocol (USDA, APHIS, 2005 June 1). *Hemagglutination-Inhibitionn Test for Subtype Identification of Influenza A Virus Antibody* (pp 1-17). Ames, IA: USDA Animal and Plant Health Inspection Service.

Woolcock, P.R. (2008): Avian influenza virus isolation and propagation in chicken eggs. In E. Spackman (Ed.), *Avian Influenza Virus Methods in Molecular Biology, 436,* (pp. 35-46). Totowa, NJ: Humana Press.

World Health Organization (WHO) (2006). Avian influenza ("bird flu"). *WHO | Avian Influenza Fact sheet*. Available from URL: http://www.who.int/mediacentre/factsheets/avian_influenza/en/index.html

World Health Organization (WHO) (2008): Update on avian influenza A (H5N1) virus infection in humans. In: *The New England Journal of Medicine, 358,* 261-73.

Word Organization for Animal Health (OIE). 2005. Chapter 2.7.12. Avian Influenza. Manual of Diagnostic Tests and Vaccines for Terrestrial Animal. Paris, France. Available from URL: http://www.oie.int/eng/normes/mmanual/A_00037.htm

World Organization for Animal Health (OIE) (2009 October). Highly pathogenic avian influenza: Aetiology; Epidemiology; Diagnosis; Prevention; and Control References. Available from URL: http://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/AVIAN_INFLUENZA_FINAL.pdf

World Organization for Animal Health (OIE) (2010c). Avian Influenza. OIE, Paris. Available from URL: http://www.fao.org/avianflu/en/clinical.html

World Organization for Animal Health (OIE) (2010d). - Terrestrial Animal Health Code: Chapter 10.4. Avian Influenza. OIE, Paris. Available from URL: http://www.oie.int

World Organization for Animal Health (OIE) (2011a). Avian Influenza-Disease Outbreak Map, 2005-2010. OIE, Paris. Available from URL: http://www.oie.int/wahis/public.php?page=disease_outbreak_map

CHAPTER 2

HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 OUTBREAKS IN THAILAND: THE BURDEN AND IMPACTS

INTRODUCTION

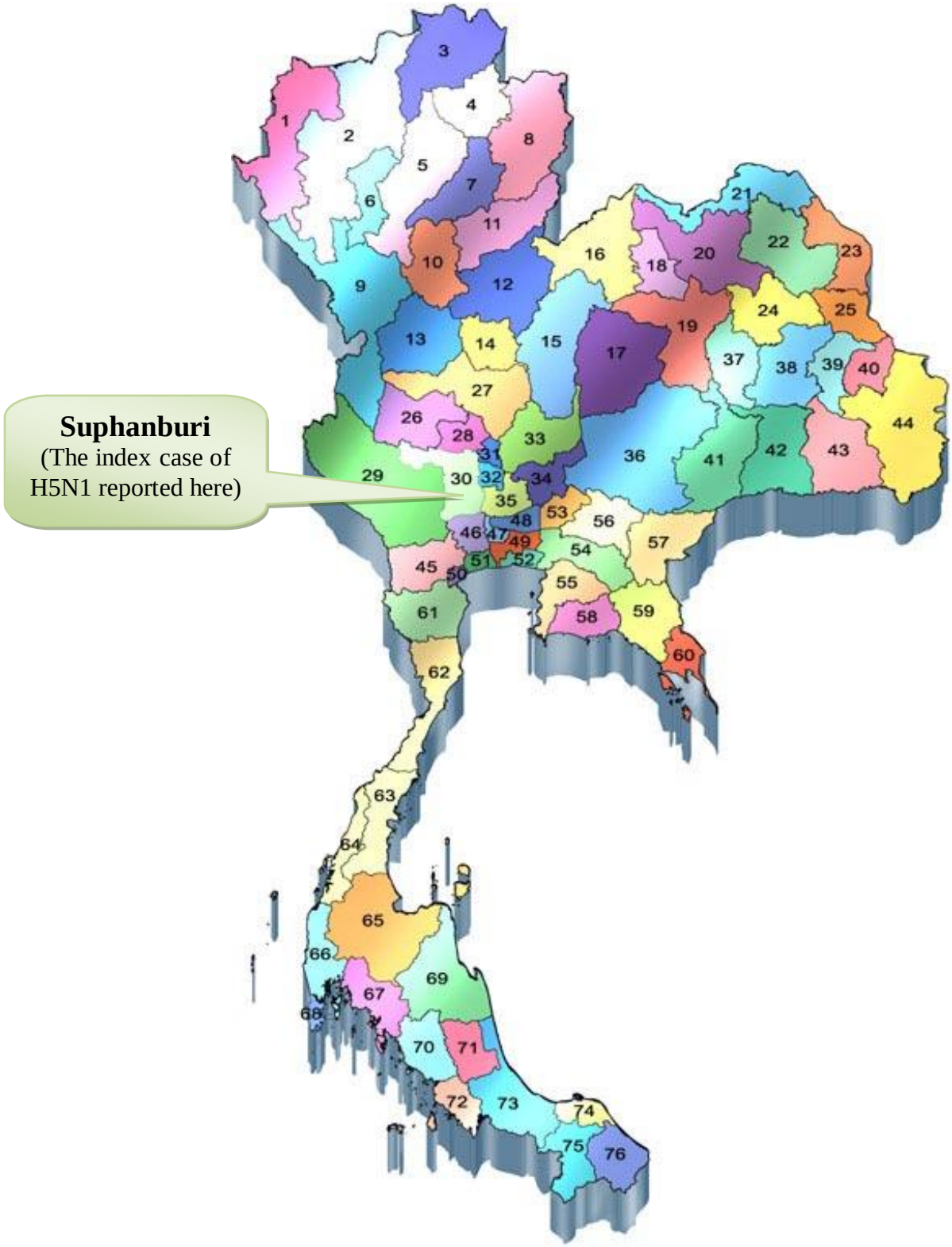
It was not until January 2004 that highly pathogenic avian influenza H5N1 virus was confirmed in Thailand. By late 2003, however, farms in many Central and Northern provinces of the country were encountering large-scale poultry deaths.

At the same time, Vietnam, a neighboring country of Thailand, reported its first confirmed human deaths caused by avian influenza A/H5N1 infection as well as widespread, large-scale poultry infection around its country. In response, Thailand Department of Livestock Development (DLD) then strengthened active surveillance for influenza virus in all kinds of poultry. Cloacal swabs of suspected cases and specimens from the dead poultry were taken from all parts of the country in order to be tested for the evidence of avian influenza infections.

On January 23, 2004, the Department of Livestock Development announced that H5N1 influenza had been identified as the cause of poultry die-offs in Thailand (DLD, 2004: OIE, 2004: Simmerman, 2004). In addition to the confirmed report of H5N1 infection in January 2004, Saito et al. (2009) revealed that the entire genomes of the 3 viruses isolated from chicken, quail, and duck during that first outbreak appeared to be genetically similar to each other; which had a very high pathogenicity, a well-known H5N1 strain.

The purpose of this review is to disclose the situation of avian H5N1 influenza outbreaks in Thailand since 2004. The burden of the disease in poultry and humans, as well as the impacts in terms of health effect both in humans and animals, economic impacts, public awareness, and social consequences in the country were subsequently revealed.

Figure 2-1: Map of Thailand shows location of the 1st HPAI A/H5N1 outbreak in the country

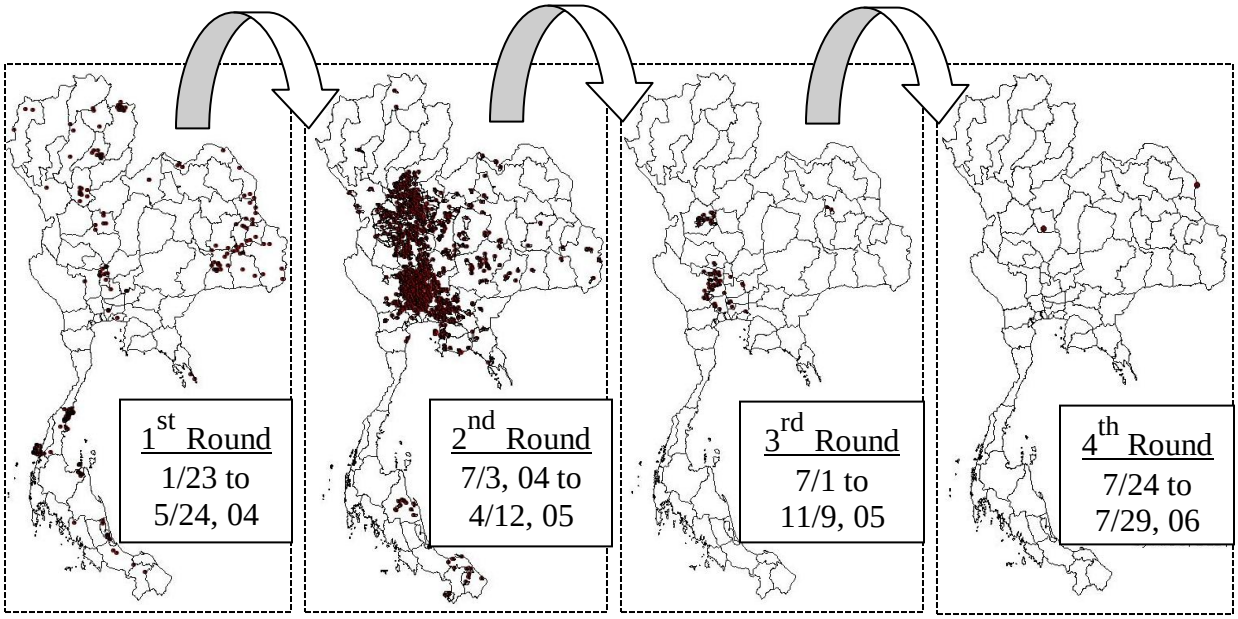


Source: URL: http://www.novabizz.com/Map/img/321.jpg

THE DYNAMICS OF AI OUTBREAKS IN THAILAND

According to OIE (2004), the index case of H5N1 infection in poultry was reported in a layer farm in Suphanburi, a province in the Central region of Thailand (#30 in Figure 2-1). Thereafter, the disease was rapidly distributed in 89 districts from 42 provinces in Thailand (more than one-half of all 76 provinces in the country); indicating that the viruses had been intensively spread throughout the country in a relatively short time (Figure 2.2).

Figure 2-2: The disease distribution during the 1st to the 4th round of H5N1 outbreaks in Thailand, 2004 – 2006



Source: Thanapongtham et al., 2007

- **HPAI A/H5N1 Outbreaks between 2004 and 2006**

There were four major rounds of outbreaks reported between 2004 and 2006: **(1)** January 23 to May 24, 2004; **(2)** July 3, 20004 to April 12, 2005; **(3)** July 1 to November 9, 2005; and **(4)** July 24 to 29, 2006. The disease occurrences for all four rounds of outbreaks were mostly reported in the Central and lower Northern regions of Thailand (Thanapongtham et al., 2007).

For each species of poultry affected during the first round of H5N1 outbreaks, 63.68% (almost two-third) of the cases and deaths were reported in native chickens, 11.58% was in broilers, 10.53% was in laying hens, 6.32% was in ducks, 4.74% was in quails, and 3.15% was in other species.

For the second round of outbreaks, 57.61% (more than half) of infected poultry was in native chickens, 28.81% (a third) was in ducks, 5.32% was in broilers, 4.71% was in laying hens, 2.02% was in quails, and 1.53% was in other species.

As for the third round of outbreaks, approximately 76.32% (more than three-forth) of infected poultry was in native chickens, 7.89 % was in quails, 6.58 % was in ducks, 5.26% was in broilers, 2.63% was in layer hens, and 1.32% was in other species.

For the fourth round, H5N1 outbreak was reported in native chickens and laying hens in Phichit and Nongkhai. All together 2,272 birds (39.2%) were infected and had finally died, while the other 3,523 were humanely destroyed (DLD, 2006a; OIE, 2006).

- **HPAI A/H5N1 Outbreaks in 2007 and 2008**

Three outbreaks were reported in 2007, and four in 2008. No outbreak was reported in 2009-2010 (OIE, 2010e).

Table 2-1: HPAI A/H5N1 outbreaks in Thailand, 2007

No.	Province	Poultry Type	No. of Cases	Confirmed Date
1.	Phisanulok	Layer Ducks	130	Jan/15/2007
2.	Nong Khai	Layer Hens	236	Jan/23/2007
3.	Ang Thong	Native Chickens	16	Jan/31/2007

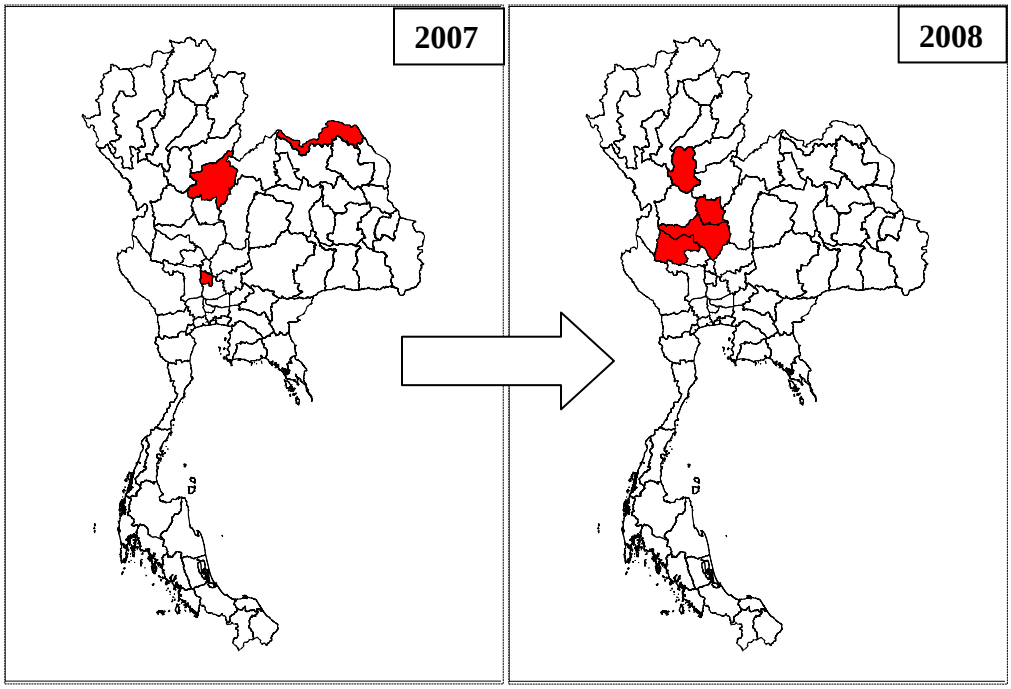
Source: Department of Livestock Development, Thailand (DLD, 2007)

Table 2-2: HPAI A/H5N1 outbreaks in Thailand, 2008

No.	Province	Poultry Type	No. of Cases	Confirmed Date
1.	Nakhon Sawan	Broilers	4,085	Jan/22/2008
2.	Phichit	Native Chickens	30	Jan/25/2008
3.	Sukhothai	Native Chickens	5	Nov/09/2008
4.	Uthai Thani	Native Chickens	5	Nov/23/2008

Source: Department of Livestock Development, Thailand (DLD, 2009a)

Figure 2-3: The outbreaks of HPAI A/H5N1 in Thailand in 2007 and 2008

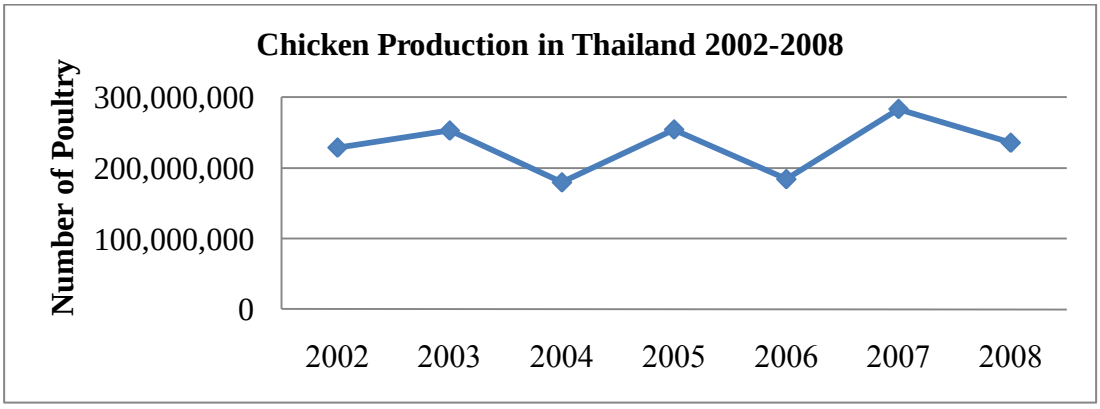


Source: Department of Livestock Development, Thailand (DLD, 2007, 2009a)

IMPACT OF AI OUTBREAKS ON POULTRY PRODUCTION

The numbers of poultry produced in Thailand had rapidly declined after the outbreaks of H5N1. The reduction in numbers of chickens and ducks was obviously reduced in 2004, while the other species had slightly changed in the years after major outbreaks. Figures 2-4 to 2-7 express the production numbers and trends of each poultry species during the outbreak period.

Figure 2-4: Chicken production numbers and trend during H5N1 outbreaks

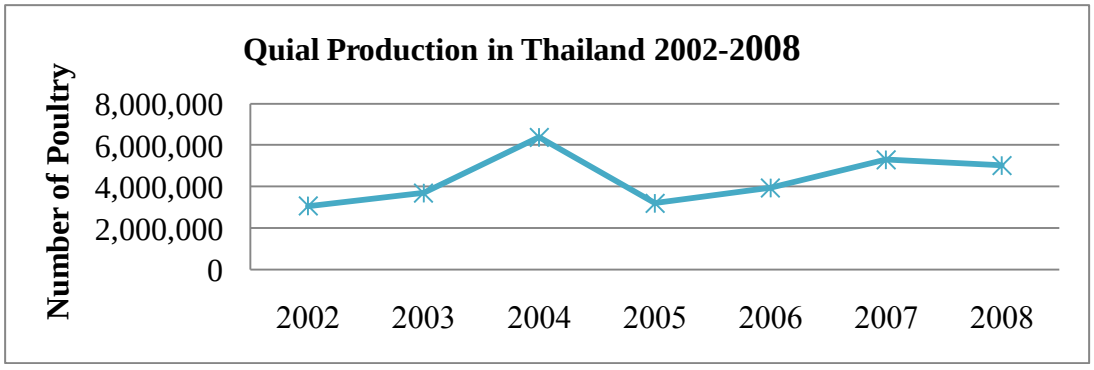


Source: Department of Livestock Development, Thailand (DLD, 2010)

Figure 2-5: Duck production numbers and trend during H5N1 outbreaks

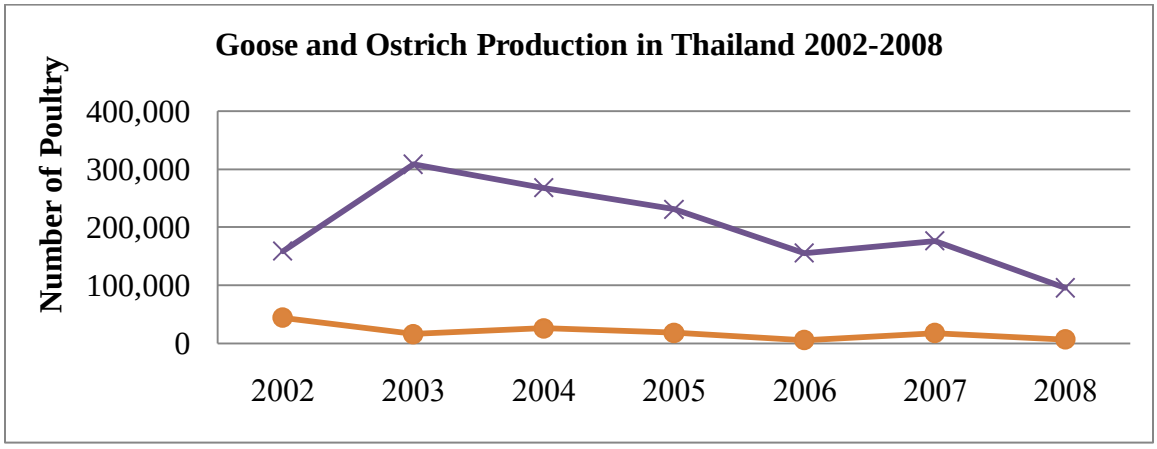
Source: Department of Livestock Development, Thailand (DLD, 2010)

Figure 2-6: Quail production numbers and trend during H5N1 outbreaks



Source: Department of Livestock Development, Thailand (DLD, 2010)

Figure 2-7: Goose and **ostrich** production numbers and trends during H5N1 outbreaks



Source: Department of Livestock Development, Thailand (DLD, 2010)

According to FAO (2006), the total amount of poultry produced reduced from 1.35 million tons in 2003 to 0.92 million tons in 2004. As for local consumption and export volumes, demands for chicken meat were considerably decreased 0.805 and 0.545 million tons in 2003 to 0.701 and 0.219 million tons in 2004.

More than 65 million birds were killed to control HPAI in Thailand in 2004 - 2006 outbreaks, which incurred costs of more than 1 billion Thai baht (or more than US$35 million) of public-money as compensation to the owners affected (NaRanong, 2008). Additionally, the significantly decreased chicken production was due mainly to the frozen broiler-meat being banned from the most important importers; especially the two largest markets, Japan and European Union (EU).

ECONOMIC IMPACT ON THE POULTRY INDUSTRY

Before the unexpected emerging of H5N1 in January 2004, Thailand was ranked in the top four of the most exporting countries in the world broiler-meat trade in 2003; only surpassed by the United States, Brazil, and European Union (EU) as presented in Table 2-3.

Table 2-3: World major chicken meat exporters in 2003

Chicken Meat World Export				
	Metric Tons			**% Market share**
Country	**2001**	**2002**	**2003**	**2002**
United States	2,521,000	2,208,000	2,472,000	44.49
Brazil	1,241,000	1,425,000	1,325,000	23.84
European Union	718,000	670,000	695,000	12.50
Thailand	**425,000**	**415,000**	**435,000**	**7.82**
China (PRC)	489,000	400,000	400,000	7.19
Others	213,000	216,000	229,000	4.16
World	**5,607,000**	**5,334,000**	**5,556,000**	**100**

Source: *Foodmarketexchange.com* (2003a).

The two largest markets for Thailand's broiler meat were Japan and EU, where the primarily value-added parts (semi-cooked and cooked products) were supplied to Japan and frozen parts were exported to the EU. The top 5 largest markets for chicken exported from Thailand are illustrated in Table 2-4.

Table 2-4: Leading markets for Thai's frozen chicken exports in 2001 and 2002

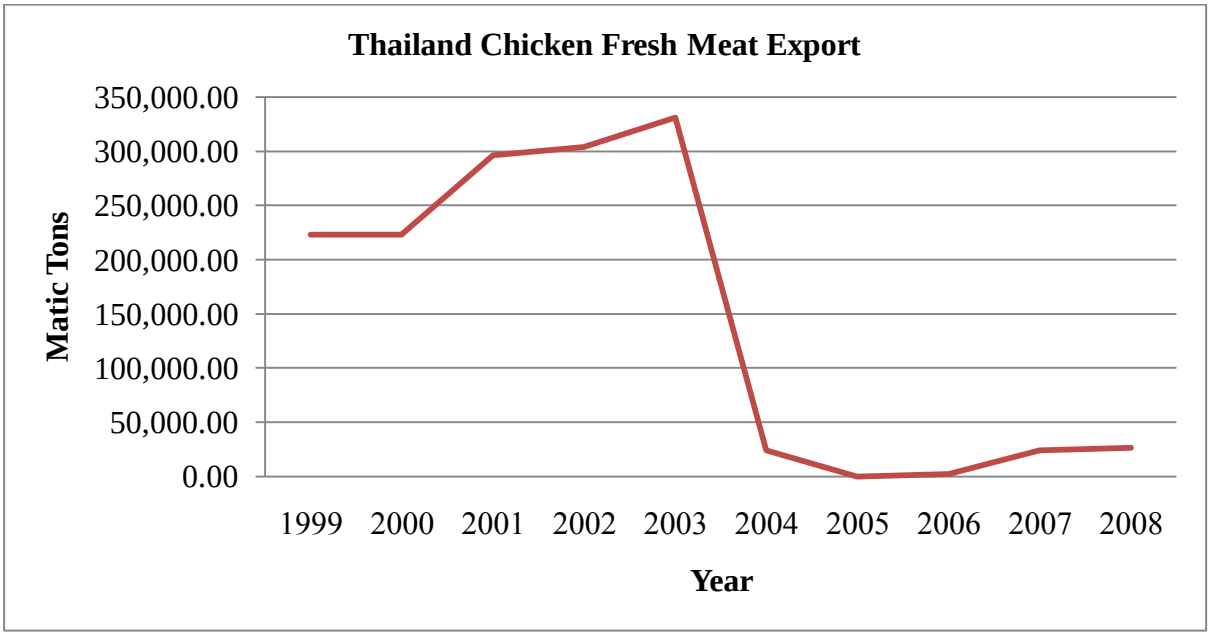
Major Markets for Frozen and Chilled Chicken				
	January- December 2001		January - November 2002	
Country	Quantity	Value	Quantity	Value
Japan	**156,008.76**	**11,842.20**	**1,740,828.96**	**108,640.54**
Germany	47,507.62	4,777.28	171,650.37	11,536.68
Netherlands	21,67.72	2,161.97	128,590.85	8,055.81
United Kingdom	17,483.83	1,800.48	98,965.50	8,548.76
Korea, Republic of	22,674.73	1,304.89	345,279.87	17,326.22
Others	38,510.61	1,607.27	351,088.75	14,873.57
Total	**282,185.55**	**23,494.09**	**3,109,404.30**	**168,981.58**

Remark: Quantity: tons; Value: million Thai Baht

Source: *Foodmarketexchange.com* (2003b).

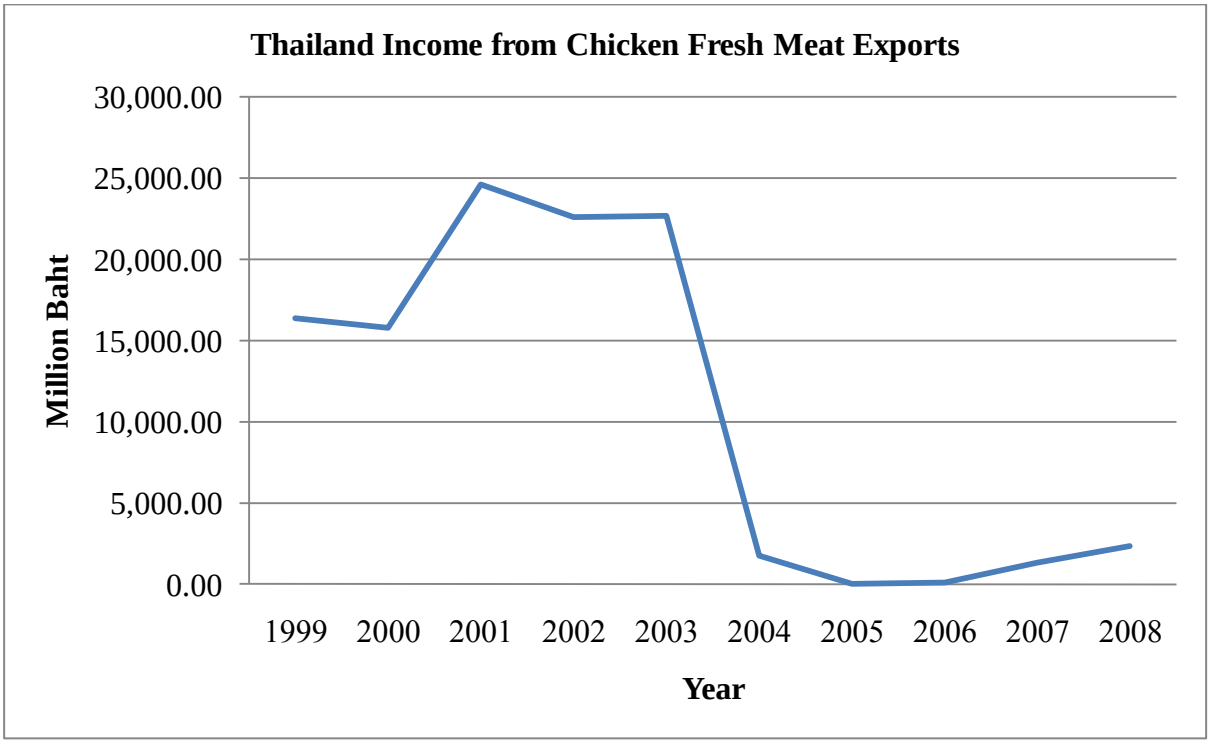
The senior executive of Thai Broiler Processing Exporters Association revealed that almost 100,000 million baht (around US$3,330 million) was lost during the outbreaks of H5N1 in Thailand (FAO, 2006). According to the DLD (2009b), the chicken fresh-meat exports (consequently the country income) were precipitously decreased since the first outbreak of H5N1. The reduction in terms of chicken fresh meat exports and the country income are presented in Figures 2-8 and 2-9.

Figure 2-8: Reduction of chicken fresh-meat exports



Source: Department of Livestock Development, Thailand (DLD, 2009b)

Figure 2-9: Reduction of country income from chicken fresh-meat exports

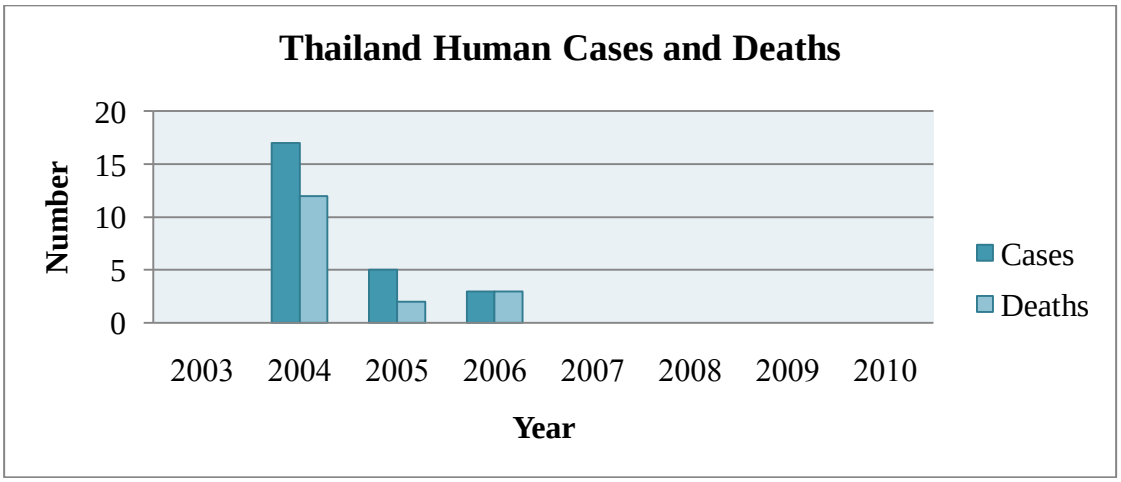


Source: Department of Livestock Development, Thailand (DLD, 2009b)

IMPACT OF AI OUTBREAKS ON HUMANS

As of December 29, 2010, there are 512 human cases from 15 countries reported to the World Health Organization. Of the 512 human cases, 304 cases have been fatal. In Thailand, 25 human cases (17 deaths) were reported thus far (WHO, 2010a). The overall human cases and deaths reported in Thailand are illustrated in Figure 2-10 and Table 2-5.

Figure 2-10: The overall number of H5N1 human cases and deaths in Thailand



Source: Adapted from the World Health Organization (WHO, 2010a)

In summary, the descriptive statistics of confirmed human cases in Thailand is as follows: Of all 25 cases, 16 (64%) are male and more than 50% are children aged less than 15 years. The median age is 17 years old, and the mean is 22.4; which range from 1.5 – 59 years. Of the 25 cases for which H5N1 infection is finally confirmed, 17 had died (case fatality = 68%). In the 18 provinces for which the data are available, Kanchanaburi (a province located in Western Thailand) had the highest, which are four confirmed human cases. The exact occupation of each case was not provided. However, the history of direct contact to the chicken carcasses appeared to be related to H5N1 infections in humans reported in Thailand. Thus far, there is no human-to-human transmission reported in the country.

Table 2-5: Chronology of H5N1 infections in humans in Thailand since 2004

No.	Date Reported	Case	Death	Age (y/o)	Sex	Status/ Occupation	Province
1.	Jan. 23, 04	1st	1st	7	M	Child	Suphanburi
2.	Jan. 25, 04	2nd	2nd	6	M	Child	Kanchanaburi
3.	Jan. 26, 04	3rd	3rd	6	M	Child	Sukhothai
4.	Feb. 2, 04	4th	4th	58	F	n.a.	Suphanburi
5.	Feb. 5, 04	5th	5th	6	M	Child	Kanchanaburi
6.	Feb. 12, 04	6th	6th	13	M	Child	Chaiyaphum
7.	Feb. 13, 04	7th	-	2	M	Child	Suphanburi
8.	Feb. 13, 04	8th	-	27	F	n.a.	Uttaradit
9.	Feb.18, 04	9th	7th	5	M	Child	Khonkaen
10.	Feb.27, 04	10th	-	46	F	n.a.	Lopburi
11.	Mar.9, 04	11th	-	31	M	n.a.	Nakhon Ratchasima
12.	Mar.17, 04	12th	8th	39	F	n.a.	Ayudhaya
13.	Sep.9, 04	13th	9th	18	M	n.a.	Prachinburi
14.	Sep.28, 04	14th	-	32	F	n.a.	Kamphaengphet
15.	Sep.28, 04	15th	10th	26	F	n.a.	Nontaburi
16.	Oct.4, 04	16th	11th	9	F	n.a.	Petchabun
17.	Oct. 25, 04	17th	12th	14	F	Child	Sukhothai
18.	Oct.24, 05	18th	13th	48	M	n.a.	Kanchanaburi
19.	Oct.24, 05	19th	-	7	M	Child	Kanchanaburi
20.	Nov.1, 05	20th	-	50	F	n.a.	Nonthaburi
21.	Nov.14, 05	21st	-	1.5	M	Child	Bangkok
22.	Dec.9, 05	22nd	14th	5	M	Child	Nakhon Nayok
23.	Jul.23, 06	23rd	15th	17	M	n.a.	Phichit
24.	Aug.24, 06	24th	16th	27	M	n.a.	Uthai Thani
25.	Sep.27, 06	25th	17th	59	M	Farmer	Nongbualamphu

Remarks: F = Female, M = Male, n.a. = not available, y/o = years old

Source: the World Health Organization (WHO, 2010a)

PUBLIC AWARENESS AND SOCIAL CONSEQUENCES

Direct effects from H5N1 outbreaks in Thailand (e.g., domestic poultry died from disease infection; the poultry production had rapidly decreased due mainly to massive culling, and the international trade barriers) were remarkably observed. On the other hand, indirect effects for the Thai society were also noticed. For instance, sales of feed and animal health products had gone down, so contract farmers had no more work; therefore, leaving the industry all together. Some broiler farmers switched to other poultry farming or other businesses. Less of them continued to operate the broiler farm, but with fewer numbers of birds (NaRanong, 2008).

Consumer panic of the disease was also the main problematic issue for the Thai government. During the outbreaks of bird flu, there was no chicken or egg product on the menu in restaurants or even in school luncheons. Many chicken restaurants, including catering businesses, were completely closed or switched to another kind of food retail. As such, many people were jobless.

Before H5N1 outbreaks, the per capita consumption of broiler meat in Thailand was estimated to be about 13-14 kg per annum (USDA, 2004). Per capita consumption reduced from 14 kg to 8 kg in 2004 (FAO, 2006). However, in 2010 the per capita consumption of chicken meat in Thailand has been substantially increased to 12-13 kg, and the consumption trend is estimated to be 14 kg per capita in 2011 (USDA, 2010).

The emergence and spreading of H5N1 in Thailand have affected not only the poultry industry and human health, but also awakened the public awareness and concerns among Thai people from the basic unit, as the family section, to the nationwide society in the country as a whole.

THE LESSONS LEARNED FROM DEALING WITH H5N1 OUTBREAKS

It has become apparent lately that high poultry population density, especially large free-grazing duck populations and the production of other backyard poultry with minimal biosecurity, represent the major risk factors for maintenance and transmission of HPAI. Evidently, domestic ducks and backyard poultry have played a key role in the persistence of infection because they can be infected silently, and the disease could unfortunately be underestimated (Gilbert et al., 2006, 2007; Songserm et al., 2006a; Tiensin et al., 2005, 2007).

During the first phase of the outbreaks in Thailand, it was difficult to achieve effective progress on reducing the incidence of HPAI since the potential risk factors and dynamics of disease transmission were not well defined for public perception. As a result, the disease rapidly spread within the susceptible poultry in more than half of the country (42 out of 76 provinces) during the first 3-month-period. Additionally, more than 12 human cases were affected within a relatively short time in the first 2 months. Therefore, public education on the full information, especially the preventive and control strategies, of the disease should be conducted first to increase public awareness and to delay or limit spreading of the disease. Moreover, attempts at controlling HPAI by culling, movement control, and decontamination in areas of high HPAI incidence in the country have had limited success. Improved outbreak response needs to be promoted, and sustainable reduction in HPAI incidence in entrenched areas requires more attention to risk reduction measures (FAO, 2010).

In summary, strong collaboration between the Thai government and other related authorities, such as the poultry industry sectors, was a key component of the successful campaign in Thailand; leading to a situation in which the disease was essentially under control with only infrequent isolated outbreaks of HPAI.

REFERENCES

REFERENCES

Department of Livestock Development (DLD, 2004). Avian influenza situations and control measures: Available from URL: http://www.dld.go.th/home/bird_flu/history_47.html

Department of Livestock Development (DLD, 2006a). Avian Influenza: Daily Report on August 2, 2006. Available from URL: http://www.dld.go.th/home/bird_flu/AICOC/AICOC_06/2_8_06.jpg [Cited on 2006 August 02]

Department of Livestock Development (DLD, 2007). Avian Influenza: Daily Report on March 15, 2007. Available from URL: http://www.dld.go.th/home/bird_flu/AICOC/AICOC_07/15_3_07.html [Cited on 2007 March 15]

Department of Livestock Development (DLD, 2009a). Avian Influenza Control Center: Daily Situation; January 1, 2009. Available from URL: http://www.dld.go.th/home/bird_flu/birdflu.html [Cited on 2009 January 01]

Department of Livestock Development (DLD, 2009b). Poultry export by destination, 2008. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=65:--2551&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2010). Statistics of chicken and duck, classified by region (2010). Available from URL: http://www.dld.go.th/ict/th/images/stories/stat_web/yearly/2553/chickenduck_ket.pdf [Cited on 2010 October 27]

Food and Agricultural Organization of the United Nations (FAO, 2006). CPF Compartmentalization (Broiler Integration): Boonpeng Santiwattanatam. In: *Report of the Twenty-ninth Session of the Animal Production and Health Commission for Asia and the Pacific (APHCA)*. Bangkok: 2006; 89-91.

Food and Agricultural Organization of the United Nations (FAO, 2010). FAO Regional Strategy for Highly Pathogenic Avian Influenza and other Emerging Infectious Diseases of Animals in Asia and the Pacific, 2010–2015. Bangkok: 2010; p. 23.

Food market exchange.com. (2003a). World Broiler Meat Trade Forecast, Outlook and Situation for 2003. Available from URL: http://www.foodmarketexchange.com/datacenter/product/poultry/chicken/detail/dc_pi_pt_chicken0805_01.php [Cited on 2008 May 01]

Food market exchange.com (2003b). Broiler Trade, Leading markets for Thai frozen chicken exports in 2002**.** Available from URL: http://www.foodmarketexchange.com/datacenter/product/poultry/chicken/detail/dc_pi_pt_chicken0403_01.php [Cited on 2004 March 01]

Gilbert, M., Chaitaweesup, P., Parakamawongsa, S., T., Premashthira, Tiensin, T., Kalpravitdh, W., Wagner, H., et al. (2006, February). Free-grazing ducks and highly pathogenic avian influenza, Thailand. *Emerging Infectious Diseases Journal, 12(2),* 227-34.

Gilbert, M., Xio, X., Chaitaweesup, P., Kalpravitdh, W., Premashthira, S., and Boles, S., et al. (2007). Avian influenza, domestic ducks and rice agriculture in Thailand. *Agriculture, Ecosystems, and Environment Journal, 119*, 409–415.

Map of Thailand (2010): Available from URL: http://www.novabizz.com/Map/img/321.jpg

NaRanong, V. (2008). Structural changes in Thailand's poultry sector: avian influenza and its aftermath. *TDRI Quarterly Review, 23*, 3-10.

Saito, T., Watanabe, C., Takemae, N., Chaisingh, A., Uchida, Y., Buranathai, C., et al. (2009): Pathogenicity of highly pathogenic avian influenza viruses of H5N1 subtype isolated in Thailand for different poultry species. *Journal of Veterinary Microbiology, 2009 Jan 1, 133(1-2),* 65-74.

Simmerman, J.M., Thawatsupha, P., Kingnate, D., Fukada, K., Chaising, A., and Dowell, S.F. (2004): Influenza in Thailand: a case study for middle income countries. *Vaccine Journal, 23 (2004),* 182–7.

Songserm ,T., Jam-on, R., Sae-Heng, N., Meemak, N., Hulse-Post, D.J., Sturm-Ramirez, K.M., et al. (2006a, April). Domestic ducks and H5N1 influenza epidemic, Thailand. *Emerging Infectious Diseases Journal, 12(4)*, 575-81.

Thanapongtham, W., Hongchumpol, N., & Phungjiem, P. (2007). The outbreaks of avian influenza in Thailand, 2004-2006 [Thai version]. *The Department of Livestock Development, Thailand*. Available from URL: www.dld.go.th/home/bird_flu/16-10-49KAN/file/3-1-50.doc

Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., et. al. (2005, November). Highly pathogenic avian influenza H5N1, Thailand, 2004. *Emerging Infectious Diseases Journal, 11(11),* 1664-72.

Tiensin, T., Nielen, M., Songserm, T., Kalpravidh, W., Chaitaweesub, P., Amonsin, A., et al. (2007). Geographic and temporal distribution of highly pathogenic avian influenza A virus (H5N1) in Thailand, 2004-2005: an overview. *Avian Diseases Journal, 51,* 182-8.

United States Department of Agriculture, Foreign Agricultural Service (USDA, 2004). World Poultry Trade Overview - October 2004. Available from URL: http://www.thepoultrysite.com/articles/250/world-poultry-trade-overview-october-2004 [Cited on 2004 October 25]

United States Department of Agriculture, Foreign Agricultural Service (USDA, 2010). Thailand: Production, Supply and Demand for Broiler Meat In: *Thailand Poultry and Product; 2010 Annual, September 1, 2010*, 8 pages.

World Health Organization (WHO) (2010a). Cumulative number of confirmed human cases of avian influenza A/(H5N1) Reported to WHO: 29 December 2010. *WHO | Avian Influenza Archives*. Available from URL: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_12_29/en/index.html

World Organization for Animal Health (OIE, 2004). Avian Influenza: Disease information. Available from URL: http://www.oie.int/downld/AVIAN%20INFLUENZA/A2004_AI.php [Cited on 2004 January 23]

World Organization for Animal Health (OIE, 2006). Avian Influenza: Disease information. Available from URL: http://www.oie.int/downld/avian%20influenza/A2006_AI.php [Cited on 2006 November 07]

World Organization for Animal Health (OIE, 2010e). Update on Highly Pathogenic Avian Influenza in Animals (Type H5 and H7) 2009 and 2010. Available from URL: http://www.oie.int/downld/AVIAN%20INFLUENZA/A2010_AI.php [Cited on 2010 December 09]

CHAPTER 3

POULTRY PRODUCTION SYSTEMS IN THAILAND: THE IMPORTANT ROLES OF TRADITIONAL BACKYARD CHICKENS AND OPEN FIELD-REARED DUCKS

INTRODUCTION

The structure of poultry production systems in Thailand is roughly divided into two sectors according to the scale and purposes of the production, i.e. the commercial and the smallholder sectors. However, according to the Food and Agricultural Organization of the United Nations (FAO, 2004), the poultry production systems can be categorized into four sectors based on the level of biosecurity and marketing of birds and bird products.

Sector 1: Industrial integrated system with high level biosecurity, and birds/products marketed commercially (e.g. farms that are part of an integrated broiler production enterprise with clearly defined and implemented standard operating procedures for biosecurity).

Sector 2: Commercial poultry production system with moderate to high biosecurity, and birds/products usually marketed commercially (e.g. farms with birds kept indoors continuously strictly preventing contact with other poultry or wildlife).

Sector 3: Commercial poultry production system with low to minimal biosecurity, and birds/products entering live bird markets (e.g. a caged layer farm with birds in open sheds, a farm with poultry spending time outside the shed, and a farm producing chickens and waterfowl).

Sector 4: Village or backyard production with minimal biosecurity, and birds/products consumed locally (e.g. a backyard native chicken farm and a free-raging, meat or egg type, duck farm produced for household or for local consumption).

The poultry production systems defined by the FAO are presented in Table 3-1.

Table 3-1: The definition of poultry production sectors according to the FAO, 2004

Sectors FAO/ Definition	Systems			
	Industrial and integrated	Commercial Poultry Production		Village or Backyard
		Biosecurity		
		High	Low	
	Sector 1	Sector 2	Sector 3	Sector 4
Biosecurity	High	Mod-high	Low	Low
Market outputs	Export and urban	Urban/rural	Live urban/rural	Rural/urban
Dependence on market for input	High	High	High	Low
Dependence on goods roads	High	High	High	Low
Location	Near capital and major cities	Near capital and major cities	Smaller towns and rural areas	Everywhere. Dominates in remote areas
Bird kept	Indoors	Indoors	Indoors/Part-time outdoors	Out most of the day
Shed	Closed	Closed	Closed/Open	Open
Contact with other chickens	None	None	Yes	Yes
Contact with ducks	None	None	Yes	Yes
Contact with other domestic birds	None	None	Yes	Yes
Contact with wildlife	None	None	Yes	Yes
Veterinary Service	Own veterinarian	Pays for veterinary Service	Pays for veterinary Service	Irregular, depend on govt. vet service
Source of medicine/vaccine	Market	Market	Market	Government and market
Source of technical information	Company and associated	Sellers of inputs	Sellers of inputs	Govt. extension service
Source of finance	-	-	-	-
Breed of poultry	Commercial	Commercial	Commercial	Native
Food security of owner	High	Ok	Ok	From ok to bad

Source: Rushton et al., 2007

POULTRY PRODUCTION SYSTEMS IN THAILAND

- **Structure of Poultry Production in Thailand**

Over two decades before the unexpected emergence of H5N1 in 2004, the structure of poultry production in Thailand had been moving toward greater industrialization and increased vertical integration (NaRanong, 2008). Although the intensive farming was very high biosecurity and semi-vertical, the traditional systems of raising backyard poultry with minimal biosecurity (Sectors 3 and 4) were still widely practiced. Almost 99% of poultry producers were segregated in the latter two, as illustrated in Table 3-2.

Table 3-2: Classification of poultry production systems in Thailand

Sector 1 Industrial and Integrated	**Sector 2 Commercial with HIGH Biosecurity**	**Sector 3 Commercial with LOW Biosecurity**	**Sector 4 Village or Backyard**
70% Production	20% Production	10% Production	
3% Producers		36% Producers	61% Producers
Export Market		Local, National Market	

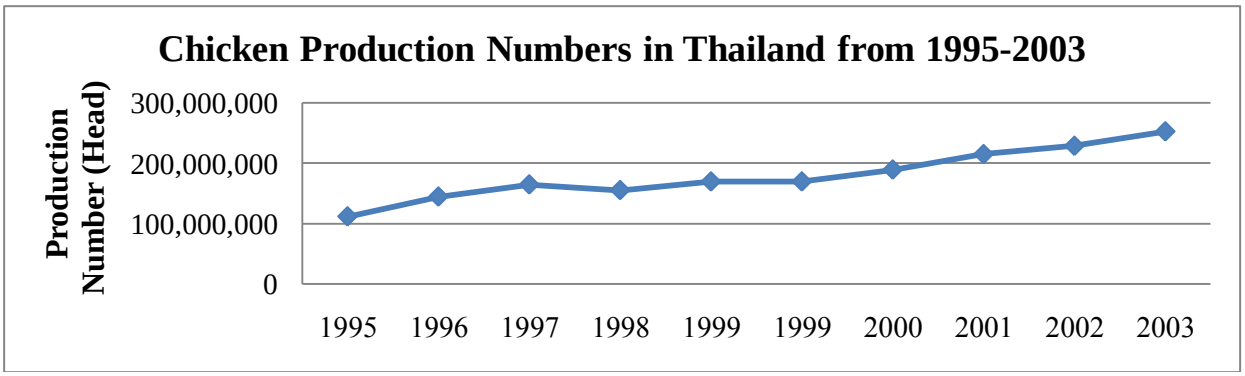
Source: Rushton et al., 2007

- **Numbers of Poultry Production: Before the Emergence of H5N1**

Generally, there are five major species of poultry predominantly produced in Thailand. The frequently raised species is chicken, secondly is duck, while the other species (goose, quail, and ostrich) are also commercially produced but in lesser extent. Since the information of other species is rarely reported, the most commonly produced species, chicken and duck, are the main focus in this education.

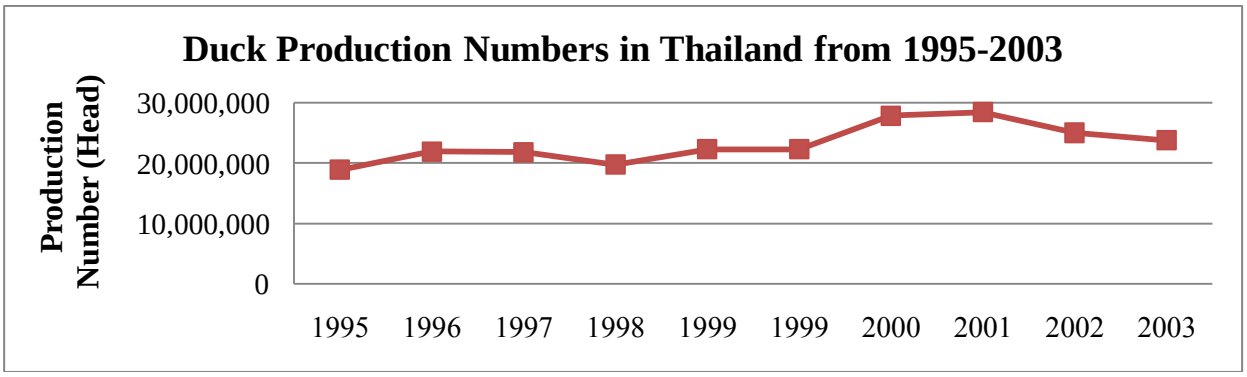
Obviously, the production trends of chickens and ducks had been significantly increased during the 1995-2003 period (Figures 3-1 and 3-2). Such changes were considered to be the results of structural changes in the poultry sector as mentioned earlier.

Figure 3-1: Chicken production numbers and trends (before the emergence of H5N1)



Source: Department of Livestock Development, Thailand (DLD, 2009c)

Figure 3-2: Duck production numbers and trends (before the emergence of H5N1)



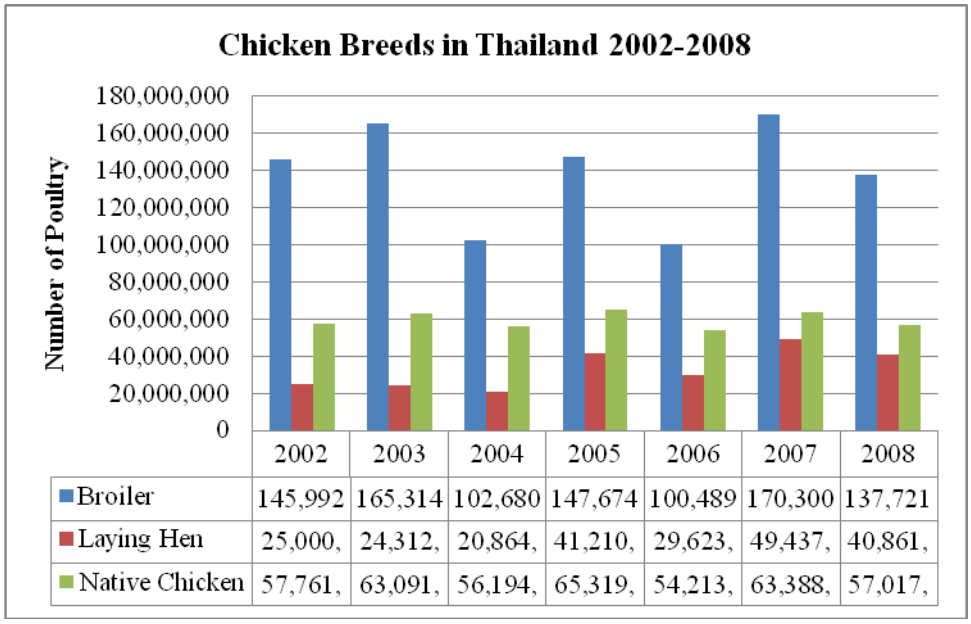
Source: Department of Livestock Development, Thailand (DLD, 2009d)

POULTRY INDUSTRY IN THAILAND

The Thailand poultry industry has been depending mainly on the meat-type breed chickens (broilers), which mostly are produced for international markets. The other breeds (laying-type hens, egg ducks, and native poultry) are obviously less in production numbers, and are mostly produced for local and national consumption.

The broiler meat industry in Thailand is made up of the western breeds (Aviagen, Cobb, and Hubbard) and mixed-native backyard. Most of the breeds are bred by the large integrators who have their own facilities and are raised by contract farmers who are strictly controlled by the integrators (Jullabutradee, 2005). The varieties of chicken and duck breeds produced in Thailand are presented in Figures 3-3 and 3-4.

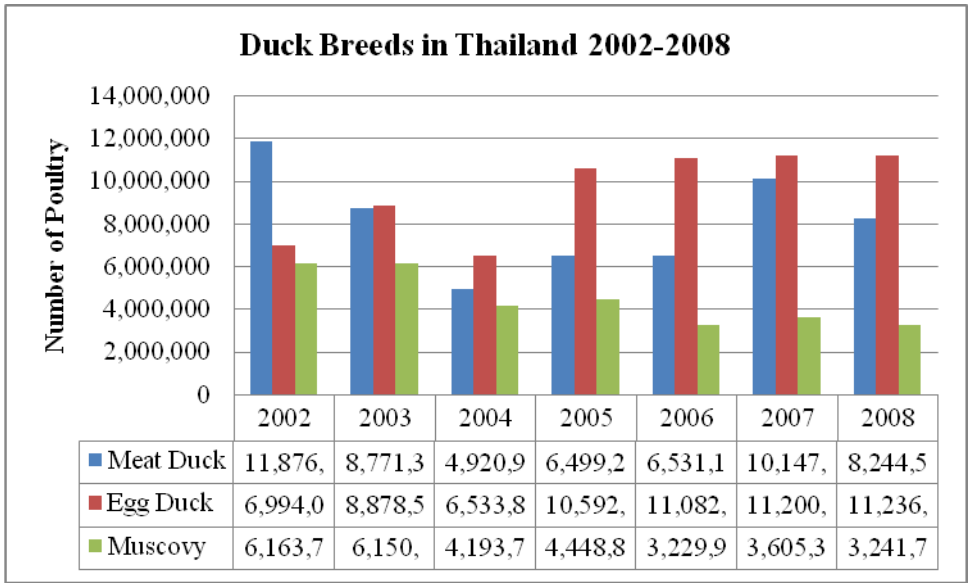
Figure 3-3: Chicken production numbers in Thailand classified by breed type



	2002	2003	2004	2005	2006	2007	2008
Broiler	145,992	165,314	102,680	147,674	100,489	170,300	137,721
Laying Hen	25,000,	24,312,	20,864,	41,210,	29,623,	49,437,	40,861,
Native Chicken	57,761,	63,091,	56,194,	65,319,	54,213,	63,388,	57,017,

Source: Department of Livestock Development, Thailand (DLD, 2009e)

Figure 3-4: Duck production numbers in Thailand classified by breed type



	2002	2003	2004	2005	2006	2007	2008
Meat Duck	11,876,	8,771,3	4,920,9	6,499,2	6,531,1	10,147,	8,244,5
Egg Duck	6,994,0	8,878,5	6,533,8	10,592,	11,082,	11,200,	11,236,
Muscovy	6,163,7	6,150,	4,193,7	4,448,8	3,229,9	3,605,3	3,241,7

Source: Department of Livestock Development, Thailand (DLD, 2009f)

TRADITIONAL POULTRY RAISING SYSTEM

Backyard poultry and free-range husbandry is traditionally practiced and is commonly found in several countries in Southeast Asia, including Thailand. The birds freely roam around the gardens/property; mixed and close proximity with humans and other animals, such as, pigs, geese, and natural, free-living birds. They are fed regularly by the owner(s) at least once a day but they mostly rely on the natural feeds, e.g. earth worms, insects, snails, rice, grains, grasses, household vegetable or leftover human foods. The backyard poultry were typically classified by a small number of native chickens and free-ranging, egg, meat, or Muscovy ducks (less than 100 birds per a household). Most chicken species classified in this sector are native and mixed breed (i.e., 3-blood-breed) chickens.

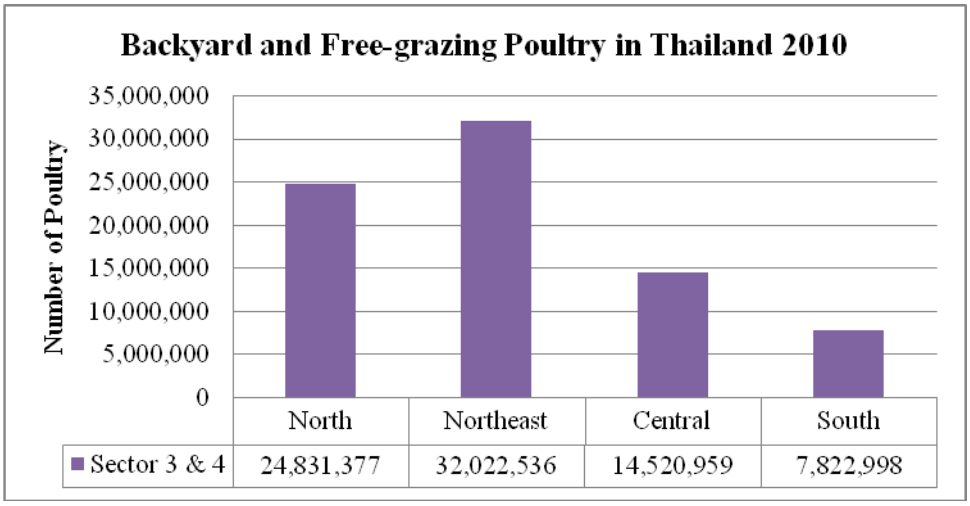
In 2006, Songserm et al. and Gilbert et al. indicated that the domestic ducks raised in open flocks, which were freely ranging and counting mostly more than 1,000 birds, were classified as the "free-grazing ducks". This kind of widespread-raising ducks composed of egg-laying ducks (khaki Campbell) or a crossbreed of khaki Campbell and native laying ducks, including a small number of "meat" ducks, such as Pekin and white Cherry Valley is common. Traditionally, the ducks were raised in the open on rice fields for 5 to 6 months. During that period, the ducks were moved by truck from one field to another to feed on leftover rice grains after the harvest. They were finally brought back to the farms in the open system for egg production and for meat purposes.

The specific information concerning the numbers of traditional poultry was not provided until 2010 when the Department of Livestock Development (DLD, 2010) revealed the number of poultry as it is classified in Sectors 3 and 4. The most commonly raised poultry in these sectors were native chickens (89.91%), while the rest of the species were egg-type and meat-type free grazing ducks (8.99% and 1.10%).

The largest number of traditional poultry production was corresponded to the largest area, the Northeastern region (40.43%). This region is approximately one-third of the overall area in Thailand (*Thailand.com*, 2010). The largest number of native chickens was also reported in the Northeastern region (44.76%), while the highest number of egg- and meat-type free grazing ducks was reported in the Northern region (54.65%).

Approximately 18.34% of this kind of poultry was reported in the Central region, and the least number of poultry (12.06%) had been reported in the Southern region (Figure 3-5).

Figure 3-5: Backyard poultry numbers (native chickens and open field-reared ducks)



Source: Department of Livestock Development, Thailand (DLD, 2010)

BACKYARD POULTRY AND FREE-GRAZING DUCKS:

THE ASSOCIATED RISK FACTORS OF AI IN THAILAND

In 2006, Gilbert et al. revealed that the spatial distribution of HPAI outbreaks in Thailand did not correspond to the areas with high densities of chickens, and the geographic pattern of H5N1 outbreaks mostly found in native chickens was not primarily driven by long-distance transmission between chicken productions units or villages. The authors concluded that the national disease distribution in Thailand showed the strongest association with the distribution of the free-grazing ducks. The additional information revealed that the free-grazing ducks were highly associated with the paddy fields in the Central region of Thailand (Gilbert et al., 2007).

Teinsin et al. (2005) and Songserm et al. (2006) also indicated that open-flock, grazing system, and backyard poultry (namely the backyard chickens and open field-reared ducks) played essential roles as the hosts of H5N1, and evidently, the free-grazing ducks had been identified as the risk factors for the occurrence of H5N1outbreaks in Thailand.

With the high susceptibility and ability of movement, ducks were presumed to be the pandemic threat of the HPAI endemic in Asia (Hulse-Post et al., 2005). The infected ducks showed few clinical signs of disease, but were capable of shedding the H5N1 avian influenza virus for a prolong period of time. The virus circulated in native chickens may have remained unnoticed because disease presence was not prominent, i.e., asymptomatic or low attack rate (Chen et al., 2004; Gilbert et al., 2006).

NATIONWIDE SURVEILLANCE AND CONTROL PROGRAMS

There was no specific surveillance program for traditional poultry raising systems; however, in late 2004 and several times later, the Thai Government launched a thorough surveillance program in all poultry sectors (including other animals and humans), which was called the "X-ray Campaign". The campaign was implemented in collaboration with many authorities under the Thai government, such as, the Ministry of Agriculture and Cooperatives, the Ministry of Public Health, the CEOs, and the Provincial Governors. The new and improved features of this surveillance system included an establishment of over 1,000 Surveillance and Rapid Response Teams (SRRT), 12 Regional, and 1 Central Networking Laboratory. In strong collaboration with the higher authorities and expertise, the Village Health Volunteers (VHV) and the well-trained livestock workers (under the DLD control) played the important roles to detect, investigate, report, support, control, and operate the "influenza-like" cases (WHO, 2007b).

The control measures and actions implemented in poultry sectors in Thailand compose of: Stamping out, Cleaning and Disinfection, Surveillance, Movement Control or Quarantine, Campaign to increase awareness and reduce panic, and other measures (e.g. Biosecurity and Poultry Compartmentalization). Notice that vaccination was not allowed to be applied in any poultry sector in Thailand, up until the presence time (Rushton et al., 2007).

REFERENCES

REFERENCES

Chen, H., Deng, G., Li, Z., Tian, G., Li, Y., Jiao, P., et al. (2004). The evolution of H5N1 influenza viruses in ducks in southern China. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 10452–7.

Department of Livestock Development (DLD, 2009c). Statistics of chicken in Thailand, 1994-2003. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=60:--2546&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2009d). Statistics of duck in Thailand, 1994-2003. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=60:--2546&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2009e). Statistics of chicken in Thailand, 1999-2008. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=65:--2551&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2009f). Statistics of duck in Thailand, 1999-2008. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=65:--2551&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2010). Statistics of chicken and duck, classified by region (2010). Available from URL: http://www.dld.go.th/ict/th/images/stories/stat_web/yearly/2553/chickenduck_ket.pdf [Cited on 2010 October 27]

Food and Agricultural Organization of the United Nations (FAO, 2004). FAO Recommendations on the prevention, control and eradication of highly pathogenic avian influenza in Asia. *FAO Position Paper, September 2004*. FAO Rome Italy, 59 pages

Gilbert, M., Chaitaweesup, P., Parakamawongsa, S., T., Premashthira, Tiensin, T., Kalpravitdh, W., Wagner, H., et al. (2006, February). Free-grazing ducks and highly pathogenic avian influenza, Thailand. *Emerging Infectious Diseases, 12(2)*, 227-34.

Gilbert, M., Xio, X., Chaitaweesup, P., Kalpravitdh, W., Premashthira, S., Boles, S., et al. (2007). Avian influenza, domestic ducks and rice agriculture in Thailand. *Agriculture, Ecosystems & Environment Journal, 119*, 409-15.

Hulse-Post, D.J., Sturm-Ramirez, K.M., Humberd, J., Seiler, P., Govorkova, E.A., Krauss, S., et al. (2005): Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 10682–7.

Jullabutradee, S. (2005). Limitations in the application of control measures during the first avian influenza outbreaks in Thailand [MSc Thesis]. Chiang Mai University, Chiang Mai, Thailand, 89 pages.

NaRanong, V. (2008). Structural changes in Thailand's poultry sector: avian influenza and its aftermath. *TDRI Quarterly Review, 23,* 3-10.

Rushton, J., Viscarra, R., Bleich, E.G., & McLeod, A. (2007): Impact of avian influenza outbreaks in the poultry sectors of five South East Asian countries (Cambodia, Indonesia, Lao PDR, Thailand, Viet Nam) outbreak costs, responses and potential long term control. *TCP/RAS/3010: FAO, Rome, Italy, 10,* 25 pages.

Songserm ,T., Jam-on, R., Sae-Heng, N., Meemak, N., Hulse-Post, D.J., Sturm-Ramirez, K.M., et al. (2006, April). Domestic ducks and H5N1 influenza epidemic, Thailand. *Emerging Infectious Diseases Journal, 12(4)*, 575-81.

Tiensin, T., Nielen, M., Songserm, T., Kalpravidh, W., Chaitaweesub, P., Amonsin, A., et al. (2007). Geographic and temporal distribution of highly pathogenic avian influenza A virus (H5N1) in Thailand, 2004-2005: an overview. *Avian Diseases Journal, 51*, 182-8.

Thailand.com. Overview of the Northeastern Region Available from URL: http://www.thailand.com/travel/overview/overview_northeast.htm [Cited on 2010 November 28]

World Health Organization (WHO, 2007), Regional Office for South-East Asia. Role of village health volunteers in Avian Influenza surveillance in Thailand. New Delhi: 2007; 27 pages.

CHAPTER 4

EPIDEMIOLOGICAL STUDY OF AVIAN INFLUENZA IN BACKYARD CHICKENS IN NORTHERN THAILAND

INTRODUCTION

Even though the major outbreaks of avian influenza A H5N1 in Thailand have declined, the occurrence of the disease remains reported in the domestic poultry and human population of Thailand's neighboring countries, as well as in many countries in Southeast Asia thus far. Avian influenza could probably be circulating in the poultry flocks in Thailand, but remains underestimated or unreported. The main reason for the disease to persist in the country is the existence of traditional backyard chickens and free-range ducks raising systems. These traditional poultry raising systems are particularly strong in Thailand, but limited research has been conducted to determine their role in maintaining and spreading the AI virus. This study was, therefore, designed to test the **hypothesis** that the backyard chickens harbor the avian influenza virus, and the risk factors associated with avian influenza virus or antibody-positive birds will be the history of H5N1 outbreaks in the area, the large flock size, farm location, management practices, and human activities, such as live bird markets, fighting cock arenas, and/or movement of poultry in the area.

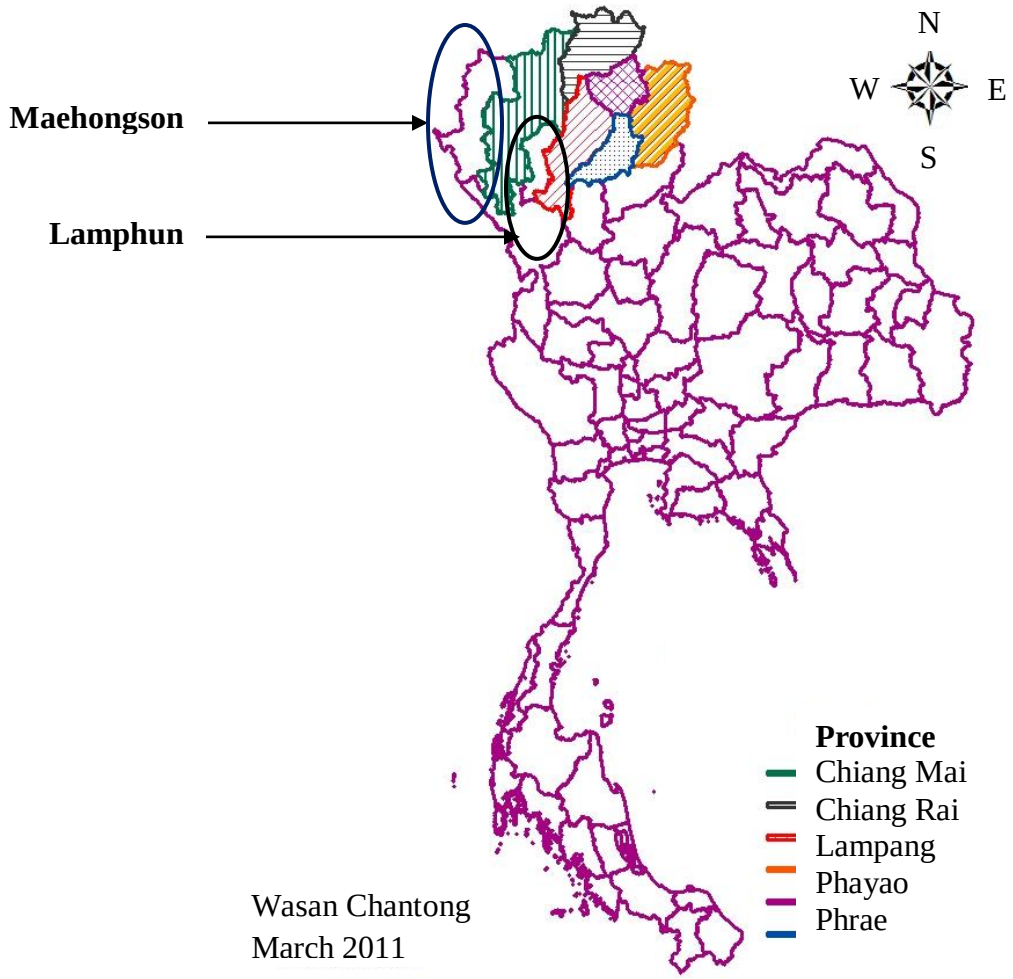
Three objectives were designed to test the stated hypothesis: (1.) Determine the prevalence of the avian influenza occurrence in backyard chickens by a combination of virus isolation and antibody test; (2.) Identify the major risk factors associated with the occurrence of laboratory-confirmed AI; and (3.) Generate Geographic Information System (GIS) mapping of the laboratory-confirmed AI distribution in the Northern region of Thailand.

MATERIALS AND METHODS

❖ STUDY DESIGN AND STUDY SITES

This study was designed as a cross-sectional study, which was performed in the six provinces in the upper region of Northern Thailand, i.e., Chiang Mai, Chiang Rai, Lampang, Nan, Phayao, and Phrae (Figures 4-1). Because of the very low numbers of poultry population **(<5%)** in the province (< **14,775** for ducks **OR** < **559,760** for chickens), Maehongson and Lamphun were not included in this study (Table 4-1).

Figure 4-1: Study area in the 6 provinces of Northern Thailand; excluding Maehongson and Lamphun



Source: The map generated using ArcGIS Desktop 10 (ESRI, 2010)

❖ SAMPLE SIZE DETERMINATION

According to the Department of Livestock Development (DLD, 2004), the expected prevalence of avian influenza H5N1 in Thailand is 25%. With 99% confidence interval and 10% accepted error, the study samples were determined using the program Win Episcope version 2.0 (University of Edinburgh, 2007). The crude sample size determination for all provinces was performed to be 1,000 birds and adjusted to the proportion of poultry produced in each province, excluding Maehongson and Lamphun (Table 4-1). Using the same procedure, the sample size determination at the district level (which was adjusted to the probability proportional to the numbers of each poultry species in a district) was calculated and shown in **Appendix C**.

Table 4-1: The sample size determination of Backyard Chickens in Northern Thailand

Province	Chicken Population	Crude Size	Excluding the 2 provinces	Proportion	Final Size
Chiang Mai	2,587,833	125	2,587,833	2,587,833/10,097,937 = **0.25627**	**256**
Chiang Rai	2,223,321	125	2,223,321	2,223,321/10,097,937 = **0.22018**	**220**
Lampang	1,305,004	125	1,305,004	1,305,004/10,097,937 = **0.12923**	**129**
Lamphun	***780,249***	***125***	***0***	***0***	***0***
Maehongson	***317,003***	***125***	***0***	***0***	***0***
Nan	827,506	125	827,506	827,506/10,097,937 = **0.08195**	**82**
Phayao	1,824,421	125	1,824,421	1,824,421/10,097,937 = **0.18067**	**181**
Phrae	1,329,852	125	1,329,852	1,329,852/10,097,937 = **0.13170**	**132**
Total	**11,195,189**	**1,000**	**10,097,937**	**1.00000**	**1,000**

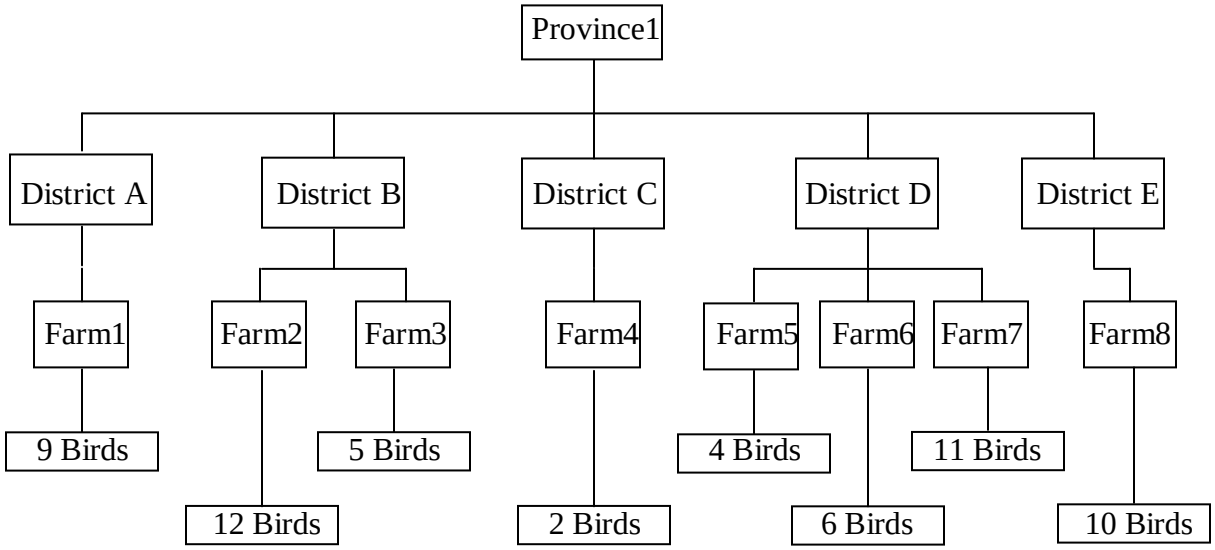
Source: Thailand Department of Livestock Development (DLD, 2008).

SELECTION OF STUDY DISTRICTS; FARMS; AND POULTRY

- **Hierarchy of Thailand's Infrastructure and Multistage Sampling**

According to Thailand's infrastructure administration, the highest authority under the Thai government is the 'Province'. Under the umbrella of each provincial administration, 'District' is at the second highest level. For instance, there are 24 districts administrated under Chiang Mai province. 'Chom Thong' is a district among those 24 and there are 6 'sub-districts' under its district administration. For this reason, the sampling of study population was designed as the Multistage Sampling based on this infrastructure (Figure 4-2).

Figure 4-2: Hierarchy of poultry population and example of multistage sampling



- **Selection of Districts**

There are 87 districts constituted in the 6 provinces of Northern Thailand and every individual district was intentionally selected for this study. The number of study population sampled within each district was proportional to the total number of backyard poultry raised in each district.

At the time of study, numbers of backyard poultry (chickens & ducks) were mostly produced in certain districts of Chiang Mai, Chiang Rai, and Phayao (Figure 4-3). The higher numbers of backyard chickens were consequentially obtained from those districts.

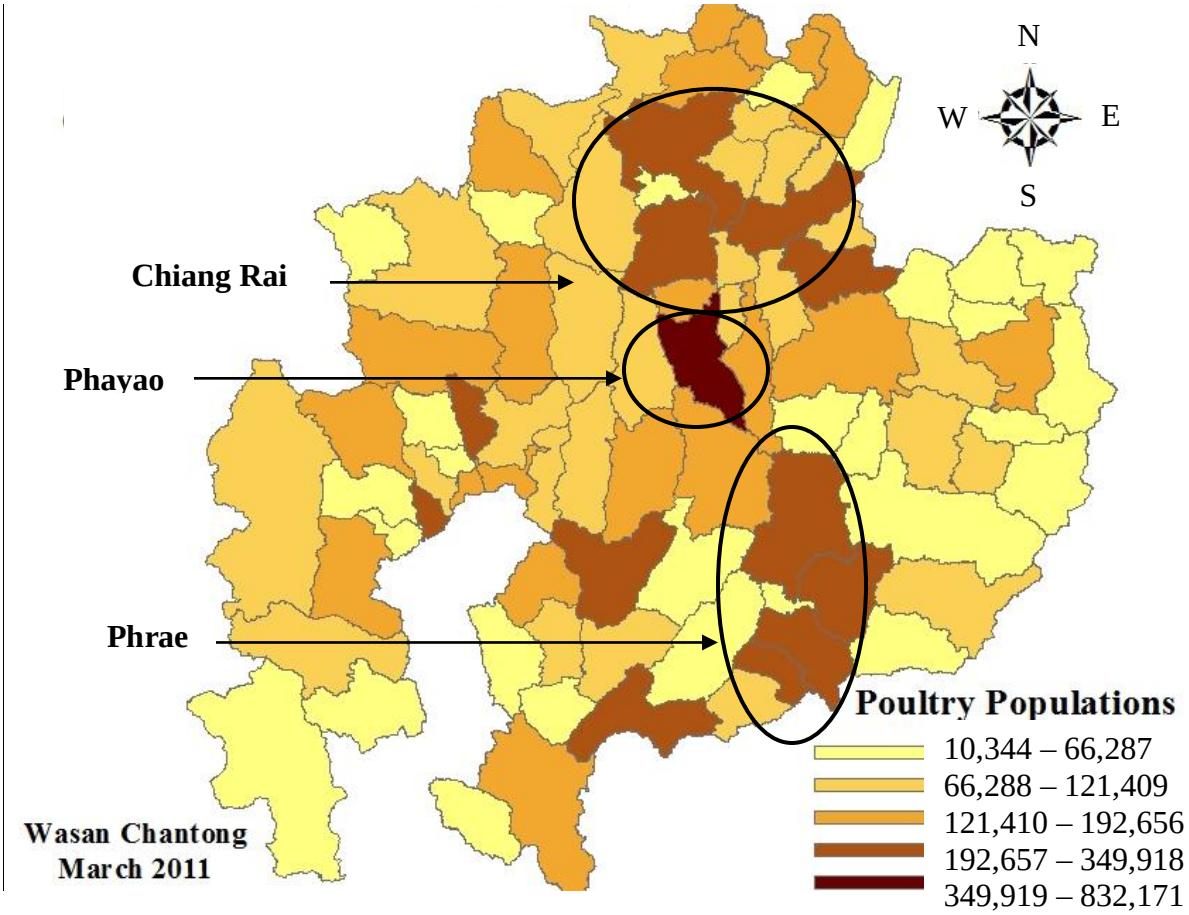
- **Selection of Farms or Households**

For the ease of practical administration, a study farm was any farm randomly selected from the full name list of the sub-districts; 1, 2, or 3 sub-districts were selected for a district.

- **Selection of Poultry**

The backyard chickens with a fixed number (1 - 29 birds) were then randomly selected.

Figure 4-3: Study area in 87 districts and density of backyard poultry population in each one



Source: Thailand Department of Livestock Development (DLD, 2008)

❖ SAMPLE COLLECTION AND QUESTIONNAIRE DESIGN

The collection, handling, storage, and transportation of the poultry specimens were conducted followed the regulations outlined in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: Avian Influenza (OIE, 2005).

➢ Collection of Swab Specimens

A swab specimen was collected from the oropharyngeal area of the backyard chickens, using a wooden cotton swab. The specimen was placed into 3-4 ml of viral transport medium immediately following collection. The transport medium used to maintain the collected specimens is a buffered balanced-salt with antibiotics that composed of (1) Isotonic Phosphate Buffered Saline (PBS), pH 7.0 – 7.4; (2) Penicillin 2,000 units/ml; (3) Streptomycin 2 mg/ml; and (4) Gentamycin 50 μg/ml.

➢ Collection of Serum Samples

Three to four ml of chicken blood was collected from a wing vein of each individual bird into a 5-ml syringe. Following collection, the syringe plunger was pulled all the way out to allow air into the barrel and to maximize the surface area, as this can allow for the most serum to be collected. To separate the serum from the clot, the syringe was allowed to sit at room temperature for a few hours (4 hours if placed at 37^{o}C) and stored at 4^{o}C overnight. After separation, the serum was poured off of the syringe into a clean tube and was centrifuged at a low speed to remove red blood cells before testing.

➢ Transportation of Specimens to the Laboratory

Following collection, the specimens were stored on wet ice in the ice-box (2-4^{o}C), and transported to a laboratory as soon as possible. The collected specimens were held at refrigeration temperature for no longer than 48 hours before testing.

In case the laboratory could not be processed within 48 hours, the specimens were kept frozen at -70^{o}C instead.

- **Questionnaire**

The questionnaire interview was conducted at the time of visit at each farm. The major potential risk factors postulated for the AI virus, or antibody positive in backyard chicken- and open field-reared duck specimens were designed and fit into the questionnaire, which were;

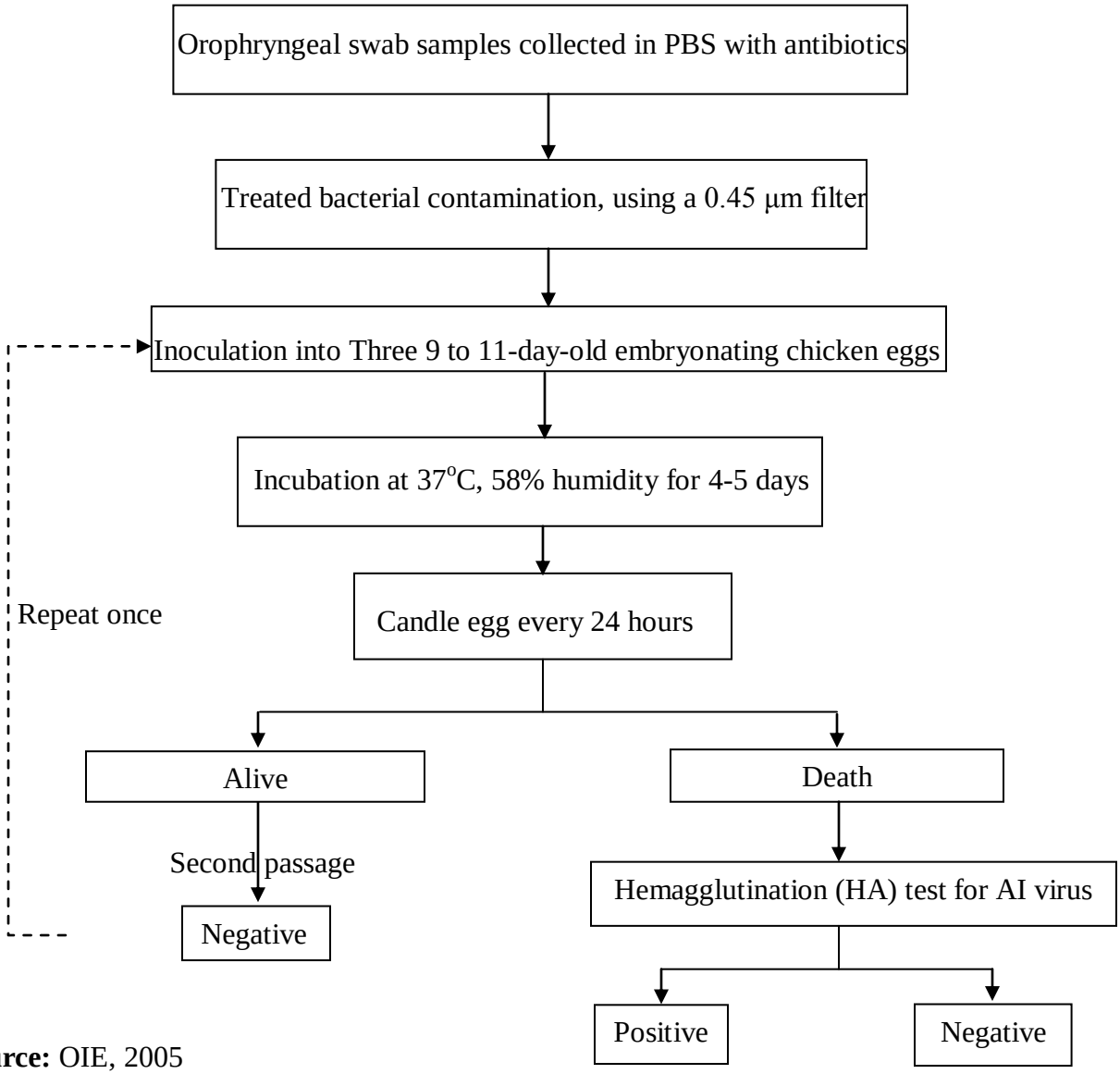
1. Basic information of the owner and farming
2. Numbers of poultry, pigs, and other animals in a farm
3. History of other diseases and vaccination of a flock
4. History of avian influenza outbreaks in the area
5. Prevention and control measures done, in case of avian influenza outbreak
6. Poultry housing and raising system
7. Feed and feeding
8. Types of land use in the area (poultry farms, rice fields, or crop production)
9. Proximity to wetlands (natural reservoirs and rivers)
10. Human activities (poultry movement, fighting-cock arena, and live bird markets)

The questionnaire was designed as a bilingual check list; formatted in both English and Thai. A copy of the questionnaire is shown in **Appendix D.**

LABORATORY PROCEDURES AT CMU (CHIANG MAI, THAILAND)

The primary virus isolation (Figure 4-4) was performed at the Animal Diagnostic Laboratory of the Chiang Mai University (CMU) in Thailand. To confirm the results carried out in Thailand, the serum specimens were packed on the ice packs and shipped to the National Veterinary Services Laboratories (NVSL) in Ames, Iowa. More information concerning the shipment and laboratory processes are illustrated under "**Laboratory Procedures at NVSL**".

Figure 4-4: Flow diagram of laboratory procedures for virus isolation conducted in Thailand



Source: OIE, 2005

1.1 AVIAN INFLUENZA VIRUS ISOLATION

❖ PRINCIPLE

Avian influenza (AI) viruses have been isolated from a wide range of avian species, including poultry, waterfowl, ratites, passerines, and psittacines (Woolcock, 2008). Despite the diverse avian host range, the AI virus can usually be isolated in embryonating chicken eggs when inoculated into the chorioallantoic sac (CAS).

❖ MATERIALS

➢ General Laboratory Equipments

1. Class III biosafety cabinet (for Asian HPAI H5N1)
2. Egg incubator
3. Egg candling light
4. Centrifuge; preferably refrigerated
5. Refrigerator
6. -70^{o}C freezer
7. 1-, 5-, 10-ml pipettes (sterile)
8. Pipette aid or a suction bulb
9. Latex gloves
10. Laboratory coat or gown
11. Biohazard bags
12. Autoclave
13. Egg punch; an 18 gauge needle punched with a rubber stopper suffices

- **Specimens and Equipments for Swab Processing**

    1. Preferred specimens: Oropharyngeal for chickens and cloacal swabs for ducks
    2. Virus Transport medium: a buffered balanced-salt solution with antibiotics (*see* the compositions under; "**Collection of Swab Specimens**")
    3. Reference antigen for positive control:
        - Avian influenza **A/H5N1** virus (A/chicken-2 /NP/Thailand//2004)
    4. Vortex mixer
    5. 15- and 50-ml propylene centrifuge tubes (sterile)
    6. 0.45-μm syringe filters
    7. 3-cc and 12-cc syringes; and 1.5-inches x 20 gauge syringe needles (sterile)

- **Materials for Egg Inoculation**

    1. 9-11-day-old embryonating chicken eggs (obtained from commercial, AI infection & vaccination-free sources)
    2. 70% Ethanol
    3. Egg punch
    4. 1-cc tuberculin syringes (sterile)
    5. 5/8-inches x 25 gauge syringe needles (sterile)
    6. Glue tape or equivalent to seal inoculation hole in eggshell
    7. 3-cc or 5-cc syringe with 18-gauge needle
    8. 0.45-μm syringe filters

❖ METHODS

➢ The Methods for Swab Processing

1. Mix the swabs in up to 5 ml of transport medium by briefly vortexing.
2. Treat the supernatant for bacterial contamination; using 0.45- μm syringe filter.

➢ The Primary Avian Influenza Virus Isolation

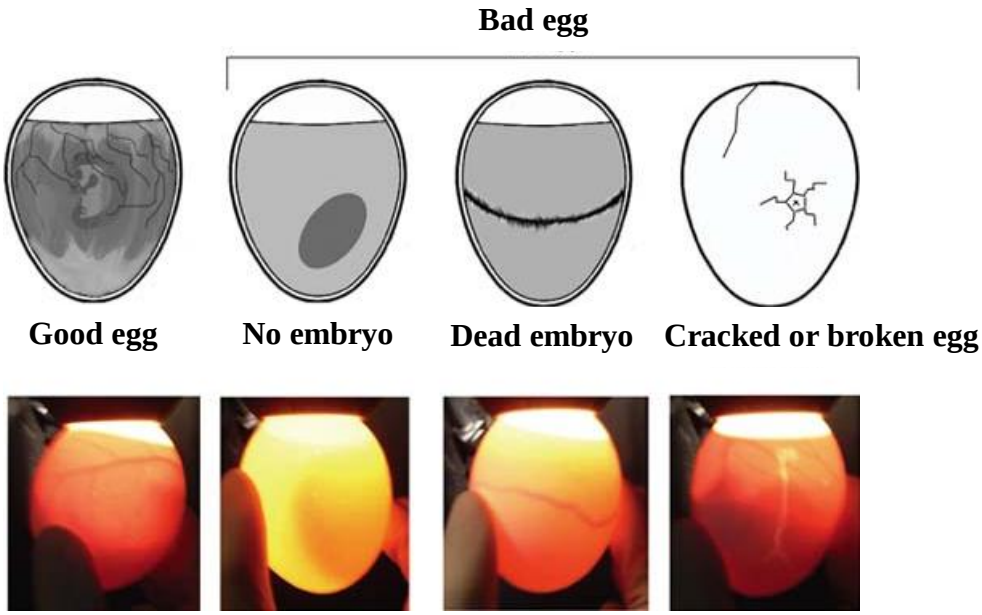
1. Inoculate three 9-11 day-old embyonating chicken eggs per specimen by the chorioallantoic sac (CAS) route with 0.2 ml of inoculum per egg.
2. Mark the eggs with pencil, mist with 70% ethanol, and allow to dry. Carefully make a hole in the eggshell with the egg punch, and inject 200 μl (0.2 ml) of inoculum into the allantoic sac using a 1-cc syringe.
3. Seal eggs with glue tape and incubate at 37^{o}C with 58% relative humidity.
4. The eggs are candled daily for a period of 4 to 5 days (Figure 4-5). Remove eggs with dead embryos within 24 hours, and chill at 4^{o}C. Note that eggs with dead embryos within 24 h post-inoculation (PI), are discarded as nonspecific.
5. After chilling, place the eggs in a class III biosafety hood and mist with 70% ethanol. Remove the portion of the shell above the air cell with sterile scissors and forceps, and harvest the amnioallantoic fluid (AAF) with a syringe and a 20 gauge 1.5-inches needle, harvest as much AAF as possible (Figure 4-6).
6. At 4-5 days' PI, remaining eggs with live embryos are chilled to 4^{o}C for 24 h.
7. AAF from each egg is tested individually for the presence of HA activity.
8. The passage II (P2) was performed before determining that a specimen is negative for the AI virus.

Note: Influenza A/H5N1 (A/chicken-2 /NP/Thailand//2004) was used for the positive control.

Figure 4-5: Eggs being candled to evaluate embryo viability

From left to right – (1) Healthy egg (notice the prominent blood vessels);

(2) No embryo; (3) Dead embryo; and (4) Cracked or Broken shell



Source: Szretter, Balish, & Katz, 2006

Figure 4-6: Example picture of AAF collecting from a 10-day-old embryonating egg

Source: Roth, 2005

1.2 HEMAGGLUTINATION (HA) ASSAY

The hemagglutination (HA) assay was used to screen amnioallantoic fluid (AAF) harvested from embryonating chicken eggs for the presence of hemagglutinating agents (Killian, 2008b). The HA assay is not an identification assay, as other agents (e.g. paramyxoviruses and adenovirus) and certain bacteria also have hemagglutinating properties.

❖ PRINCIPLE:

The hemagglutinin protein on the surface of influenza virus particles is capable of binding to *N*-acetylneuraminic acid-containing proteins on avian and mammalian red blood cells (RBCs). When combined, if the influenza virus is present in a high enough concentration, there is an agglutination reaction and RBCs link together to form a diffuse lattice.

❖ MATERIALS

1. Sterile tube (sufficient size to hold blood and anticoagulant)
2. Anticoagulant: Alsever's solution

   Preparation: Weigh out reagents into a conicol flask: 0.55 g of citric acid, 0.8 g of sodium citrate, 2.05 g of D-glucose, and 0.42 g of sodium chloride. Dissolve in distilled water and make up volume to 100 ml. Dispense into sterile 10–ml bottles, and sterilize by autoclaving at 116^{o}C for 10 minutes. Use slow exhaust. Allow to cool, then tighten the lids and label the bottles. Store at 4^{o}C.
3. U-bottomed microtiter plates
4. Single and multichanel pipettes and pipette tips to deliver 50-µl volumes
5. 0.01 Molar Phosphate Buffered Saline (PBS), pH 7.3 (*see* preparation methods below)
6. Chicken erythrocytes (0.5 – 1.0% RBCs in PBS) (*see* preparation methods below)

- **Preparation of 0.01 Molar Phosphate Buffered Saline (PBS)**

  - Combine the following ingredients:

    - 1 g of sodium chloride
    - 1.33 g of sodium phosphate dibasic, and
    - 0.22 g of sodium phosphate monobasic

  - Dissolve in distilled water, and make volume up to 1 Liter.

  - Mix thoroughly and check pH. The final pH should be 7.2 ± 0.1.

- **Preparation of 1.0% Erythrocyte Suspension**

  - Whole blood taken from specific pathogen-free (SPF) chickens is collected in an equal volume of Alsever's solution for preservation of erythrocytes.

  - Wash the erythrocytes to remove buffy coat and Alsever's solution by adding 20 ml of blood to a 50-ml centrifuge tube, and fill the tube with PBS. Gently invert the tube several times to wash the erythrocytes.

  - Centrifuge at 800Xg for 10 minutes at 4^{o}C. Aspirate the PBS and buffy coat from the tube, and refill the tube with fresh PBS, mix by inversion.

  - Repeat the wash and centrifugation cycle two additional times for a total of three washes.

  - Prepare a 1.0% suspension of erythrocytes.

❖ **METHODS**

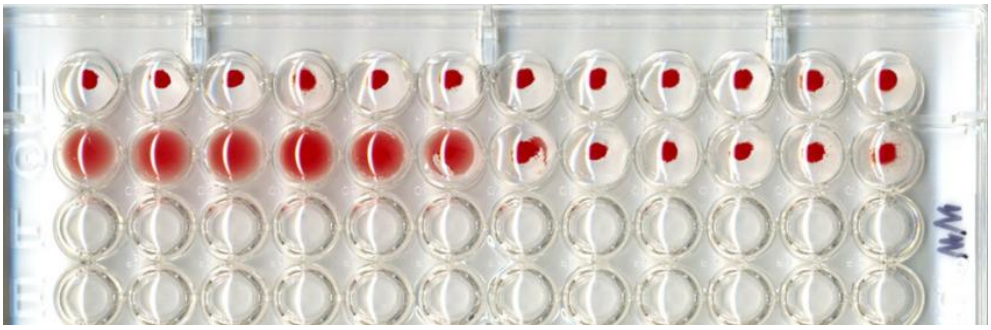
1. Dispense 50 µl of PBS into each well of a plastic U- bottomed microtiter plate.
2. Place 50 µl of virus suspension (i.e. AAF) in the first well that this will result in a 1:2 dilution of test material.
3. Dilute the test material: Mix the contents of the first well by pipetting up and down. Pipette 50 µl from the first well and place into the second well. Continue to make two-fold dilutions of the virus suspension across an entire row. Discard the excess 50 µl after the last row. All wells should have a final volume of 50 µl. The dilutions, from the first well, will be 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, etc.
4. Dispense 50 µl of 1.0% (volume/volume) chicken RBCs to each well.
5. Mix by tapping the plate gently, and then allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, by which time control RBCs should be settled into a distinct button.
6. HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs.
7. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

   For example: For 6 wells of complete HA with an end point dilution of 1: 64 where the initial dilution is 1:2, the number of HAUs/50 µl is 64 (Figure 4-7).

   Note: The HA test was calibrated by using AAF containing avian influenza A/H5N1 virus antigen (A/chicken-2 /NP/Thailand//2004) to be tested for the positive control.

Figure 4-7: Hemagglutination test plate

From left to right: 1) First row – negative
2) Second row – positive with 64 HAUs/50 µl



Source: University of Manibota, 2008

❖ THE QUALITY CONTROL FOR LABORATORY TESTING AT CMU

The sufficient test for the common procedures performed in the laboratory was conducted at least twice a year. The efficiency of technicians and effectiveness of facilities was tested by the standard operating practices (SOP) with the reference results that randomly provided by the national reference laboratory under the Thailand Department of Livestock Development (DLD). The final results were confirmed and approved by the well-trained technicians at the reference laboratory in order for the laboratory workers, equipment, and testing procedures to be standardized, up-to-date, and improved for current laboratory testing.

1.2 AGAR GEL IMMUNODIFFUSION (AGID) TEST

❖ PRINCIPLE

AGID test is performed to visualize the immunoprecipitation reaction of antibody to AI virus in the poultry serum after diffusion in an agar matrix.

❖ MATERIALS

1. Glass flask
2. Vacuum pump
3. Vacuum flask with pipette or 1-cc syringe fitted to end of tubing
4. Humidified chamber
5. Agarose
6. 8% NaCl and 1.0 Molar HCl
7. Glycin/sarcosyl buffer: 1% (weight/volume) sodium lauroyl sarcosinate buffered to pH 9.0 with 0.5 Molar glycin
8. Influenza A antibody in reference serum; Mouse Antibody-Influenza A Monoclonal Antibody (CHEMICON® International, Inc., Billerica, MA)
9. AI viral antigen; avian influenza A/H5N1 virus (A/chicken-2 /NP/Thailand//2004) (Center for Animal Disease Diagnosis, Chiang Mai University, Thailand).
10. AGID template for cutting agarose

➢ Preparation of AGID Agar and Plates

1. Weigh 8.0 g of NaCl and 0.9 g of agarose and add PBS to 100 ml in a glass flask.
2. Mix well to dissolve NaCl.
3. Microwave for 5 minutes at 100% power. Mix solution well while hot.

4. Let solution cool, but not harden, then dispense 17-ml aliquots into tubes with caps. Store in a refrigerator up to several months.
5. Reheat tube at low power in microwave to melt agarose (or place in a boiling water bath) when ready to use.
6. Plate preparation: Pour 17 ml into a 15 x 100-mm Petri dish, place on a level surface, and allow to cool.
7. Immediately before use, prepare wells in the agarose by pressing the pattern template into the hardened agarose. Remove agar plugs with a syringe or Pasteur pipette attached to a vacuum flask.

❖ **METHODS**

1. The AGID plates can be prepared in Petri dishes with 0.9% agarose in 0.01 Molar; pH 7.2 PBS with an additional 8% Sodium Chloride (NaCl).
2. A seven-well template with a center well surrounded by six evenly spaced wells is used. The wells are 2.4 mm apart and 3.5 mm in diameter.
3. Place 50 µL of **reference antigen (Ag)** [avian influenza A/H5N1 virus (A/chicken-2 /NP/Thailand//2004)] to the center well; 50 µL the **test serum (TS)** into well 1, 3, and 5; and 50 µL of **known positive serum (KP)** [Positive Control: Mouse Antibody-Influenza A Monoclonal Antibody] into well 2, 4, and 6.
4. Cover and place the plates in a humidified chamber for 24 hours at room temperature.
5. The plates were examined for the presence of precipitin lines. If negative, incubate for an additional 48 hours.

❖ INTERPRETATION

The lines must be formed between the wells containing reference antigen and antiserum. If the line is present between an unknown serum sample and known antigen (influenza A virus) and is continuous with the adjacent lines, the antibody contained in the serum can be identified as antibody against type A influenza virus. The negative result was identified when there was no precipitin line formed between the test serum and antigen. The weak positive reaction can also be identified by the test as indicated in Figure 4-8 for the well labeled as KP1.

Figure 4-8: Example of an Agar Gel Immunodiffusion (AGID) test plate

Along the Clockwise Direction:

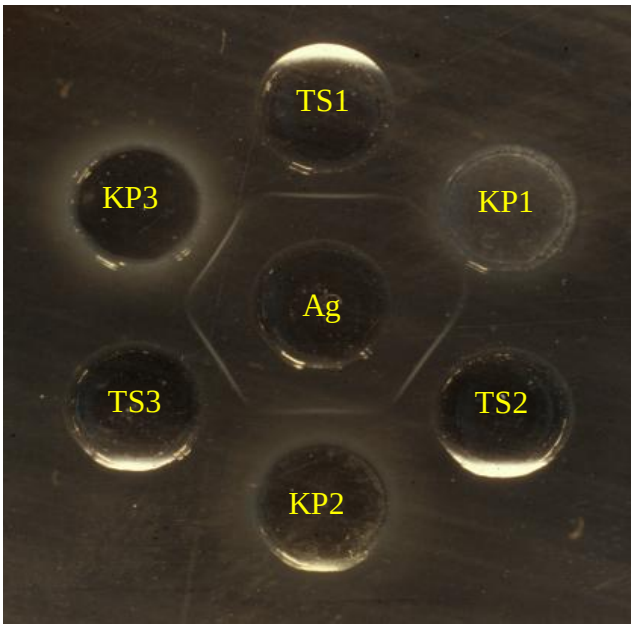
TS1 = Test Sample 1 KP1 = Known Positive Sample 1-weak

TS2 = Test Sample 2 KP2 = Known Positive Sample 2-strong

TS3 = Test Sample 3 KP3 = Known Positive Sample 3-strong

At the Center Point: Ag = Antigen

Avian influenza A/H5N1 virus (A/chicken-2 /NP/Thailand//2004)



Source: Michigan State University Diagnostic Center for Population and Animal Health (DCPAH): The figure retrieved from Professor Dr. Roger K. Maes

LABORATORY PROCEDURES AT USDA NVSL (AMES, IOWA, USA)

❖ SAMPLE PREPARATION FOR SHIPMENT

The individual backyard chicken and open field-reared duck serum was collected separately in an autoclaved microcentrifuge tube. The specimens were labeled in the ordinal number IDs on the top of the hinged lid of the tube. For examples, the sample IDs C1 to C1000 were labled for the serum collected from the first to the thousandth chickens (**C**hicken samples labled as a '**C**'), and the sample ID numbers D1 to D1000 were labeled for the serum collected from the first to the thousandth open field-reared duck ('**D**' as a **D**uck).

The serum samples were packed on the sufficient ice packs and delivered to the USDA NVSL. The transportation of the diagnostic serum specimens was followed the regulations outlined by the US Department of Agriculture (USDA).

❖ THE LABORATORY PROCEDURES

Antibody to influenza A viruses, hemagglutin (HA) subtyping, and AGID test were conducted by the microbiologists at the USDA National Veterinary Services Laboratories (NVSL) in Ames, Iowa in order to confirm the test results carried out in Chiang Mai, Thailand.

The commercially available FlockCheck® MultiS-Screen ELISA (IDEXX Laboratories, Inc., Westbrook, ME, USA) was used to screen for type A influenza antibody in the poultry sera. The H-subtype identification and AGID test were conducted using the samples that were positive to ELISA test and had adequate serum quantity for the tests. AGID test protocol was the same as that has done in Chiang Mai. The NVSL testing protocol for Hemagglutination-inhibition (HI) test for subtype identification of influenza A virus antibody was used. Information regarding IDEXX ELISA and HI tests carried out at the NVSL was elucidated in **Appendix E**.

DATA MANAGEMENT AND STATISTICAL ANALYSES

Laboratory results and data from the questionnaire interviews were manipulated as the computer spreadsheets in Microsoft Office Excel® 2007 (Microsoft Corporation, Redmond, WA, USA). The program Statistical Analysis System (SAS® version 9.2) (SAS Institute Inc., Cary, NC, USA) was used to generate the descriptive statistics and logistic regression models.

❖ DESCRIPTIVE STATISTICS

Avian influenza prevalence was calculated by the number of specimens testing positive for avian influenza (based upon the testing methods) divided by the total number of specimens tested in the respective unit; classified by sample type, tested method, and study province.

❖ LOGISTIC REGRESSION ANALYSES AND MODELING

The logistic regression modeling was performed in order to assess the association between the binary outcome variable and the variety of potential associated risk factors.

- **Outcome Variable** is classified as positive or negative for poultry specimens tested for AI; either by virus isolation and/or serological antibody test.
- **Risk Factor Variables** compose of;
  - Close Proximity to Other Animals (e.g. pig) or Other Poultry
  - History of AI Outbreaks in the Study Area or in the Neighboring Areas
  - Flock Size (small, large) and Farming Types (semi-confined or fully open)
  - Farm Close to Other Farms; Rice paddy field; Crop and Grain Production)
  - Farm Close to Wetland (reservoir; stream or river)
  - Farm Close to Fighting Cock Arenas; Live Poultry Market; and Poultry Movement and Transportation

GIS MAPPING AND MANTEL-HAENSZEL CHI-SQUARE TEST FOR GIS DATA

❖ PRINCIPLE

Geographic Information System (GIS) relies on the computer-based technology to produce, organize and analyze spatial information in the form of maps (Environmental Systems Research Institute [ESRI], 2010). GIS encompasses database management, mapping, image processing, and statistical analysis tools. GIS maps were generated using a commercial computer software program ArcGIS® Desktop 10 (ESRI, Inc., Redlands, CA, USA). Mantel-Haenszel Chi-square test was performed in order to evaluate the association between the areas with influenza A antibody positivity and areas with other predictive variables obtained from the questionnaire.

❖ PURPOSES OF GIS MAPPING

GIS mapping was used to determine the distribution of laboratory-confirmed avian influenza in relation to certain associated risk factors. That is, to create the point features of district where avian influenza positive samples were collected from in 2009. The spatial dependence between the AI positive areas and the varying relationship across space were then assessed as the maps associated to certain pre-exposing risk factors, i.e., the poultry population and density, the areas with the history of AI outbreaks in 2004-2006, the wetland areas, and the main roads (assumed to be close proximity to the poultry movement).

Once the areas that had been infected with avian influenza and the associated risk factors were identified, the distribution maps could be used to communicate the findings to the policy makers or communicate the associated risk factors to people who reside in those risk areas. So that the preventive or control measures can be implemented in the infected zones to prevent the flocks or even to prevent people themselves from risk of AI exposure, if any administration is available or if it is possible.

❖ GIS MAPPING METHODS

The summary procedures for GIS mapping are as the followings.

1. The AI positive file was first created in SAS® version 9.2, where the laboratory tested results and covariates were calculated for each district area of all study provinces.
2. The data were then exported from the SAS® Program and saved as a dBase file (.dbf).
3. Using the ArcMap® Application program contained in the ArcGIS® software, the dBase file can be joined to the existing attribute table of the map layers or the shapefiles (.shp) on map of Thailand's administration at province and district levels, which already saved as the ESRI® Map File (.mxd).
4. The ArcGIS® program was finally used to create the **new maps** from the map layers related to the avian influenza positive areas.
5. The maps were finally exported from the ArcGIS® Program and saved as the picture files (.jpg) in order to be reported in the Results Section; accompanied with the other findings for this dissertation.

❖ MANTEL-HAENSZEL CHI-SQUARE TEST FOR GIS DATA

Mantel-Haenszel Chi-square (MH χ^2) is a statistical analysis that is used to determine how likely it was that AI antibody positivity (an outcome) was simply due to a predictive risk factor, i.e. the areas with history of H5N1 outbreaks, the backyard poultry population in the district, wetlands, and main roads in the study area. The measurement of probability for a variable to affect the outcome expresses as a p-value. The p-value < 0.05 represents the significant association and $p \geq 0.05$ means no effect of the test variable on the outcome.

RESULTS

❖ AI VIRUS ISOLATION AND SEROLOGICAL TESTING

Of 1,000 oropharyngeal swab samples collected from the backyard chickens, no sample (0) was positive to AI virus isolation by egg inoculation. This could be confirmed with the negative test results obtained from the hemagglutination (HA) assay; following the collection of amnioallantoic fluid (AAF) from the death embryonating eggs inoculated with the test sample.

Likewise, among 1,000 serum samples collected from the same poultry, zero (0) was positive when tested by Agar Gel Immunodiffusion (AGID) Test. The results of the tests conducted at Chiang Mai University in Thailand are presented in the Table 4-2.

However, when the same serum samples were tested by the commercial AI Virus Antibody Test Kit ELISA (IDEXX MultiS-Screen) at the NVSL in Iowa; 15 out of 1,000 sera (1.5%) were positive to avian influenza (AI) antibodies (Table 4-3). Out of 3 serum samples that were tested positive to ELISA and had adequate quantity to be tested by AGID test, only 1 serum was tested positive to influenza A antibody (Table 4-4). Classified by province, the AI antibody positivity is more likely to be found in Nan and Phayao (Table 4-5 and Figure 4-9).

Table 4-2: Results of AI virus isolation and AGID test conducted in Chiang Mai, Thailand

Sample Type / Test	Number of Samples	Number of AI Positive	% Positive	95% CI
Oropharyngeal Swab / Virus Isolation	1,000	0	0	0, 0
Amnioallantoic Fluid / Hemagglutination	2,000 (1 sample x 2 passages)	0	0	0, 0
Chicken Serum / AGID Test	1,000	0	0	0, 0

Table 4-3: Results of serological testing for AI conducted at the National Veterinary Services Laboratories in Ames, Iowa

Sample Type / Test	**Frequency**	**%**
Chicken Serum /		
IDEXX ELISA (+)	15	1.5
IDEXX ELISA (-)	985	98.5
Total	**1000**	**100.00**

Table 4-4: Results of AGID test for the ELISA test positive sera; conducted at NVSL

Sample Type / Test	**Frequency**	**%**
Chicken Serum /		
AGID Test (+)	1	33.33
AGID Test (-)	2	66.67
Total	**3**	**100.00**

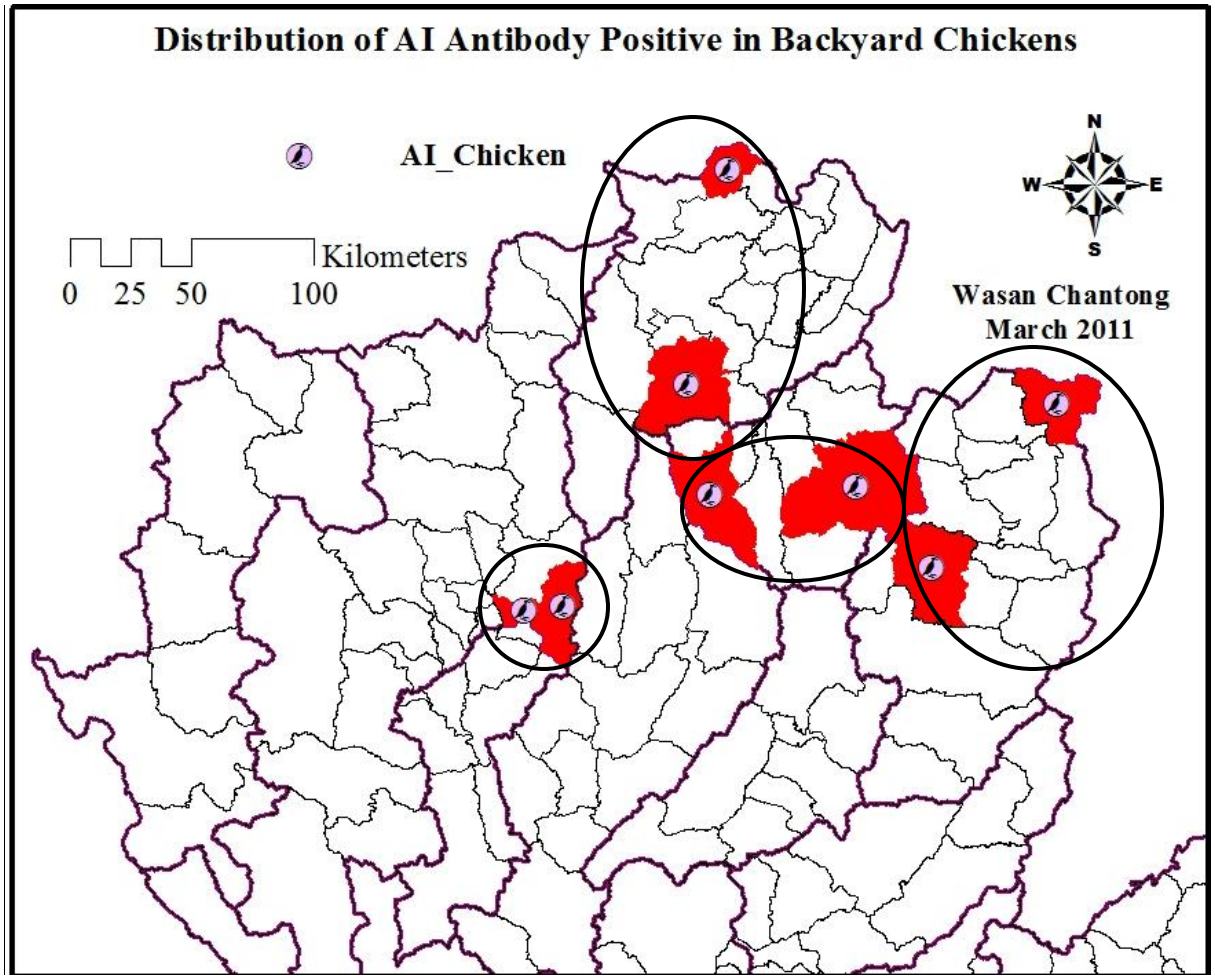
Table 4-5: Results of AI antibody testing positive in Backyard Chickens; classified by **province**

Province	**Number of Samples**	**AI Antibody Positive**	**% Positive**	**95% CI**
Chiang Mai	256	2	0.78	0, 1.87
Chiang Rai	220	2	0.91	0, 2.17
Lampang	129	0	0	0
Nan	**82**	**4**	**4.88**	**0.12, 9.64**
Phayao	**181**	**7**	**3.87**	**1.03, 6.70**
Phrae	132	0	0	0
Total	**1000**	**15**	**1.5**	**0.75, 2.25**

❖ DISTRIBUTION OF AI ANTIBOY POSITIVITY IN BACKYARD CHICKENS

The backyard chickens with AI antibody positivity are distributed in 8 districts among all 87 districts within the 6 provinces. The AI antibody positive birds appeared to be highly observed in the far East and Middle areas of Thailand's Northern region, i.e., certain districts in Nan, Phayao, Chiang Rai and Chiang Mai (from right to left; Figure 4-9).

Figure 4-9: Distribution of AI antibody positivity in Backyard Chickens



Source: Map generated using ArcGIS Desktop 10 (ESRI, 2010)

❖ AVIAN INFLUENZA VIRUS SUB-TYPING

Of 7 chicken serum samples that were positive to ELISA and had sufficient quantity to be tested for the H1-H16 virus subtypes by Hemagglutination-inhibition (HI) test, 3 serum samples were tested positive for H5 avian influenza antibody (Sample ID C133, C134, and C135). When those 7 samples were tested individually with 23 HI reference antigens (Tables 4-6 to 4-9); the same positive samples were positive to H5N1 and H5N9 (≥1:32 dilution of antibodies).

Table 4-6: Results of AI virus antibody sub-typing in Backyard Chickens

ID	Flock #	NJ/8/76 - EQUINE-1 H1N7	A/Mall/ OH/351-6/08 H1N3	A/PINTAIL/ALB/293/77 H2N9	A/DK/UKR/1/63 H3N8	A/DK/CZECH/56 H4N6	A/TY/WI/68 H5N9	Mute Swan/MI/451072-2/06 H5N1
C133	18	-	-	-	-	-	-	**≥1:32**
C134	18	<1:8	<1:8	<1:8	<1:8	<1:8	**≥1:32**	**≥1:32**
C135	18	-	-	-	-	-	-	**≥1:32**

Table 4-7: Results of AI virus antibody sub-typing in Backyard Chickens (continued 1)

ID	Flock #	A/TY/ONT/63 H6N5	Mallard /OH/464497/06 H6N1	A/TY/ORE/71 H7N3	TY/NE/505577/07 H7N9	A/TY/VA/1/02 H7N2	A/TY/ONTARIO/6118/67 H8N4	A/TY/WISC/66 H9N2
C133	18	<1:8	-	-	<1:8	-	-	-
C134	18	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
C135	18	<1:8	-	-	<1:8	-	-	-

Table 4-8: Results of AI virus antibody sub-typing in Backyard Chickens (continued 2)

ID	Flock #	A/TY/CA/6889/80 H9N2	A/GULL/MD/4435/80 H9N5	A/CK/GERM/49 H10N7	A/DK/ENG/56 H11N6	A/DK/ALB/60/76 H12N5	A/GULL/MD/704/77 H13N6	A/MAL/ GURJEV 263/82 H14N5
C133	18	-	-	-	-	-	-	-
C134	18	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
C135	18	-	-	-	-	-	-	-

Table 4-9: Results of AI virus antibody sub-typing in Backyard Chickens (continued 3)

ID	Flock #	A/DK/AUST/341/83 H15N8	A/Shorebird/DE/172/06 3/10/10 H16N3
C133	18	-	-
C134	18	<1:8	<1:8
C135	18	-	-

❖ DESCRIPTIVE STATISTICS

➢ Prevalence of Avian Influenza Infection

When the poultry sera were tested and positive to antibodies against influenza A viruses, it can be assumed that the poultry had been infected at least once by avian influenza virus, and so is summarized for the 1.5% of the backyard chickens tested positive to IDEXX® Multi-S Screen ELISA test kit.

➢ Continuous Predictive Variables for AI Infection in Backyard Chickens

At the time of study in 2009-2010, there were 135,326 backyard chickens on average raised by 4,952 households per a study district. The density of backyard chickens per household estimated at the district level was approximately 29 chickens per household (Table 4-10).

Table 4-10: Descriptive statistics of CONTINUOUS variables for Backyard Chickens

Variable	Mean	Std Dev	Median	Minimum	Maximum
Chicken Population 2009 Classified by District	135,326.4	79,659	109,336	18,365	345,147
Numbers of Households	4,951.68	2,817	4,351	580	11,728
Density per Household	28.71	10.01	25.57	8.88	70.10

Note: Std Dev = Standard Deviation

- **Binary Predictive Variables for AI Infection in Backyard Chickens**

There was approximately two-third (66.8%) of the chicken farms (or households in this study) that had also raised other kinds of poultry, e.g., ducks, geese, or turkeys. Almost a half of these households had operated a small pig farm on their property; counting for < 10 pigs/farm. Most of the poultry raised by these households had no vaccination. Information for other variables is illustrated in Table 4-11.

Table 4-11: Descriptive statistics of BINARY variables for Backyard Chickens

Variable	Frequency	Proportion
Chicken Density per Household	High (>25) = 512 Low (≤25) = 488	High Density = 51.2% Low Density = 48.8%
History of AI Outbreak in the District (2004-2008)	Yes = 455 No = 545	History of Outbreak = 45.5% No History = 54.5%
Existence of Other Poultry in Farm	Yes = 668 No = 332	Close to Other Poultry = 66.8% Not Close to Others = 33.2%
Existence of Pigs in Farm	Yes = 424 No = 576	Close to Pig = 42.4% Not Close to Pigs = 57.6%
History of Vaccination of a Flock	Yes = 62 No = 938	No Vaccination = 93.8% Vaccination = 6.2%
History of Other Diseases in Farm	Yes = 29 No = 971	Other Diseases = 2.90% No Other Diseases = 97.1%
Existence of Fighting Cock Arena in the Study Area	Yes = 113 No = 887	Close to Fighting Cock = 11.3% Not Close = 88.7%
Existence of Live Bird Market in the Study Area	Yes = 176 No = 824	Close to Bird Market = 17.6% Not Close to Market = 82.4%
Movement of Poultry in the Area	Yes = 96 No = 904	Poultry Movement = 9.6% No Poultry Movement = 90.4%

- **Multiple-categorical Predictive Variables for AI Infection in Backyard Chickens**

Table 4-12: Descriptive statistics of MULTIPLE categorical variables for Backyard Chickens

Variable	Frequency	Proportion
Number of Poultry in the Household	< 10 = 3 10 – 50 = 491 51 – 100 = 434 > 100 = 72	Low Number = 0.3% Low to Moderate = 49.1% Moderate to High = 43.4% High Number = 7.2%
Type of Backyard Chickens	Natives = 948 Broilers = 15 Layer Hens = 2 Fighting Cocks = 35	Native Chickens = 94.8% Broilers = 1.5% Layer Hens = 0.2% Fighting Cocks = 3.5%
Farm Close to previous AI Outbreak Area	Within 1 km radius = 26 Within 10 km radius = 8 Within 20 km radius = 75 No Outbreak = 891	Very Close = 2.6% Close to Outbreaks = 0.8% Far from Outbreaks = 7.5% No Outbreaks Reported 89.1%
Housing of Poultry	No = 25 Under Human's House = 136 Outside Household = 839	No Poultry House = 2.5% Under Human's = 13.6% Outside = 83.9%
Raising System	Free Roaming = 1000 Confinement = 0	Free Roaming Poultry = 100 %
Feed and Feeding	By Owner Only = 180 By Owner & Nature = 820 By Nature Only = 0	Fed by Owner Only = 18.0% Owner & Natural = 18.0%
Land Use in the Area	Other Farms = 44 Rice Field = 702 Seed /Grain Production = 254	Close to Other Farms = 4.4% Close to Rice Fields = 70.2% Close to Crop Fields = 25.4%
Existence of Wetlands in the Area	No = 0 Reservoir = 212 River/Stream = 788	Close to the Wetlands = 100 %

During the outbreaks of HPAI between 2004 and 2006, almost one-half (45.5%) of the study areas were affected by the H5N1 subtype viruses (DLD, 2007), and approximately 3% of the farms included in this study were located close to the areas with history of H5N1 outbreaks.

The information obtained from the questionnaires revealed that most of the poultry farms were small-scale farming (< 100 chickens/farm). About 1 out of 10 households operated the farm at the large scale, in which more than 100 chickens were produced. However, all birds (100%) were raised freely in the household area (garden or property) with some having bird houses (either under- or outside human's house) and a few of them (2.5%) had not.

The native breed chickens were preferably raised in the study area (> 90%). The birds were fed regularly by the owner(s), but mostly relied on the natural feeds, such as, grains, rice, and human leftover. Over 90% of all farms were located close to the rice paddy fields and land use for commercial agricultural production (e.g., corn or pea nut production). Of all tested chickens, 4.4% were raised in close proximity to the other farms. No farm was located far away from the wetlands; at least one of them was located close to the reservoir, river, or stream.

❖ UNIVARIABLE ANALYSES-RISK FACTORS

➢ Univariable Analyses-Logistic Regression

The results obtained from univariable Logistic Regression Analyses revealed that at least 5 predicted variables appeared to be significantly associated with the AI antibody positive in backyard chickens ($p < 0.10$). Those variables are: (1) the farm located in the district with the history of H5N1 infection reported by DLD; (2) the flock had no vaccination of any kind; (3) the large-size farm (> 100 birds/flock); (4) the farm located close to the other farms; and (5) the farm located close to the rice field. The other risk factors related to, but in less significant association with AI antibody positivity in backyard chickens are presented in Table 4-13.

Table 4-13: Results of UNIVARIABLE analyses-risk factors associated with Avian Influenza antibody positivity in Backyard Chickens

Risk Factors	**OR**	**95% CI**	**Wald χ^2 P-value**
High Density/ Household	0.83	0.29, 2.31	0.7238
History of AI outbreaks in the District	**0.29**	**0.08, 1.05**	**0.0597***
Existence of Other Birds in Farm	2.01	0.56, 7.16	0.2834
Existence of Pigs in Farm	2.06	0.73, 5.84	0.1734
No Vaccination of the Flock	**3.29**	**1.08, 14.29**	**0.0381****
Other Diseases in Farm	<.001	<0.01, >999.99	0.3456
Farm Close to Fighting Cock Arena	1.99	0.55, 7.16	0.2926
Farm Close to Live Bird Market	1.72	0.54, 5.46	0.3585
Movement of Poultry in the Area	0.67	0.08, 5.15	0.6995
Flock Size (>100 chickens/flock)	**6.85**	**2.27, 20.62**	**0.0006****
Native-Breed Chickens	>999.99	<0.01, >999.99	0.9790
Farm Close to Previous Outbreak Area	7.16	0.72, 71.55	0.0937*
No Poultry House	<.001	<0.01, >999.99	0.9855
Farm Close to Other Farms	**5.76**	**1.56, 21.19**	**0.0085****
Farm Close to Rice Field	**0.37**	**0.13, 1.01**	**0.0536***
Farm Close to Crop Field	1.48	0.50, 4.36	0.4796
Farm Close to Reservoir	1.36	0.43, 4.31	0.6026
Farm Close to River	0.74	0.23, 2.33	0.6026

**Note: ** ** Highly Significant at $p < 0.05$; * Significant at $0.05 \leq p < 0.10$

- **Univariable Analyses-Mantel-Haenszel Chi-square**
  - **Mantel-Haenszel Chi-square test for the association between ELISA test positive Backyard Chickens and history of AI outbreaks in the district**

From the Mantel-Haenszel Chi-square test, the history of avian influenza (AI) outbreaks in the areas is significantly associated with AI antibody positivity in backyard chickens [MH χ^2 (degree of freedom = 1)] is 3.99 and p-value = 0.0458) (Table 4-14).

Table 4-14: The results for Mantel-Haenszel Chi-square test of AI antibody positivity in Backyard Chickens in association with the history of AI outbreaks in the district

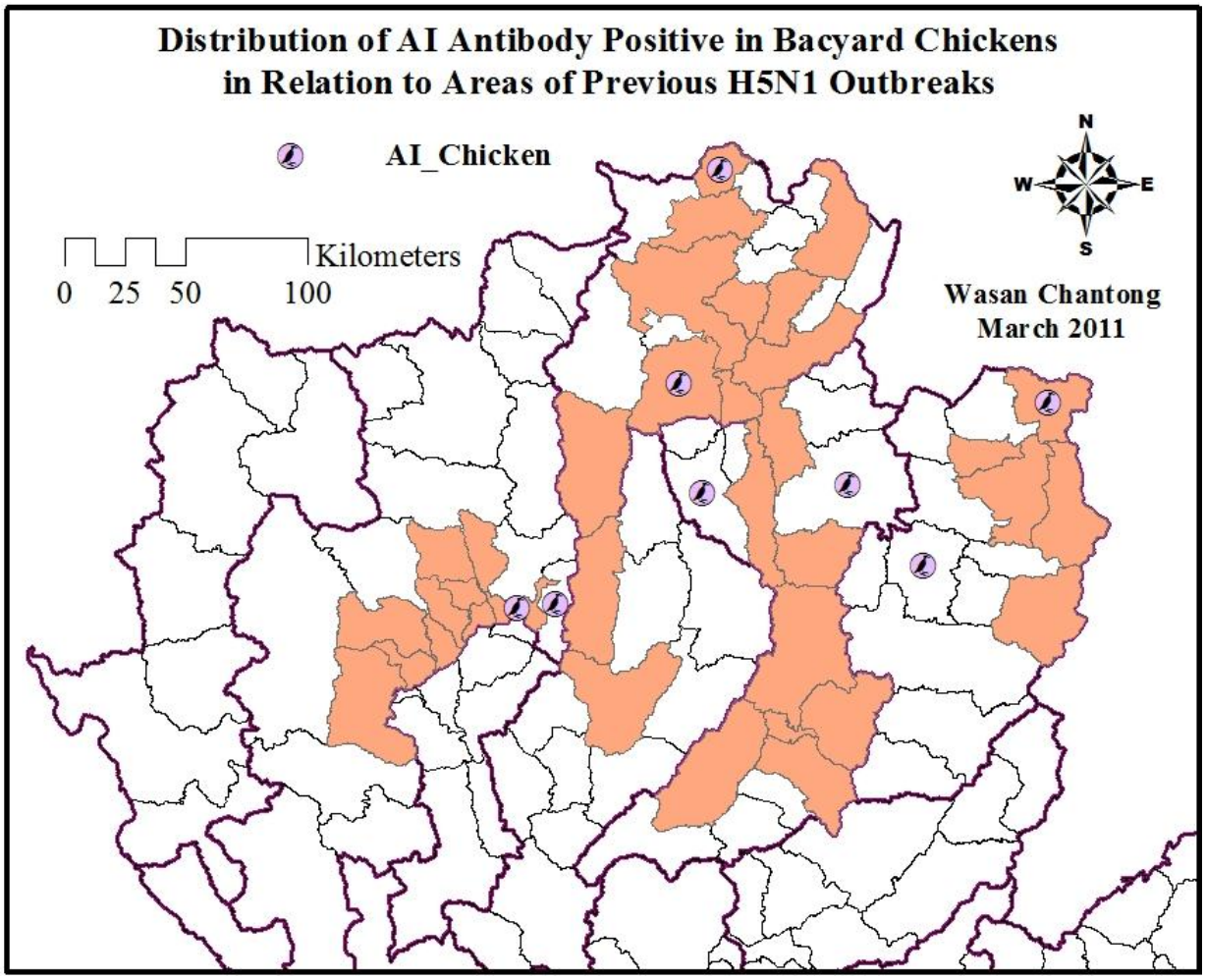
History of AI	**ELISA**		**# Total (%)**
	# Positive (+) (Row %)	**# Negative (-)** (Row %)	
AI+	**3** (0.66%)	**452** (99.34%)	**455 (45.5%)**
AI-	**12** (2.20%)	**533** (97.80%)	**545 (54.5%)**
Total (%)	**15 (1.5%)**	**985 (98.5%)**	**1000 (100%)**

Note: The percentage of AI antibody positivity tested by ELISA in backyard chickens is lower (0.66%) in the areas with previous AI outbreaks (AI+) than the positive percentage (2.20%) in the areas without previous AI outbreaks (AI-).

- **Map of AI Birds and History of AI**

From the GIS map (Figure 4-10), the occurrence of AI antibody positivity in backyard chickens is near or located in the areas with AI outbreaks in 2004-2006 reported by the Thailand Department of Livestock Development (DLD, 2007). This finding is compatible with the significant association obtained from Mantel-Haenszel Chi-square test and Logistic Regression.

Figure 4-10: Distribution of AI antibody positivity in Backyard Chickens in relation to Previous H5N1 Outbreak Areas



Source: Map generated using ArcGIS Desktop 10 (ESRI, 2010)

- **Mantel-Haenszel Chi-square test for trend for the ordinal levels of Backyard Chicken population in the district and AI antibody positivity**

The occurrence of AI antibody positivity in backyard chickens is significantly associated with the population of backyard chickens in the district reported by the Thailand Department of Livestock Development (DLD, 2010).

The Cochran-Armitage Trend Test Statistic was 1.98 and the two-sided p-value = 0.0475. The results for MH χ^2 test for trend are presented in Table 4-15.

Table 4-15: The results for Mantel-Haenszel Chi-square test for trend of AI antibody positivity in association with Backyard Chicken population in 2009

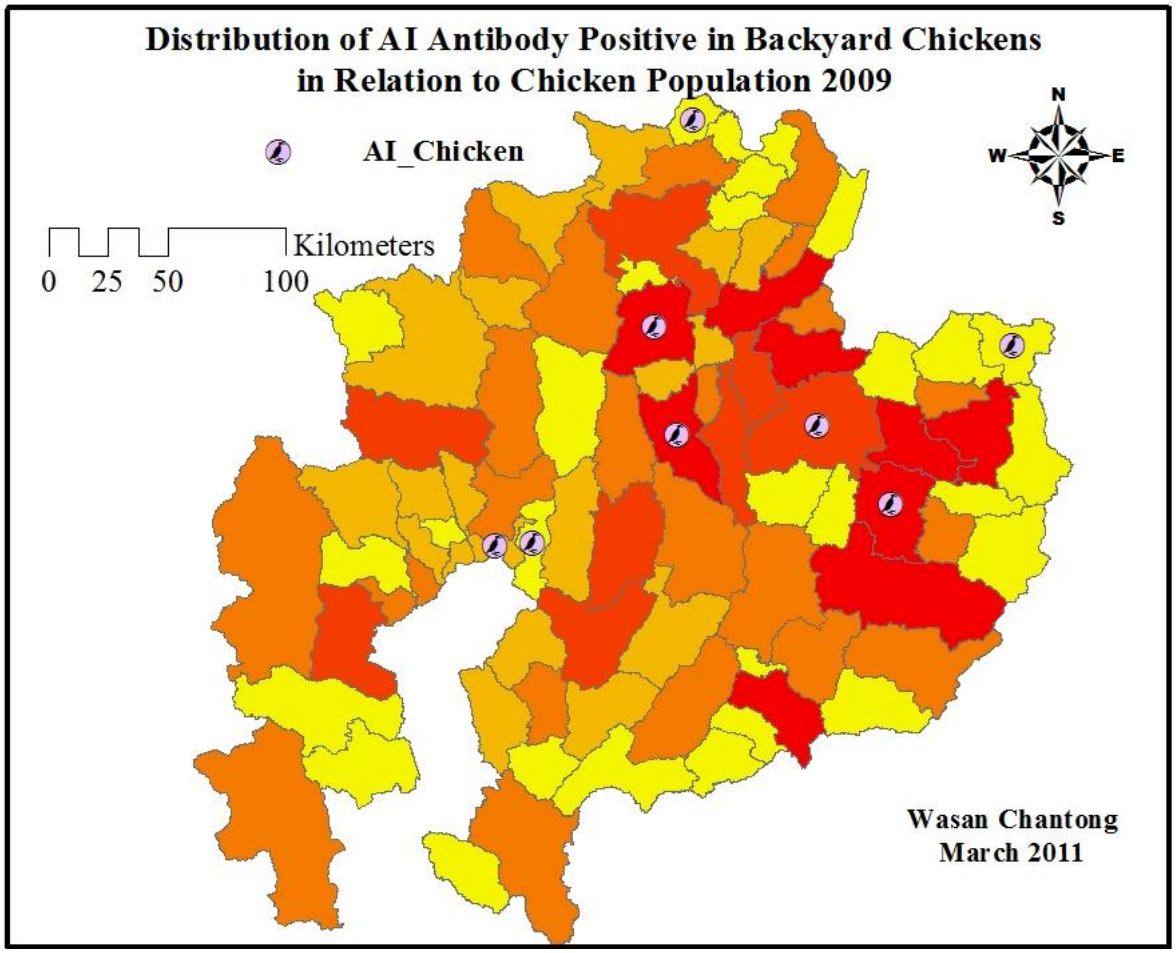
Backyard Chicken Population in 2009	**ELISA**		**Total (%)**
	# Positive (+) (Row %)	**# Negative (-)**	
18,365 – 58,813	**3** (1.71%)	**172** (98.29%)	**175 (17.5%)**
58,854 – 94,325	**1** (0.52%)	**190** (99.48%)	**191 (19.1%)**
94,326 – 136,722	**0** (0.00%)	**283** (100.00%)	**283 (28.3%)**
136,723 – 186,317	**4** (3.10%)	**125** (96.90%)	**129 (12.9%)**
186,318 – 345,147	**7** (3.15%)	**215** (96.85%)	**222 (22.2%)**
Total (%)	**15 (1.50%)**	**985 (98.50%)**	**1000 (100.0%)**

Source: Thailand Department of Livestock Development (DLD, 2010)

- **Map of AI Birds and District Population of Backyard Chickens**

Considering the relationship in terms of place and time, the AI antibody positivity in backyard chickens were relatively close to, or located in, the areas with high backyard chicken population in 2009 (Figure 4-11).

Figure 4-11: Distribution of AI antibody positivity in Backyard Chickens in relation to Total Backyard Chicken Population in 2009



Source: Choropleth map generated in ArcGIS Desktop 10 (ESRI, 2010)

❖ MULTIVARIABLE ANALYSES-RISK FACTORS

➢ Random Effect of the Flock on the Multiple Logistic Regression Model

The Random Effect of Flock was added into the Multiple Logistic Regression model in order to test for the clustering. The model after adjusted for flock is provided as the following.

Logit $\pi(X_i) = \ln[\pi(X_i) / \{1 - \pi(X_i)\}]$

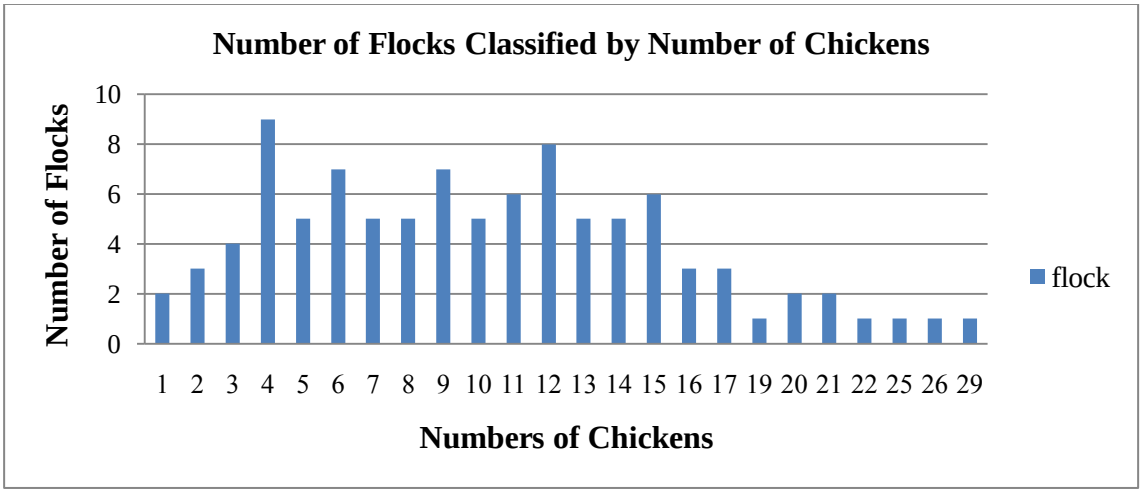
$= \alpha + \beta_1 (\text{History of Outbreaks in District})_{1i} + \beta_2 (\text{No vaccination})_{2i} + \beta_3 (\text{Flock Size})_{3i}$

$+ \beta_4 (\text{Farm Close to Other Farms})_{4i} + \beta_5 (\text{Close to the Rice Fields})_{5i} + \mathbf{u}_{\text{flock (i)}}$;

where $\mathbf{u}_{\text{flock (i)}} \sim N(0, \delta^2)$, the X_iS are the predictor values for the i^{th} bird, and the relationship between the probability p_i and the binary outcome Y_i is unchanged : $p(Y_i=1) = p_i$.

SAS PROC GLIMMIX was used to determine the random effect of the flock on the model. Unfortunately, the SAS program could not calculate the output since the data at the flock level are relatively sparse. Notice from the Figure 4-12 that almost 20 flocks contained only 1, 2, 3, or 4 birds per flock.

Figure 4-12: Distributions in numbers of flock when classified by the number of birds per flock



Source: Data obtained from the questionnaire conducted by Wasan Chantong, 2009-2010

- **Multivariable Analyses-Logistic Regression**

Taking into account the variables that were significant with univariable analyses, the predictive model for risk factors associated with AI antibody positivity in backyard chickens was generated and presented in Table 4-16.

Table 4-16: Final MULTIVARIABLE logistic regression model for risk factors associated with Avian Influenza antibody positivity in Backyard Chickens

Risk Factors	**OR**	**95% CI**	**Wald χ^2 P-value**
Flock Size (> 100 birds/flock)	6.10	1.69, 22.04	0.0058
Farm Close to Other Farms	5.22	0.99, 27.61	0.0517

- **Conclusion of the Final Model**

The final model for the predictive risk factors associated with avian influenza antibody positive in backyard chickens is concluded as the following.

π (X) = Probability (AI infection in Backyard Chickens | Flock Size; Farm Close to Other Farms)

Logit π (X) = ln [π (X) / {1 - π (X)}]

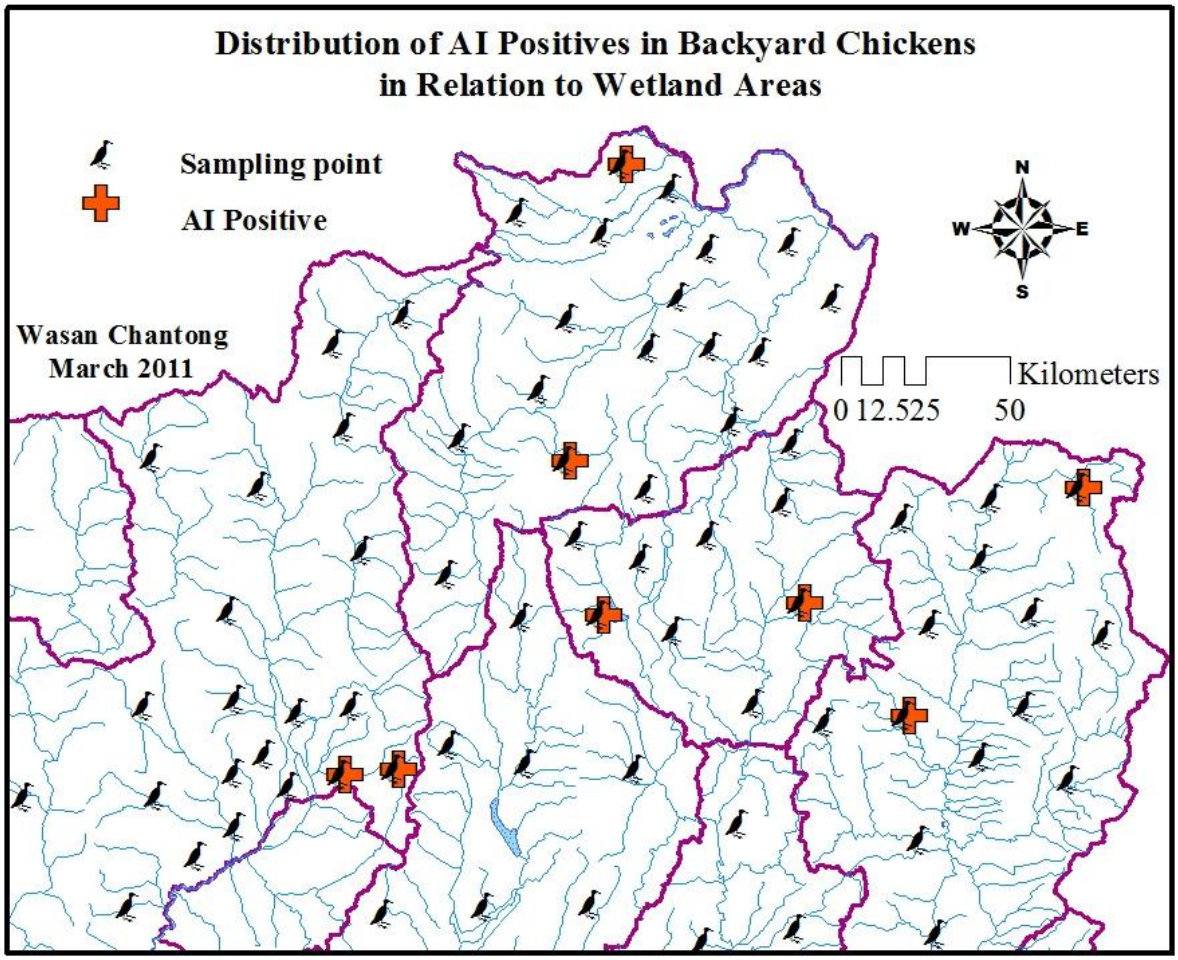
= $\alpha + \beta_1$ (Flock Size) + β_2 (Farm Close to Other Farms)

OTHER MAPS

❖ AI ANTIBODY POSITIVITY AND WETLAND AREAS

Avian influenza antibody positive in backyard chickens were mapped in association with the wetland areas existed in the study provinces (Figure 4-13). The map, however, cannot differentiate the areas with AI antibody positivity and other areas without AI since the wetlands, especially the streams and rivers, are widely distributed in all areas where the samples were collected from.

Figure 4-13: Distribution of AI antibody positivity in Backyard Chickens in relation to the Wetlands

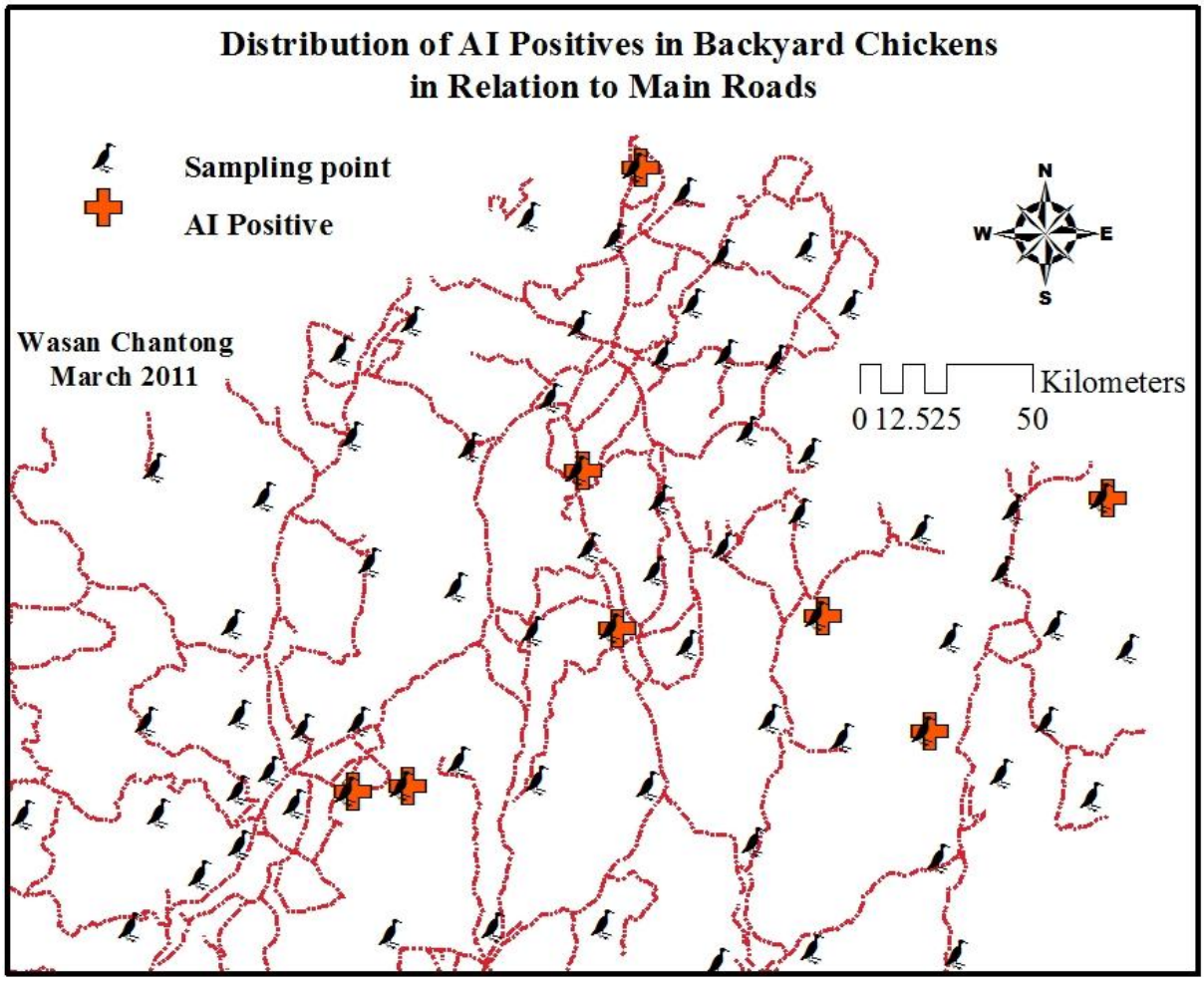


Source: Map generated using ArcGIS Desktop 10 (ESRI, 2010)

❖ AI ANTIBODY POSITIVITY AND MAIN ROADS IN THE STUDY AREA

The same explanations can be stated as well for the areas with AI antibody positivity and the association with the main roads, by which the poultry movement and transportation were assumed (Figure 4-14). The association however cannot be differentiated since the sampling areas and the main roads appeared to be existed in all areas. Therefore, the main roads (poultry movement in the area) are not obviously associated with the avian influenza antibody positivity.

Figure 4-14: Distribution of AI antibody positivity in Backyard Chickens in relation to the Main Roads



Source: Map generated using ArcGIS Desktop 10 (ESRI, 2010)

DISCUSSION

❖ PREVALENCE OF AVIAN INFLUENZA IN BACKYARD CHICKENS

Influenza A antibody positivity found in this study was assumed to be the evidence of avian influenza virus infection in the flocks of backyard chickens obtained from 8 out of 87 study districts. Although the prevalence of the disease is relatively low, less than 2 out of 100 birds (1.5%), the results were considered significant since the influenza A/H5 subtype was definitely identified (by HI test) in 3 of 7 ELISA tested positive and were sufficient for 16 H-sub-typing. The other four sera those were positive by ELISA, but provided the negative results for H-subtype and were considered as the low antibody concentration contained in the serum samples (<1:8 dilution). From the findings, we are able to conclude that the influenza A/H5 subtype remains circulating in the backyard chickens in Northern region of Thailand.

❖ ASSOCIATED RISK FACTORS

The predictive risk factors associated with avian influenza in the backyard chickens in Northern Thailand had appeared to be (1) the large flock size (> 100 chickens/flock) and (2) the chicken farm located close to the other farms.

From this study, the flock size of the backyard poultry does affect the influenza A infection of the flock; the lager the flock size, the higher the risk. The variable “large flock size” was highly associated with the AI antibody positive (p-value = 0.0058). Comparing to the birds produced in the smaller farm, the farm with the larger size had by far the higher risk (OR = 6.10). As for the chickens produced close to the other farms, the association with the AI antibody positive was quite strong (OR = 5.22; p-value = 0.0517). It is absolutely true for the farm located close to the other farms that the infected birds are able to come in close proximity to the healthy flock and bring about the infection.

The confinement of open flock seems to be the better way to cope with above problems.

However, confinement is not always the best. As an example, the birds have no freedom to express their natural behaviors; which can lead stress to the flock inevitably. When certain infectious disease was introduced into the flock, the birds might get infection easily. With the intensive farming, the virulent disease such as HPAI may cause the high death rates; and/or the high numbers of healthy birds confined in the same compartment may be inevitably destroyed.

❖ GIS MAPPING

The areas in which influenza A antibody positivity was identified are significantly associated with the history of H5N1 outbreaks in the areas. This finding is compatible with the results obtained from other statistical analyses (both logistic regression and Chi-square tests). However, the prevalence antibody positivity was more likely to be found in the flock without the history of H5N1 outbreaks. The high prevalence was supposed to be found in the areas **with** the history, not the areas **without** the outbreaks. This finding is reasonable for this study since the H5N1 outbreaks occurred over the last five years, and had been affected mostly the poultry in farming Sector 3 (the commercial, open flocks with minimal biosecurity). This study, in particular, has been conducted in the poultry classified in Sector 4 (backyard or village poultry).

The high prevalence of antibody positivity in backyard chickens is also related to the areas with the high chicken population. This was considered to be resulted from the larger sample sizes that had been selected from the high chicken population areas and the high density of poultry led to the higher infection rate. Therefore, we can conclude that the larger sample size and the higher the poultry density, the more likely the antibody positivity to be found in that area. It could be mentioned here also that the wetlands and the main roads had no association with the occurrence of influenza A/H5 identified in the study areas.

CONCLUSIONS

The postulated hypothesis that the backyard chickens harbor the avian influenza virus was finally tested to be true for this particular study. The avian influenza subtype identified in this study is definitely H5. However, this H5 subtype could be the highly pathogenic H5N1, or other low pathogenic H5 subtypes. Moreover, the most significant risk factors associated with AI infection in backyard chickens determined from this study are the large flock size (>100 birds/flock) and farms located close to other farms. Although the avian influenza subtype, such as H5N1, could not yet be definitely identified in this study, the sustaining ongoing surveillance for early disease detection and preparedness for rapid disease response are still strongly recommended.

REFERENCES

REFERENCES

Department of Livestock Development (DLD, 2004). Avian influenza situations and control measures: Available from URL: http://www.dld.go.th/home/bird_flu/history_47.html

Department of Livestock Development (DLD), Thailand. (2007). Avian influenza situations and control measures: Available from URL: http://www.dld.go.th/home/bird_flu/birdflu.html

Department of Livestock Development (DLD, 2008). Statistics of Livestock in Thailand 2007. Available from URL: http://www.dld.go.th/ict/yearly/yearly50/stock50.html [Cited on 2008 January 1]

Department of Livestock Development (DLD, 2010). Statistics of chicken and duck, classified by region (2010). Available from URL: http://www.dld.go.th/ict/th/images/stories/stat_web/yearly/2553/chickenduck_ket.pdf [Cited on 2010 October 27]

Environmental Systems Research Institute, Inc. (ESRI, 2010). ArcGIS Desktop 10. ESRI Headquarters 380 New York Street, Redlands, CA 92373-8100 USA

Killian, M.L. (2008a). Avian influenza virus sample types, collection, and handling. In E. Spackman (Ed.), *Avian Influenza Virus Methods in Molecular Biology, 436,* (pp. 7-18). Totowa, NJ: Humana Press.

Killian, M.L. (2008b): Hemagglutination assay for the avian influenza virus. In E. Spackman (Ed.), *Avian Influenza Virus Methods in Molecular Biology, 436,* (pp. 47 – 52). Totowa, NJ: Humana Press.

Michigan State University, Diagnostic Center for Population and Animal Health (DCPAH) (2008). The example figure of AGID test plate. Retrieved from Prof. Dr. Roger Maes in April, 2008.

Microsoft Corporation, Microsoft Office Excel (2007). Corporate Headquarters**,** Microsoft Corporation, One Microsoft Way, Redmond, WA 98052-6399 USA

Pedersen, J.C. (2008): Hemagglutination-inhibition test for avian influenza virus subtype identification and the detection and quantification of serum antibodies to the avian influenza virus. In E. Spackman (Ed.), *Avian Influenza Virus Methods in Molecular Biology, 436,* (pp. 53 – 66). Totowa, NJ: Humana Press.

Roth, W.G. (2005). A photo of 10-day-embryonating chicken egg. Available from URL: http://www.science-art.com/image/?id=2051 [Cited on 2008 April 22]

SAS Institute Inc. (2010). SAS® 9.2. SAS Institute Inc. 100 SAS Campus Drive, Cary, NC 27513-2414 USA

Spackman, E. & Suarez, D.L. (2008). Detection and identification of the H5 hemagglutinin subtype by real-time PCR. In: E. Spackman (Ed.), *Methods in Molecular Biology, Avian Influenza Virus, 436,* (pp. 27 – 33). Totowa, NJ: Humana Press.

Szretter, K.J. Balish, A.L., & Katz, J.M. (2006). Influenza: Propagation, Quantification, and Storage. *Current Protocols in Microbiology*. DOI: 10.1002/0471729256.mc15g01s3 [Cited in 2006 December]

United States Department of Agriculture, National Veterinary Services Laboratories Testing Protocol (USDA, APHIS, 2005 June 1). *Hemagglutination-Inhibitionn Test for Subtype Identification of Influenza A Virus Antibody* (pp 1-17). Ames, IA: USDA Animal and Plant Health Inspection Service.

University of Edinburgh, Computer-aided Learning in Veterinary Education (2007). Win Episcope version 2.0. Available from URL: http://www.clive.ed.ac.uk/cliveCatalogueItem.asp?id=B6BC9009-C10F-4393-A22D-48F436516AC4

University of Manibota, Department of Microbiology (2008). Hemagglutination test. Available from URL: http://www.umanitoba.ca/science/microbiology/staff/cameron/60_401.htm [Cited on 2008 April 23]

Woolcock, P.R. (2008): Avian influenza virus isolation and propagation in chicken eggs. In: E. Spackman (Ed.), *Methods in Molecular Biology, Avian Influenza Virus* (pp. 35 – 46). Totowa, NJ: Humana Press.

Word Organization for Animal Health (OIE). 2005. Chapter 2.7.12. Avian Influenza. Manual of Diagnostic Tests and Vaccines for Terrestrial Animal. Paris, France. Available from URL: http://www.oie.int/eng/normes/mmanual/A_00037.htm

CHAPTER 5

EPIDEMIOLOGICAL STUDY OF AVIAN INFLUENZA IN OPEN FIELD-REARED DUCKS IN NORTHERN THAILAND

INTRODUCTION

Having been incriminated as the major source of HPAI A/H5N1 spreading in Thailand, the open field-reared ducks have played a key role since then for disease prevention and control in the country over the past several years. In order for the effectiveness of disease control and eradication, the occurrence of avian influenza in these traditional-raising poultry has to be first identified. Therefore, this epidemiological study has been ultimately aimed to identify the prevalence of HPAI H5N1 in the Northern region of Thailand. The study could be able to demonstrate the occurrence of avian influenza by a combination of virus isolation and antibody detection methods. By ways of logistic regression analysis and GIS mapping, certain risk factors could be determined. Information gained from this study could be useful for further avian influenza prevention, and control in this particular bird species and for the specific study areas.

The objectives for this study was designed as stated for the similar study conducted in backyard chickens. To repeat, the objective of this epidemiological study in the open field-reared ducks are: (1.) Determine the prevalence of the avian influenza occurrence by a combination of virus isolation and antibody test; (2.) Identify the risk factors associated with the laboratory-confirmed AI by Logistic Regression Analysis; and (3.) Generate the Geographic Information System (GIS) mapping of the laboratory-confirmed avian influenza.

This dissertation chapter is therefore mainly focused on the results of virus isolation and antibody detection for avian influenza, as well as the associated risk factor determination in the open field-reared ducks produced in Northern Thailand, in particular.

MATERIALS AND METHODS

The materials and methods of this study are similar as what had been described in Chapter 4. The reader is referred to Chapter 4 for the full details of Materials and Methods. However, the sample size determination specific for the open field-reared ducks in each study province and district using the program Win Episcope version 2.0 (University of Edinburgh, 2007) is mentioned here. Based on the AI expected prevalence of 25% (DLD, 2004) and 99% confidence interval with 10% accepted error, the crude and adjusted sizes after dropping Lamphun and Maehongson were calculated following the probability proportional to numbers of the birds produced in each province (Table 5-1) and at the district level (**Appendix C**).

Table 5-1: The sample size determination of Open Field-reared Ducks in Northern Thailand

Province	**Duck Population**	**Crude Size**	**Excluding the 2 provinces**	**Proportion**	**Final Size**
Chiang Mai	83,395	125	59,500	59,500/278,088 = **0.21396**	**215**
Chiang Rai	82,707	125	82,707	82,707/278,088 = **0.29741**	**297**
Lampang	27,058	125	27,058	27,058/278,088 = **0.09730**	**97**
Lamphun	***11,087***	***125***	***0***	***0***	***0***
Maehongson	***6,307***	***125***	***0***	***0***	***0***
Nan	24,283	125	24,283	24,283/278,088 = **0.08732**	**87**
Phayao	54,152	125	54,152	54,152/278,088 = **0.19473**	**195**
Phrae	30,388	125	30,388	30,388/278,088 = **0.10927**	**109**
Total	**295,482**	**1,000**	**278,088**	**1.00000**	**1,000**

Source: Thailand Department of Livestock Development (DLD, 2008).

RESULTS

❖ AI VIRUS ISOLATION AND SEROLOGICAL TESTING

According to the virus isolation and AGID test methods conducted at Chiang Mai University in Thailand, zero (0) virus isolate tested sample was positive to AI virus isolation and antibody (Table 5-2). However, when the same 1,000 serum samples were tested at the National Veterinary Services Laboratories in Ames, Iowa, using the commercial test kit ELISA (IDEXX® FlockCheck MultS-Screen; IDEXX Laboratories, Inc., Westbrook, ME, USA); 2.1% of the tested samples were found positive to avian influenza antibodies (Table 5-3).

Table 5-2: Results of AI virus isolation and AGID test conducted in Chiang Mai, Thailand

Sample Type / Test	Number of Samples	AI Positive	% Positive	95% CI
Cloacal Swab / Virus Isolation	1,000	0	0	0, 0
Amnioallantoic Fluid / Hemagglutination	2,000 (1 sample x 2 passages)	0	0	0, 0
Duck Serum / AGID Test	1,000	0	0	0, 0

Table 5-3: Results of serological testing for AI conducted at the National Veterinary Services Laboratories in Ames, Iowa

Sample Type / Test	Frequency	%
Chicken Serum /		
IDEXX ELISA (+)	21	2.1
IDEXX ELISA (-)	979	97.9
Total	**1000**	**100.00**

Table 5-4: Results of AGID test for the ELISA test positive sera; conducted at NVSL

Sample Type / Test	**Frequency**	**%**
Chicken Serum /		
AGID Test (+)	0	0.00
AGID Test (-)	10	100.00
Total	**10**	**100.00**

From 10 serum samples that were tested positive to the ELISA test, no sample (0) was tested positive to avian influenza antibody using AGID test (Table 5-4). For the province-specific prevalence, the high avian influenza antibody positivity (13.8%) was more likely to be determined in Nan (Table 5-6). This finding is definitely compatible with the results of the same test obtained from the similar study conducted in backyard chickens; i.e., 4.9% prevalence of AI antibody positive for serum samples were collected from **Nan** province (Tables 4-5 and 5-5).

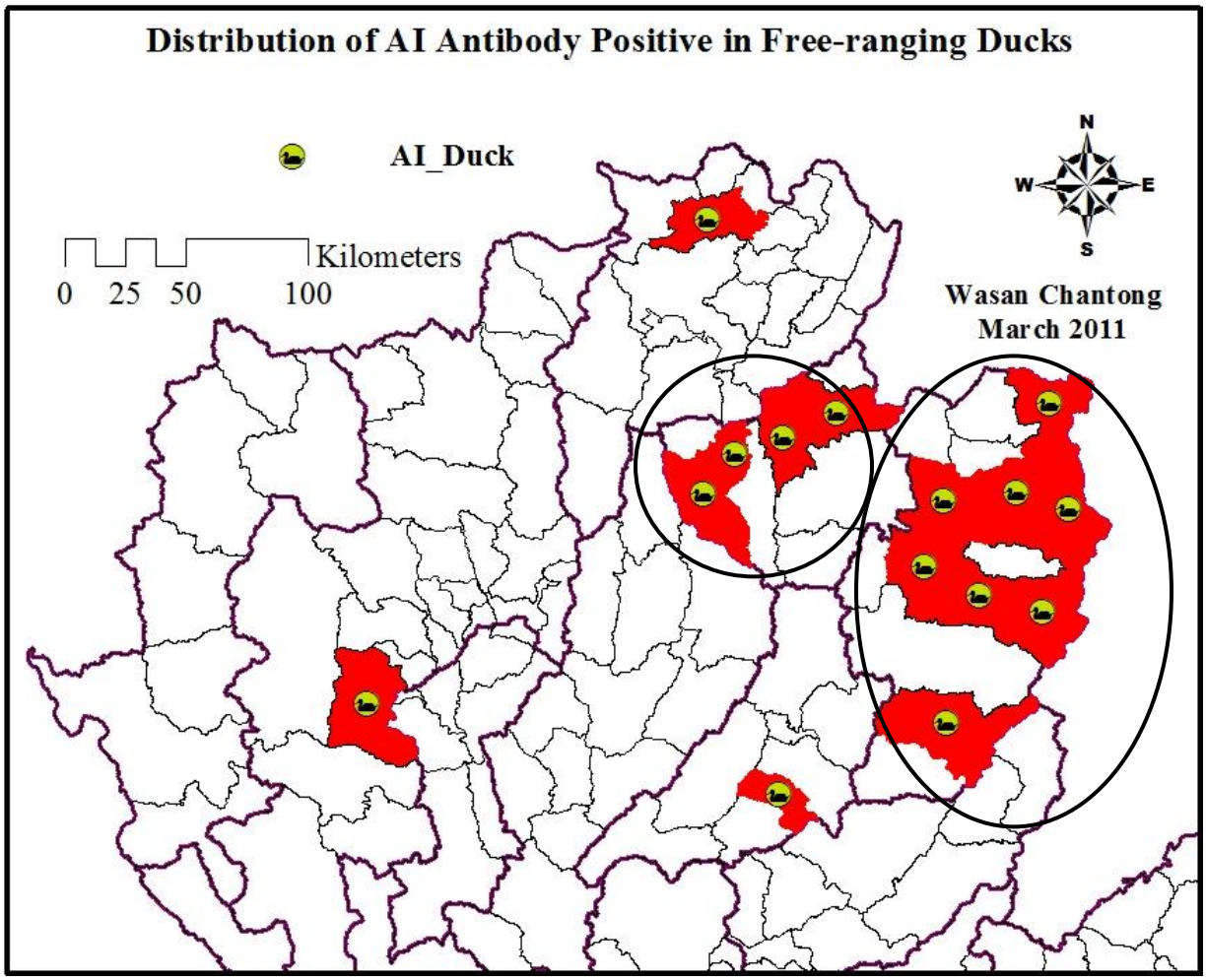
Table 5-5: Results of AI antibody testing positive in Open Field-reared Ducks; classified by province

Province	**Numbers of samples**	**AI positive**	**% positive**	**95% CI**
Chiang Mai	125	1	0.80	0, 1.38
Chiang Rai	297	1	0.34	0, 1.00
Lampang	97	0	0	0
Nan	**87**	**12**	**13.79**	**6.40, 21.18**
Phayao	195	6	3.08	0.63, 5.52
Phrae	109	1	0.92	0, 2.74
Total	**1000**	**21**	**2.1**	**1.21, 2.99**

❖ **DISTRIBUTION OF AI ANTIBOY POSITIVITY IN OPEN FIELD-REARED DUCKS**

The distribution of AI antibody positive in the open field-reared ducks is illustrated in Figure 5-1. There are 15 among 87 districts that avian influenza antibodies were definitely identified. The high prevalence of avian influenza could be described according to the high areas predicted by the antibody positive, which is prominently observed in certain districts of Nan and Phayao.

Figure 5-1: Distribution of AI antibody positivity in Open Field-reared Ducks
(**In the circles: Left;** Phayao: **Right;** Nan)



Source: Map generated using ArcGIS Desktop 10 (ESRI, 2010)

❖ AVIAN INFLUENZA VIRUS SUB-TYPING

Out of fourteen serum samples, which each had sufficient specimen for the H1-H16 subtypes testing, all of them is tested negative for the AI antibodies (dilution of antibodies to each reference antigen <1:8). The results of all 14 serum samples are presented in the Tables 5-6 to 5-8.

Table 5-6: Results of AI virus antibody sub-typing in Open Field-reared Ducks

ID	Flock #	NJ/8/76-EQUINE-1 H1N7	A/Mall/OH/351-6/08 H1N3	A/PINTAIL/ALB/293/77 H2N9	A/DK/UKR/1/63 H3N8	A/DK/CZECH/56 H4N6	A/TY/WI/68 H5N9	Mute Swan/MI/451072-2/06 H5N1
D056	7	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D058	7	-	-	-	-	-	-	<1:8
D059	7	-	-	-	-	-	-	<1:8
D067	8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D098	13	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D101	14	-	-	-	-	-	-	<1:8
D107	16	-	-	-	-	-	-	<1:8
D141	21	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D142	21	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D229	30	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D471	57	-	-	-	-	-	-	<1:8
D658	66	-	-	-	-	-	-	<1:8
D670	66	-	-	-	-	-	-	<1:8
D892	83	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8

Table 5-7: Results of AI virus antibody sub-typing in Open Field-reared Ducks (continued 1)

ID	Flock #	A/TY/ONT/63 H6N5	Mallard /OH/464497/06 H6N1	A/TY/ORE/71 H7N3	TY/NE/505577/07 H7N9	A/TY/VA/1/02 H7N2	A/TY/ONTARIO/6118/67 H8N4	A/TY/WISC/66 H9N2	A/TY/CA/6889/80 H9N2
D056	7	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D058	7	<1:8			<1:8				
D059	7	<1:8			<1:8				
D067	8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D098	13	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D101	14	<1:8			<1:8				
D107	16	<1:8			<1:8				
D141	21	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D142	21	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D229	30	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D471	57	<1:8			<1:8				
D658	66	<1:8			<1:8				
D670	66	<1:8			<1:8				
D892	83	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8

Table 5-8: Results of AI virus antibody sub-typing in Open Field-reared Ducks (continued 2)

ID	Flock #	A/GULL /MD/443 5/80 H9N5	A/CK/ GERM/ 49 H10N7	A/DK/ ENG/5 6 H11N6	A/DK/A LB/60/7 6 H12N5	A/GUL L/MD/7 04/77 H13N6	A/MAL /GURJ EV 263/82 H14N5	A/DK/ AUST /341/8 3 H15N 8	A/Shore bird/DE /172/06 3/10/10 H16N3
D056	7	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D058	7	<1:8	-	-	-	-	-	-	-
D059	7	<1:8	-	-	-	-	-	-	-
D067	8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D098	13	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D101	14	<1:8	-	-	-	-	-	-	-
D107	16	<1:8	-	-	-	-	-	-	-
D141	21	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D142	21	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D229	30	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8
D471	57	<1:8	-	-	-	-	-	-	-
D658	66	<1:8	-	-	-	-	-	-	-
D670	66	<1:8	-	-	-	-	-	-	-
D892	83	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8

❖ DESCRIPTIVE STATISTICS

➢ Prevalence of Avian Influenza Infection

Two point one percent of laboratory confirmed-antibody to avian influenza observed in the open field-reared duck sera is considered as the results of disease infection in these particular poultry. Taking into account this positive result, 2.1% of AI antibody positive is identified as an outcome variable for the logistic regression modeling in order for determining the associated risk factors of AI infection in the open field-reared ducks raised in the Northern region of Thailand at the time of study.

➢ Continuous Predictive Variables for AI Infection in Open Field-reared Ducks

In 2009, there were approximately 6,380 ducks produced at the district level in Northern Thailand. The mean number of households in which these ducks were produced was 365 raisers per district, while the optimum density of the ducks per a household in 2009 was only 15 birds (Table 5-9).

Table 5-9: Descriptive statistics of CONTINUOUS variables for Open Field-reared Ducks

Variable	Mean	Std Dev	Median	Minimum	Maximum
Duck Population 2009 Classified by district	6,380.12	5423	4,215	25	16,601
Numbers of Households	364.38	300.25	281	2	1,039
Density per Household	18.93	14.88	15.56	3.06	124

Note: Std Dev = Standard Deviation

➢ Binary Predictive Variables for AI Infection in Open Field-reared Ducks

There were approximately one-half of the study districts (52%) that had been infected by the HPAI H5N1 subtype during 2004-2006 outbreaks (DLD, 2007). Sixty-five percent of the ducks raised in the study areas was within close proximity to other poultry, and one-third of those ducks was raised close to other pigs in a farm. Almost all ducks (97.4%) produced in the study area had no vaccination, but no disease problems was reported. Approximately 10 - 20 % of the ducks were produced close to the fighting cock arena, live bird market, and poultry movement operated in the area (Table 5-10).

Table 5-10: Descriptive statistics of BINARY variables for Open Field-reared Ducks

Variable	Frequency	Proportion
Duck Density per Household	High (>15) = 516 Low (≤15) = 484	High Density = 51.6% Low Density = 48.4%
History of AI Outbreak in District (2004-2008)	Yes = 521 No = 479	History of Outbreak = 52.1% No History = 47.9%
Existence of Other Poultry in a Farm	Yes = 650 No = 350	Close to Other Poultry = 65.0% Not Close to Others = 35.0%
Existence of Pigs in a Farm	Yes = 332 No = 668	Close to Pig = 33.2% Not Close to Pigs = 66.8%
History of Vaccination of a Flock	Yes = 26 No = 974	No Vaccination = 97.4% Vaccination = 2.6%
History of Other Diseases in a Farm	Yes = 0 No = 100	Other Diseases = 0% No Other Diseases = 100%
Existence of Fighting Cock Arena in the Area	Yes = 87 No = 913	Close to Fighting Cock = 8.7% Not Close = 91.3%
Existence of Live Bird Market in the Area	Yes = 162 No = 838	Close to Bird Market = 16.2% Not Close to Market = 83.8%
Movement of Poultry in the Area	Yes = 183 No = 817	Poultry Movement = 18.3% No Poultry Movement = 81.7%

- **Multiple-categorical Predictive Variables for AI in Open Field-reared Ducks**

Out of 1,000 ducks included in this study, 153 (15.3%) were produced in the large-scale farm and 85.3% of them were the Muscovy (*Cairina moschata*) and 14.7% were Mallard (*Anas platyrhynchos*). One point three percent of the study flocks was raised close to the last H5N1 outbreak areas (within 10 km radius). All ducks (100%) had their own houses, but had the ability to roam around the household. More than 90% of these ducks was produced close to the rice fields, and only 2.3% was raised close to the other farms (Table 5-11).

Table 5-11: Descriptive statistics of MULTIPLE categorical variables for Open Field-reared Ducks

Variable	**Frequency**	**Proportion**
Number of Poultry in the Farm (Household)	< 10 = 10 10 – 50 = 429 51 – 100 = 408 > 100 = 153	Low Number = 0.1% Low to Moderate = 42.9% Moderate to High = 40.8% High Number = 15.3%
Type of Open Field-reared Ducks	Mallard = 147 Muscovy = 853 Others = 0	Mallard Ducks = 14.7% Muscovy Ducks = 85.3%
Farm Close to AI Outbreak in the Area	Within 1 km radius = 0 Within 10 km radius = 13 Within 20 km radius = 79 No Outbreak = 908	Very Close to Outbreak = 0% Close to Outbreaks = 1.3% Far from Outbreaks = 7.9% No Outbreaks Reported 90.8%
Housing of Poultry	No = 0 Under Human's House = 30 Outside Household = 970	No Poultry House = 0% Under Human's = 3.0% Outside = 97.0%
Raising System	Free Roaming = 1000 Confinement = 0	Free Roaming Poultry = 100 %
Feed and Feeding	By Owner Only = 294 By Owner & Nature = 706 By Nature Only = 0	Fed by Owner Only = 29.4% Owner & Natural = 70.6%
Land Use in the Area	Other Farms = 23 Rice Field = 849 Seed /Grain Production = 128	Close to Other Farms = 2.3% Close to Rice Fields = 84.9% Close to Crop Fields = 12.8%
Existence of Wetland in the Area	No = 0 Reservoir = 233 River/Stream = 767	Close to the Wetlands = 100 %

❖ UNIVARIABLE ANALYSES-RISK FACTORS

Univariable and multivariable analyses were generated using SAS PROC LOGISTIC (SAS® Institute, Inc., Cary, NC, USA) to determine the risk factors associated with avian influenza antibody positive in the open field-reared ducks.

➢ Univariable Analyses-Logistic Regression

From univariable analyses, 2 predicted variables were highly significant associated with the antibody positive to AI in the open field-reared ducks ($p < 0.05$); (1) no vaccination applied for the flock; and (2) the farm located close to the areas with previous H5N1 outbreaks (Table 5-12).

➢ Univariable Analyses-Mantel-Haenszel Chi-square

- **Mantel-Haenszel Chi-square for the association between ELISA test positive Open Field-reared Ducks and history of AI outbreaks in the district**

The areas with AI antibody positive in open field-reared ducks are relatively close to the areas of H5N1 outbreaks in 2004-2006 reported by Thailand Department of Livestock Development (DLD, 2007).

However, the results obtained from **Mantel-Haenszel Chi-square** revealed that there is no significant association between the AI antibody positivity and the history of AI (H5N1) outbreaks in the district where the Mantel-Haenszel Chi-square = 1.68 and p-value = 0.19 (Table 5-13 and Figure 5-2).

Table 5-12: Results of UNIVARIABLE analyses-risk factors associated with Avian Influenza antibody positivity in Open Field-reared Ducks

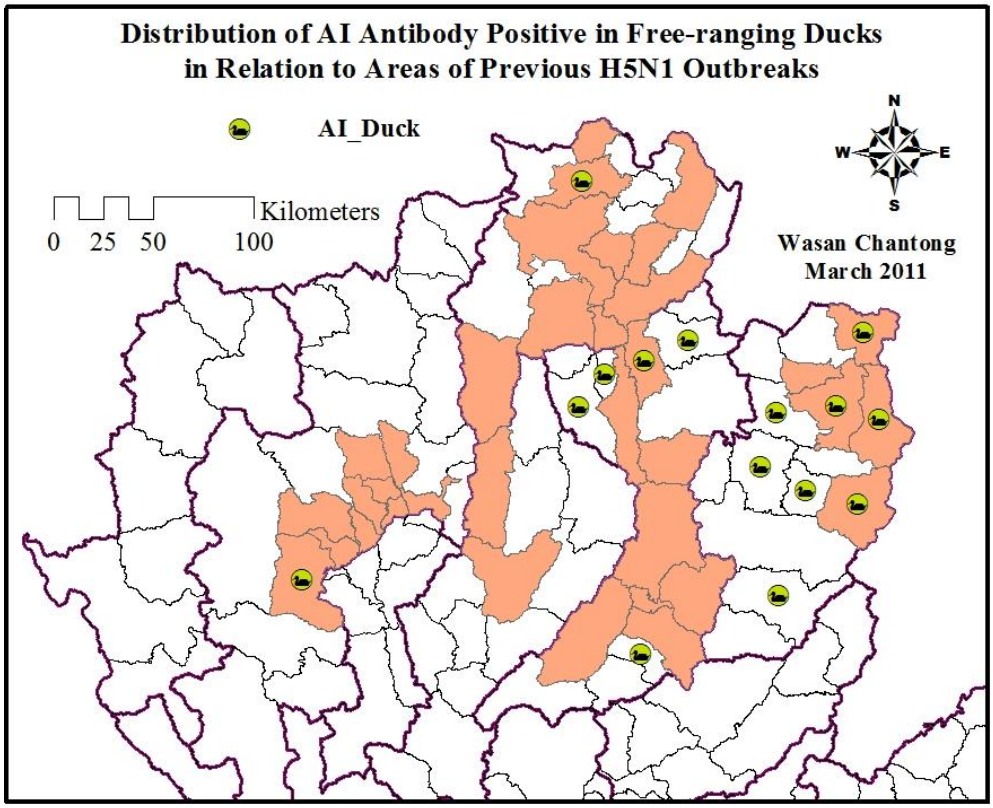
Risk Factors	**OR**	**95% CI**	**Wald χ^2 P-value**
Density/ Household (high; >15 Ducks)	0.85	0.36, 2.02	0.7125
History of AI Outbreak in District	0.56	0.23, 1.36	0.2001
Existence of Other Birds in Farm	1.08	0.43, 2.69	0.8715
Existence of Pigs in Farm	1.24	0.51, 3.03	0.6308
No Vaccination of the Flock	**10.24**	**3.18, 32.92**	**<0.001****
Farm Close to Fighting Cock Arena	<0.01	<0.01, >999.99	0.9768
Farm Close to Live Bird Market	2.11	0.80, 5.52	0.1281
Movement of Poultry in the Area	0.46	0.10, 2.01	0.3047
Flock Size (>100 ducks/flock)	1.75	0.63, 4.86	0.2796
Mallard Type	1.37	0.45, 4.15	0.5712
Muscovy Type	0.73	0.24, 2.19	0.5712
Farm Close to Outbreak Area	**34.67**	**3.48, 344.86**	**0.0025****
Farm Close to Other Farms	<0.01	<0.01, >999.99	0.9835
Farm Close to Rice Field	0.56	0.20, 1.55	0.2662
Farm Close to Crop Field	2.17	0.78, 6.04	0.1360
Farm Close to Reservoir	0.77	0.25, 2.31	0.6424
Farm Close to River	1.29	0.43, 3.89	0.6424

Note: ** significant at $p < 0.05$

Table 5-13: The results for Mantel-Haenszel Chi-square test of AI antibody positivity in Open Field-reared Ducks in association with the history of AI outbreaks in the district

History of AI	ELISA		# Total (%)
	# Positive (+) (Row %)	# Negative (-) (Row %)	
AI+	**8** (1.54%)	**513** (98.46%)	**521 (52.1%)**
AI-	**13** (2.71%)	**466** (97.29%)	**479 (47.9%)**
Total (%)	**21 (2.1%)**	**979 (98.5%)**	**1000 (100%)**

Figure 5-2: Distribution of AI antibody positivity in Open Field-reared Ducks in relation to Areas of Previous H5N1 Outbreaks



Source: Map generated using ArcGIS Desktop 10 (ESRI, 2010)

- **Mantel-Haenszel Chi-square test for trend for the ordinal levels of Open Field-reared Duck population in the district and AI antibody positivity**

There was no significant association between the population of open field-reared ducks in the district and AI antibody positivity (Table 5-14) using ELISA test (Test Statistic = -1.03 and two-sided p-value = 0.30).

Table 5-14: The results for Mantel-Haenszel Chi-square test for trend of AI antibody positivity in association with Open Field-reared Duck population in 2009

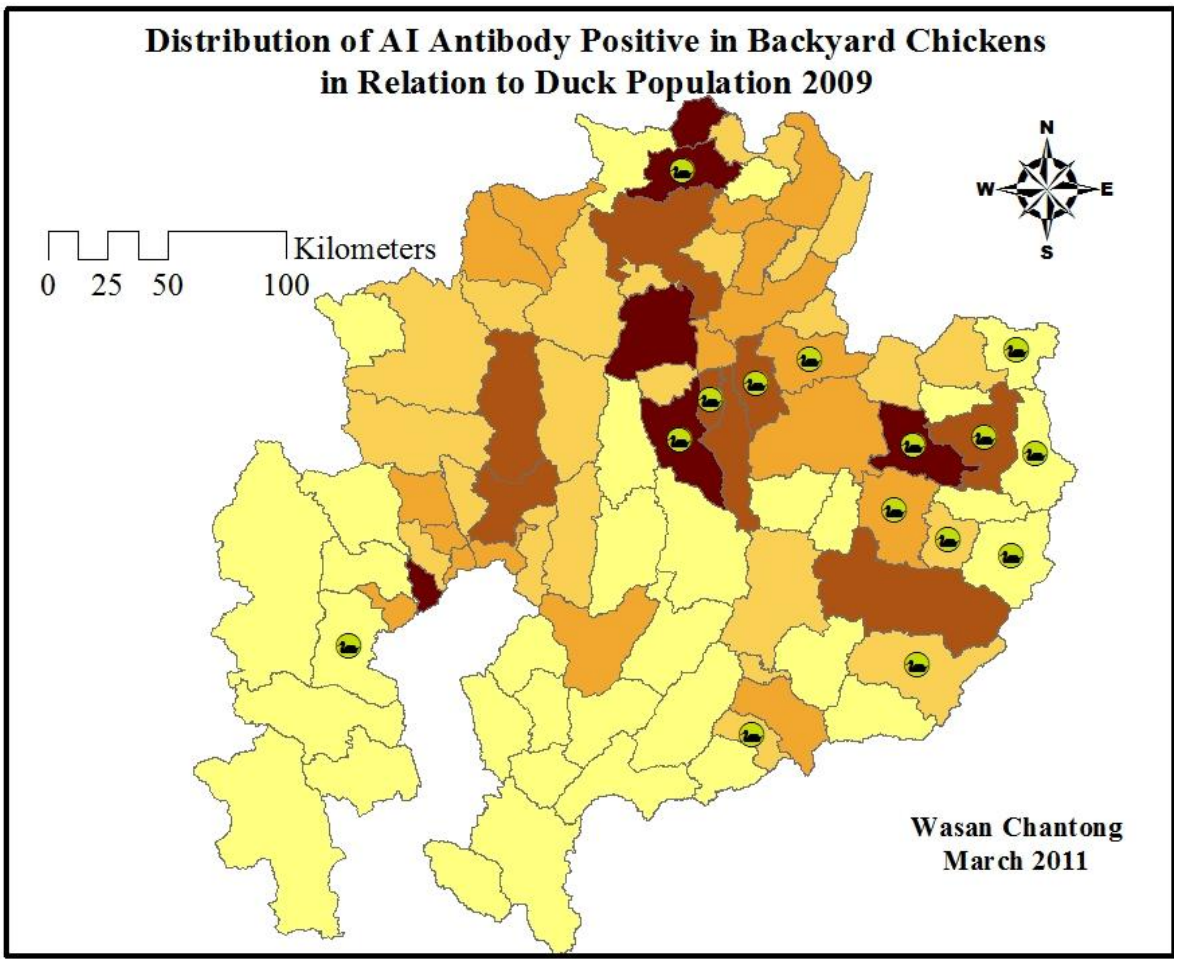
Open Field-reared Duck Population in 2009	**ELISA**		**Total (%)**
	# Positive (+) (Row %)	**# Negative (-)**	
0 – 1,119	**5** (3.47%)	**139** (96.53%)	**144 (14.4%)**
1,120 – 2,618	**3** (1.49%)	**198** (98.51%)	**201 (20.1%)**
2,619 – 5,024	**6** (2.51%)	**233** (97.49%)	**239 (23.9%)**
5,025 – 8,750	**3** (2.50%)	**117** (97.50%)	**120 (12.0%)**
8,751 – 16,601	**4** (1.35%)	**292** (96.85%)	**296 (29.6%)**
Total (%)	**21** (1.50%)	**979** (97.90%)	**1000 (100.0%)**

Source: Thailand Department of Livestock Development (DLD, 2010)

- **Map of AI Birds and District Population of Open Field-reared Ducks**

The areas with AI antibody positive in open field-reared ducks appeared to be close or located in the areas with high population of the open field-reared ducks in 2009 (Figure 5-3). However, this spatial association was not statistically significant by the Chi-square test for trend at the respective time.

Figure 5-3: Distribution of AI antibody positivity in Open Field-reared Ducks in relation to Total Population of Open Field-reared Ducks in 2009



Source: Choropleth map generated using ArcGIS Desktop 10 (ESRI, 2010)

❖ MULTIVARIABLE ANALYSES-RISK FACTORS

➢ Random Effect of the Flock on the Multiple Logistic Regression Model

Taking into account the two significant risk factors obtained from univariable analyses, no vaccination of the flock and farm located close to the AI outbreak areas (p-value < 0.05); the multivariable analyses were generated.

The random effect of flock was added into the multivariable model in order to test for the clustering. The adjusted model after controlling for the flock is provided as the following.

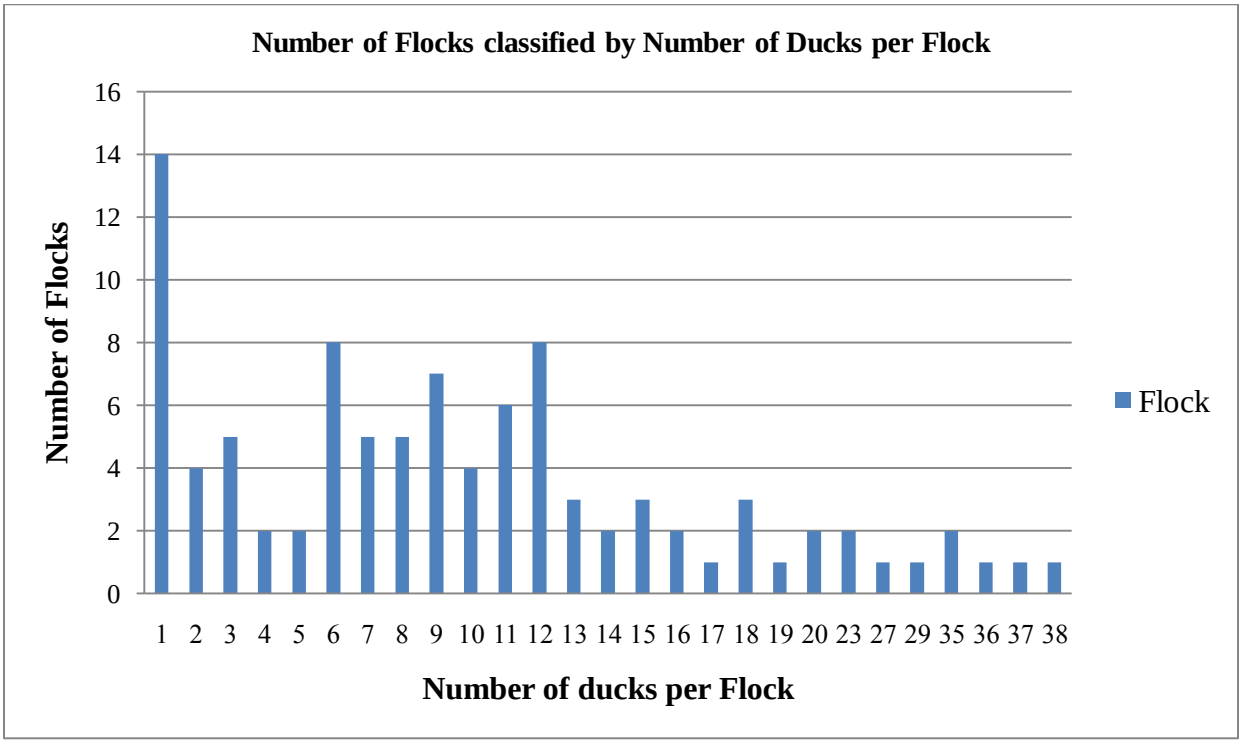
Logit $\pi(X_i) = \ln [\pi(X_i) / \{1 - \pi(X_i)\}]$

$= \alpha + \beta_1$ (No Vaccination) $_{1i} + \beta_2$ (Close to the Outbreak Area) $_{2i} + \mathbf{u_{flock\ (i)}}$;

where $u_{flock\ (i)} \sim N(0, \delta^2)$, the X_is are the predictor values for the i^{th} bird, and the relationship between the probability p_i and the binary outcome Y_i is unchanged : $p(Y_i=1) = p_i$.

SAS PROC GLIMMIX was used to determine the random effect of the flock on the model. Unfortunately, the SAS output could not be generated since the data at the flock level collected in this study are relatively sparse. Notice from the Figure 5-4 that about 20 flocks contained only 1, 2, or 3 birds per flock.

Figure 5-4: Distributions in numbers of flock when classified by the number of birds per flock



Source: Data obtained from the questionnaires conducted by Wasan Chantong, 2009-2010

- **Multivariable Analyses-Logistic Regression**

From the **Multivariable Analyses**, which included the two significant variables from univariable analyses into the model (Table 5-15), the significant confounding effect was obviously identified. The confounding effect caused more than 10% change in parameter estimates [odds ratio (OR)] in the multivariable model, as well as the significance had also changed (change in p-value). Only significant variable in the multivariable model was "Farm Close to Outbreak Areas".

Table 5-15: Results of MULTIVARIABLE logistic regression model for risk factors associated with Avian Influenza antibody positivity in Open Field-reared Ducks

Risk Factors	**OR**	**95% CI**	**Wald χ^2 P-value**
Farm Close to Outbreak Area	21.73	0.43, >999.99	0.0493
No Vaccination of the Flock	1.26	0.03, 55.71	0.8233

- **Conclusion of the Final Model**

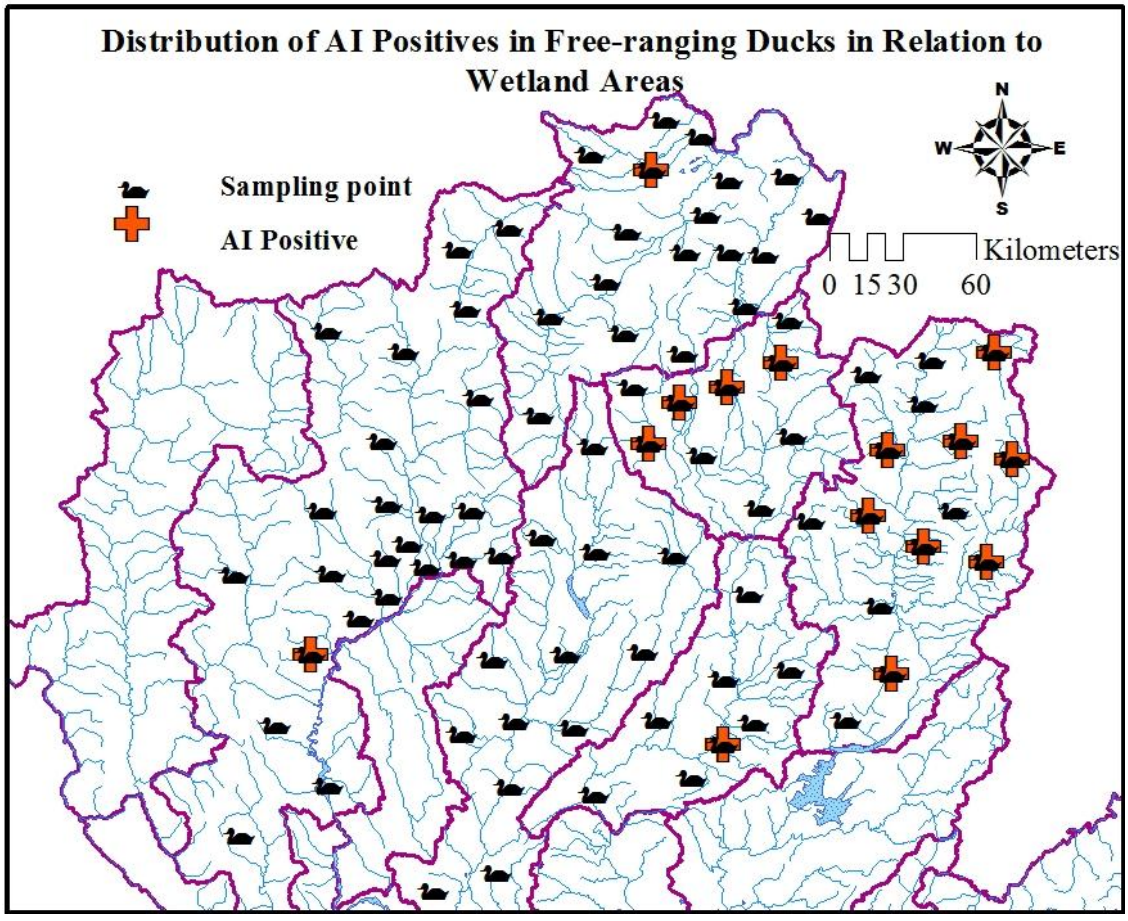
The final model for the multivariable analyses-risk factors associated with avian influenza antibody positivity in open field-reared ducks was not successfully identified due to the confounding effect identified in the model.

OTHER MAPS

- **AI ANTIBODY POSITIVITY IN OPEN FIELD-REARED DUCKS AND WETLAND AREAS**

From the Figure 5-5, avian influenza antibody positive in the open field-reared ducks were somewhat not in association with the wetland areas existed in the study provinces. The map, however, cannot distinguish the differences between the areas with AI antibody positive and other areas without AI.

Figure 5-5: Distribution of AI antibody positivity in Open Field-reared Ducks in relation to the Wetlands

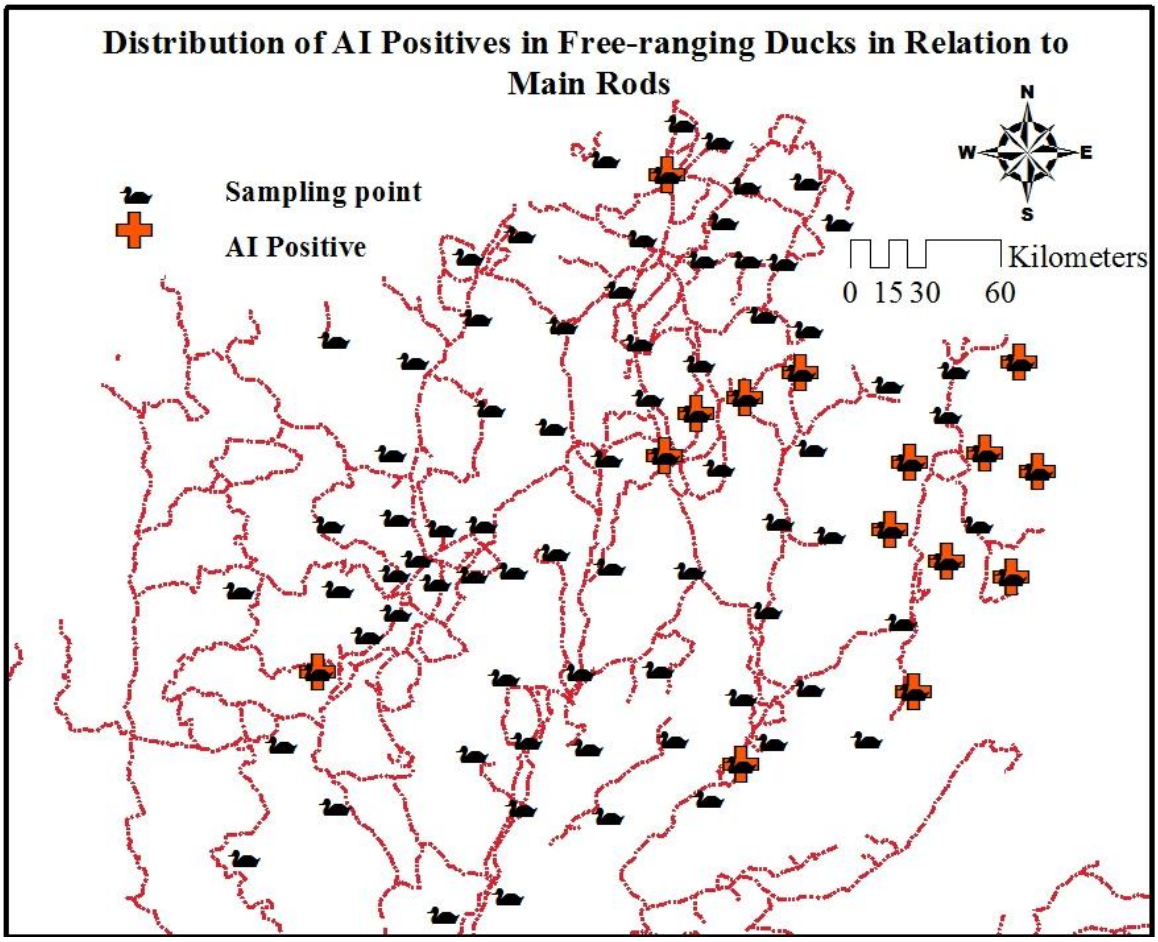


Source: Map generated using ArcGIS Desktop 10 (ESRI, 2010)

❖ AI ANTIBODY POSITIVITY AND MAIN ROADS IN THE STUDY AREA

By being close to the main roads, the poultry movement and transportation were assumed to exist in the areas. However, the association between the avian influenza infected areas and the areas without AI infection (Figure 5-6) were not well distinguished since the main roads appeared to be existed in every sampling point in the study areas. Therefore, the main roads (assumed as poultry movement existed in the area) are not obviously associated with the AI antibody positive.

Figure 5-6: Distribution of AI antibody positivity in Open Field-reared Ducks in relation to the Main Roads



Source: Map generated using ArcGIS Desktop 10 (ESRI, 2010)

DISCUSSION

❖ PREVALENCE OF AVIAN INFLUENZA

It was obviously observed that only a small amount of specimens (2.1%) collected from the open field-reared ducks was positive to influenza A antibodies. However, this amount of positive antibody is somewhat higher than that found in the backyard chickens (1.5%). The higher positive percentage found in ducks was compatible with the fact that the ducks normally get the silent infection with a high capacity of virus shedding for a long period of time. The expected prevalence of the disease is much higher in ducks rather than the virus sensitive poultry, such as chickens. However, the difference in prevalence of influenza A antibody in ducks and chickens is not statistically significant since the Chi-square test p-value > 0.30.

Considering the 95% Confidence Intervals for the positive samples, most of all positive samples, when classified by province, included 0 (zero). This means one could obtain the negative results when the samples were tested individually for each province. This could probably occur when the sample size is quite low for such a specific area. With no doubt, zero prevalence is possible for the tests conducted in Chiang Mai, Thailand. From there, we found that all samples were tested negative to virus isolation and antibody detection by AGID test.

❖ AVIAN INFLUENZA SUBTYPES

No H subtype of influenza A virus was found from the study in open field-reared ducks. This could be resulted from the low concentration of antibody in the duck serum.

❖ ASSOCIATED RISK FACTORS FOR AI ANTIBODY POSITIVE IN OPEN FIELD-REARED DUCKS

As the evidence of univariable and multivariable analyses-risk factors, it could be identified that the predictive risk factors of being AI infected in the open field-reared ducks produced in Northern Thailand are the probability of the farm that is located close to the areas with the history of H5N1 outbreaks, and no kind of vaccine applied for the flock.

➢ Farm Located Close to the Areas with the History of H5N1 Outbreaks

It is no doubt that the duck farms located in close proximity to the areas with the history of H5N1 avian influenza outbreaks were inevitably affected by the same subtype of avian influenza infection. However, since this study could not identify whether or not the antibody tested positive for AI is definitely H5N1; one cannot assume that living close to the areas with the last H5N1 outbreaks has the higher risk to be infected with any other AI virus strains.

➢ No Vaccination Applied for the Flock

The words stated that "Prevention is better than cure" is always true for any infectious disease and so is useful for this study. Having no vaccine applied in the poultry flock is a highly significant association with being infected with the AI virus. Not only could the AI virus invade the unvaccinated flock, but also the other viral and bacterial infectious diseases. However, it is not the case for avian influenza where the prevalence of disease is relatively low and the cost of vaccine implementation for each individual bird is somewhat high for the poor country. However, vaccine application in the flock is still useful for other infectious disease, such as, fowl pox, cholera, infectious bronchitis, etc., but not for avian influenza in Thailand.

- **No Final Risk Model Identified**

Since there was a confounding effect identified from the 2 significant variables when included in the multivariable analyses, the final associated risk model could not be obtained from this study. This could happen with the rare event of disease such the findings in this study. Taking together the univarible-analyses risk factors can cause the final insignificant model.

- **Other Insignificant Variables**

Being the Muscovy (*Cairina moschata*) or Mallard (*Anas platyrhynchos*) is not significant for avian influenza infection found in this study, since both duck species had no significant difference for AI infection. The similar findings can also be explained for other predictive variables, for examples, the duck farm located close to the rice field, close to seed and grain production areas, close to the fighting cock arena, or even close to the wetlands.

- **GIS MAPPING**

The commercial computer software ArcGIS® Program seemed to be useful for this particular study where the distribution of avian influenza antibody positive, and certain associated risk factors can be depicted across space and time.

From the distribution map generated using the ArcMap®, the areas with the positive AI antibody are found relatively close to or located in the areas of previous H5N1 outbreaks. However, from the statistical analysis for the history of AI in the area, and the areas with high duck population in relation to AI antibody positivity, there was no statistical association between those areas. This can be postulated that the data reported by DLD was not the same data as that obtained from this study, since the data for outbreak history were mostly reported in the poultry classified in Sector 3 but this study had mentioned only the birds in Sector 4.

CONCLUSIONS

This study could generate the information concerning the prevalence of avian influenza occurrence and its associated risk factors, as well as the simulation maps for AI infection. The hypothesis stated that avian influenza viruses are still circulating in the open field-reared ducks is tested to be true, at least for the Northern region of Thailand. Besides the bad news for people who reside in this region, the good news is that the virus infection is not yet proved to be the HPAI A/H5N1.

REFERENCES

REFERENCES

Department of Livestock Development (DLD, 2004). Avian influenza situations and control measures: Available from URL: http://www.dld.go.th/home/bird_flu/history_47.html

Department of Livestock Development (DLD), Thailand. (2007). Avian influenza situations and control measures: Available from URL: http://www.dld.go.th/home/bird_flu/birdflu.html

Department of Livestock Development (DLD, 2008). Statistics of Livestock in Thailand 2007. Available from URL: http://www.dld.go.th/ict/yearly/yearly50/stock50.html [Cited on 2008 January 1]

Department of Livestock Development (DLD, 2010). Statistics of chicken and duck, classified by region (2010). Available from URL: http://www.dld.go.th/ict/th/images/stories/stat_web/yearly/2553/chickenduck_ket.pdf [Cited on 2010 October 27]

Environmental Systems Research Institute, Inc. (ESRI, 2010). ArcGIS Desktop 10. ESRI Headquarters 380 New York Street, Redlands, CA 92373-8100 USA

SAS Institute Inc. (2010). SAS® 9.2. SAS Institute Inc. 100 SAS Campus Drive, Cary, NC 27513-2414 USA

University of Edinburgh, Computer-aided Learning in Veterinary Education (2007). Win Episcope version 2.0. Available from URL: http://www.clive.ed.ac.uk/cliveCatalogueItem.asp?id=B6BC9009-C10F-4393-A22D-48F436516AC4

CHAPTER 6

OVERALL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

The fundamental research question asked in this dissertation is whether or not the avian influenza viruses are still circulating in the backyard chickens and open field-reared ducks (Sector 4) in Northern Thailand. Results of this study demonstrate that at the time the samples were collected, there was no evidence of active highly pathogenic influenza type A (HPIA) virus, as determined by negative virus isolation findings. This study, however, provided evidence of antibodies to influenza type A subtype H5, suggesting that these birds were once exposed to the AI virus, but were not actively shedding the virus at the time the samples were collected. It is reasonable to speculate that the birds were exposed to the virus before the study time in 2009-2010, and antibodies produced by the flocks persisted for a long period of time.

Before the time of research, the majority of reported H5N1 outbreaks in Thailand occurred in the poultry classified in Sector 3, high density (> 1,000 birds/flock) and raised freely in open areas with minimal biosecurity. Chickens and ducks for this study were from Sector 4, which has minimal biosecurity and was lower in production number, < 100 birds/flock. Taking into account the low prevalence of avian influenza infection in the low number of backyard poultry in Sector 4, the very low positive results (1-2%) found in this study are not surprising.

AVIAN INFLUENZA A SUBTYPE

Only H5 subtype of influenza A virus had been identified from the chicken sera. No H subtype was found in the open field-reared ducks. These results could be due to the low concentration of antibody in the collected sera (from both chickens and ducks).

Based on the findings from this study and from the fact that H5N1 recently flared up in the study area, the H5 subtype identified in this study (by Hemagglutination-inhibition, HI) test is likely to be the highly pathogenic H5N1 avian influenza. In the low prevalence of H5, only three (chicken sera) out of 21 ELISA-positive sera obtained from both backyard chickens and open field-reared ducks was considered to be the continual results from the effectiveness of the intensive H5N1 avian influenza surveillance systems in Thailand. The strong collaboration between the Thai government and other related authorities in particular seemed also to be a key component of the successful campaign for avian influenza preventions and controls in Thailand (FAO, 2010c).

FACTORS ASSOCIATED WITH INFLUENZA A ANTIBODY POSITIVITY

From this epidemiological study, it was demonstrated that two statistically significant risk factors were associated with increased odds of having AI in backyard chickens, large flock size, and close proximity of a flock to other agricultural farms. In the open field-reared ducks, the only risk factor that appeared to significantly increase the odds of a bird being positive was the farms close proximity to the areas with a history of H5N1 outbreaks during 2004-2008.

However, history of avian influenza H5N1 outbreaks in the districts was not a significant risk factor since the previous outbreaks had affected mostly the Sector 3 poultry, but in this study the poultry farming in Sector 4 were mainly focused. Moreover, the situation of avian influenza outbreaks in the country has been changing over time.

NEGATIVE RESULTS FOUND IN CHIANG MAI

All sera tested by the AGID test at Chiang Mai University were negative. Duplicate sera were sent to the NVSL, and were tested for the presence of antibodies to AI using the AGID and a commercial IDEXX test. It is important to note that all AGID results tested at the NVSL were negative, except one sample that was classified as suspect. The results of the IDDEX test, however, showed a 1.5% and 2.1% sero-prevalence in chickens and open field reared ducks, respectively. The IDEXX test has a higher sensitivity compared to the AGID test, and should be recommended for future use in place of the AGID test.

Another explanation that could be addressed, also for AGID test, is that the sera samples collected from the poultry (particularly ducks) do not contain good precipitin antibodies to be tested by AGID, and the test method may not be sensitive enough to detect low levels of viral antibody in the serum [the sensitivity of AGID test ranging from 35 - 75%] (Brown et al., 2010; Jeong et al., 2010; Zhou et al., 2000; Suarez & Schultz-Cherry, 2000; Sullivan et al., 2009). The assumption stated for AGID that the test is fairly insensitive has been confirmed from the same procedures of the test performed on the same sera at NVSL. Only 1 out of 13 sufficient samples, which were already positive to influenza A antibody using ELISA test, was suggestive (not indicated as "**Positive**") to influenza A antibody.

The expected prevalence included for the sample size determination in this study was by far higher than the test prevalence (25% versus 1-2%). This overestimated prevalence led to the low determining sample size. With the low sample size, the lower actual prevalence could be determined accordingly.

Reasons for the negative virus isolation may include: 1) lack of active virus infection, resulting in the negative isolation results from egg inoculation and hemagglutination methods, and 2) as mentioned in the "**Materials and Methods**" section, only one swab was taken from each bird. The collected sample might have had less quantity of viruses, or the virus antigen was present in a low concentration or degraded before further replication.

When classified by province, the 95% Confidence Intervals (CI) of the positive sera collected from Chiang Mai and Chiang Rai had included 0 (zero). In other words, it is possible to find the negative results in the low samples collected from these study provinces.

Since this study could not definitely define the subtypes of avian influenza as H5N1, the positive avian influenza antibodies found in this study could be any combination of H5 and any 9 N-subtypes. If the low pathogenic H5 subtype is identified instead of H5N1, it is possible for virus isolation conducted in Chiang Mai, Thailand, to be underestimated; since the viruses did not cause the high death rate in the embryonating chicken eggs. However, when the AAF was collected and tested with the hemagglutination test, it should be positive to the hemagglutination reactions with no doubt.

In summary, the influenza A/H5 viruses identified in this study was not in the active infection form but had once infected the flocks, which were identified in the poultry sera using the high sensitive ELISA test. With the low to fair sensitivity, AGID is not a recommended test for screening of avian influenza antibody.

RECOMMENDATIONS

❖ DEVELOPMENT OF EDUCATIONAL PROGRAMS

The findings from this study could be used to develop the educational programs to the farmers in the influenza A/H5 infected areas. The risks of avian influenza, which still existed in the area, should be communicated to the farmers in order for them to prepare themselves for the re-emerging of the avian influenza infection. Measures to reduce the risks of AI occurrence for poultry, such as farming backyard poultry with low density but higher biosecurity and not too much close to the other farms should be educated. By the educational programs, the farmers can protect their flocks or even themselves from the risk of disease infection.

❖ INTENSIVE OR BATTERY FARMING

With a fear of transmission by wild birds and free-ranging backyard poultry, certain control strategies, i.e. restricted movement, confinement, and compartmentalization or battery farming, might be the preferable ways to control the outbreaks of HPAI. Paradoxically, this kind of operating practices could inevitably increase the risk of outbreaks and rapid transmission, rather than improving biosecurity or maintaining the current trend to better animal welfare resulting from the free-range operating (Graham et al., 2008).

Some may argue that operating the large-scale or high intensive farming is better than the traditional poultry raising systems. By the intensive farming practice, the owners can get the better benefits from their production and it is effortless for farming management. The large-scale farming with the high biosecurity practice can prevent the transmission of the infectious diseases. For instance, the high biosecurity can prevent the transmission of AI from the wild birds and/or from other vectors outside the farm.

By the fact that poultry produced in Sectors 3 and 4 (open flock and backyard) are generally considered susceptible to HPAI infection because of their low biosecurity, the HPAI viruses if introduced to the poultry classified in Sectors 1 and 2 (intensive, commercial with high biosecurity) are likely to spread among other poultry in the flock extremely quickly. The larger number of poultry might be killed by the disease, or more likely to be destroyed to control the transmission comparing to the smaller backyard raising systems (Beach et al., 2007).

One disadvantage of the intensive farming is that it has a higher risk for disease infection. We have found from this study that the larger the flock size, the higher the risk AI infection. This predictive risk factor is highly associated with AI for the backyard flocks that operated with the large-scale; comparing to the one that practice the farm in the lesser scale (p-value < 0.05).

However, the packed poultry population can cause spreading of the disease rapidly when it was introduced into the farm. Moreover, if the infection is severe, the poultry will have high probability to be infected or be destroyed. Then the large-scale farming is not always the best. As one may recall from this study, that being raised in the larger farm-backyard chickens are more likely to be infected with avian influenza viruses.

Since the free-grazing ducks had been incriminated as the significant sources of H5N1 spreading in Thailand, it has been forced by the Thai government that all free lance duck flocks had to be compartmentalized (DLD, 2006; Songserm et al., 2006). However, many producers disagree with the regulations and still operate the traditional poultry husbandry as they used to do. This may have the higher risk for AI infection, but it could be better than having the large intensive farm in terms of economic losses when the HPAI actually enter the small free-ranging duck farm. Then less money would be lost.

❖ MORE INTENSIVE STUDIES

The intensive studies, especially with the larger sample sizes and in the other types of poultry should be performed. With the larger sizes, the power of the study will be increased, i.e. the study has higher capability to detect the lower prevalence of disease. As from this study the prevalence of influenza A antibody was in fact 1-2%, but the expected prevalence for sample size determination was 25%. As a result, the sample sizes were relatively low (1:10,000 for chickens and 1:300 for ducks). More intensive studies should be carried out in the infected areas. In other words, the study should be specific to the areas from which the influenza A/H5 positive specimens were collected. The famous poultry, such as fighting cocks, as well as the poultry in live bird markets, should also be included in the intensive studies. Other farming sectors, i.e. poultry intensively produced in Sectors 1, 2, or 3, should be included for further studies.

❖ SUSTAINING ONGOING SURVEILLANCE

H5N1 subtype might not yet be determined in this study; however, the H5 subtype was definitely identified. Whether or not the backyard chickens and free-ranging ducks in Northern Thailand currently harbor the H5N1 viruses, HPAI H5N1 is still a major threat and remains circulating in the domestic poultry of many countries in Southeast Asia.

Although the major outbreaks of HPAI H5N1 have declined in Thailand, nobody dares to state that the virus has been completely wiped out from our country as long as the neighboring countries, such as Cambodia and Myanmar, still continue reporting HPAI cases of human beings to the World Health Organization (WHO, 2011), and reporting infected flocks of domestic poultry and wild birds to the World Organization for Animal Health (OIE, 2011b).

New human cases have been reported continually in Indonesia, up until the present time (March 2, 2011); leading the country to have the highest human prevalence of HPAI (172 cases included 142 deaths, thus far). Similar cases had been occurring in Vietnam, the second ranked country for confirmed human cases, 7 new cases (with 2 deaths) were reported in 2010 (WHO, 2010c). Interestingly, 3 human cases (finally had been fatal) are currently reported in Cambodia, the close neighboring country of Thailand (WHO, 2011). In order to protect a country and its citizens against the re-emergence and spreading of HPAI A/H5N1, it is strongly recommended that the country innovate health management with enhanced disease intelligence supported by a global network of diagnostic laboratories; facilitating early warning, early detection, and rapid response (FAO, 2010b). Since nobody knows when and how the highly pathogenic avian influenza viruses will re-entry our country, it is strongly recommended that a thorough, on-going, systemic disease surveillance and prevention should be persistently applied.

REFERENCES

REFERENCES

Beach, R.H., Poulos, C., & Pattanayak, S.K. (2007). Agricultural household response to avian influenza prevention and control policies. Presented at the Annual Meetings of the Southern Agricultural Economics Association, February 3-6, 2007, Mobile, Alabama, 2007

Brown, J.D., Luttrell, M.P., Berghaus, R.D., Kistler, W., Kleeler, S.P., Howey, A., et al. (2010). Prevalence of antibodies to type A influenza virus in wild avian species using two serologic assays. *Journal or Wildlife Diseases, 46(3),* 896-911.

Department of Livestock Development, Bureau of Animal Disease Control, Prevention, and Treatment (DLD, 2006). *The guideline for Avian Influenza control* [Thai version]. Bangkok: Agricultural Co-operative Club of Thailand.

Food and Agricultural Organization of the United Nations (FAO, 2010b). Avian Influenza: continued support is required. Available from URL: http://www.fao.org/avianflu/en/news/hpai_support.html [Cited on 2010 September 17]

Food and Agricultural Organization of the United Nations (FAO, 2010c). Key achievements and lessons learned from the HPAI program. FAO Regional Strategy for Highly Pathogenic Avian Influenza and other Emerging Infectious Diseases of Animals in Asia and the Pacific, 2010–2015. Available from http://www.fao.org/docrep/012/i1474e/i1474e00.htm [Cited on 2010 March]

Graham, J.P., Leibler, J.H., Price, L.B., Otte, J.M., Pfeiffer, D.U., Tiensin, T., et al. (2008, May-June). The animal-human interface and infectious disease in industrial food animal production: Rethinking biosecurity and biocontainment. *Public Health Reporters Journal, 123(3),* 282-99.

Jeong, O., Kim, M., Kang, H., Ha, G., Oh, G., Yoo, J., et al. (2010). Validation of egg yolk antibody based C-ELISA for avian influenza surveillance in breeder duck. *Journal of Veterinary Microbiology, 144*, 287-92.

Songserm ,T., Jam-on, R., Sae-Heng, N., Meemak, N., Hulse-Post, D.J., Sturm-Ramirez, K.M., et al. (2006, April). Domestic ducks and H5N1 influenza epidemic, Thailand. *Emerging Infectious Diseases Journal, 12(4)*, 575-81.

Suarez, D.L. & Schultz-Cherry, S. (2000). Immunology of avian influenza virus: a review. *Developmental & Comparative Immunology Journal, 2000 Mar-Apr, 24(2-3),* 269-83.

Sullivan, H.J., Blitvich, B.J., VanDalen K., & Bentler, K.T. (2009). Evaluation of an epitope-blocking enzyme-linked immunosorbent assay for the detection of antibodies to influenza A virus in domestic and wild avian and mammalian species. *Journal of Virological Methods, 161,* 141-6.

World Health Organization (WHO) (2010c). Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO. Available from URL: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_11_19/en/index.html [Cited on 2010 November 19]

World Health Organization (WHO) (2011). Situation up date archives. Available from URL: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_03_02/en/index.html [Cited on 2011 March 02]

World Organization for Animal Health (OIE) (2011b). Update on highly pathogenic avian influenza in animals (Type H5 and H7). Available from URL: http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/2011/ [Cited on 2011 February 12]

Zhou, E.M., Chan, M., Heckert, R.A., Riva, J., & Cantin, M.F. (2003; Proceeding). A competitive Enzyme-linked Immunosorbent Assay for avian influenza serological surveillance. *Journal of Avian Diseases, 47 (Special Issue)*, 305-12.

APPENDICES

APPENDIX A

COUNTRIES REPORTED H5N1 AVIAN INFLUENZA IN DOMESTIC POULTRY/WILDLIFE 2003-2010

63 Countries Report H5N1 Avian Influenza in Domestic Poultry/Wildlife 2003-2010
(OIE, 2010)

1. Afghanistan
2. Albania
3. Austria
4. Azerbaijan
5. Bangladesh
6. Benin
7. Bhutan
8. Bosnia and Herzegovina
9. Bulgaria
10. Burkina Faso
11. Cambodia
12. Cameroon
13. China
14. Côte d'Ivoire
15. Croatia
16. Czech Republic
17. Denmark
18. Djibouti
19. Egypt
20. France
21. Georgia
22. Germany
23. Ghana
24. Greece
25. Hong Kong (SARPRC)
26. Hungary
27. India
28. Indonesia
29. Iraq
30. Iran
31. Israel
32. Italy
33. Japan
34. Jordan
35. Kazakhstan
36. Korea (Republic of)
37. Kuwait
38. Laos
39. Malaysia
40. Mongolia
41. Myanmar
42. Nepal
43. Niger
44. Nigeria
45. Pakistan
46. Palestinian Autonomous Territories
47. Poland
48. Romania
49. Russia
50. Saudi Arabia
51. Serbia and Montenegro
52. Slovakia
53. Slovenia
54. Spain
55. Sudan
56. Sweden
57. Switzerland
58. Thailand
59. Togo
60. Turkey
61. Ukraine
62. United Kingdom
63. Vietnam

APPENDIX B

CUMULATIVE NUMBER OF CONFIRMED HUMAN CASES OF AVIAN INFLUENZA A/H5N1 REPORTED TO WHO

Table A-1: Cumulative Number of Confirmed Human Cases of Avian Influenza A/H5N1 Reported to WHO

29 December 2010

Country	2003		2004		2005		2006		2007		2008		2009		2010		Total	
	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	0	0	8	5
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	1	0	1	1	10	8
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	2	1	40	26
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	39	4	25	11	115	38
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	21	19	9	7	171	141
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	3	2
Lao People's Democratic Republic	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	2	2
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Nigeria	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1
Pakistan	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	3	1
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	0	0	25	17
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	0	0	12	4
Viet Nam	3	3	29	20	61	19	0	0	8	5	6	5	5	5	7	2	119	59
Total	**4**	**4**	**46**	**32**	**98**	**43**	**115**	**79**	**88**	**59**	**44**	**33**	**73**	**32**	**44**	**22**	**512**	**304**

Remark: C = Cases and D = Deaths

Total number of cases includes number of deaths.

WHO reports only laboratory-confirmed cases.

All dates refer to onset of illness.

Indonesia numbers indicate cumulative total of sporadic cases and deaths which occurred during 2009.

APPENDIX C

POULTRY POPULATIONS AND SAMPLE SIZES CLASSIFIED BY DISTRICT

Table A-2: Backyard Chicken and Open Field-reared Duck Populations and Sample Sizes in Chiang Mai Classified by District

District	Chicken_pop	Chicken_size	Duck_pop	Duck_size
Chai Prakarn	38,900	4	375	1
Chiang Dao	88,861	9	2,516	9
Chom Thong	172,200	17	2,830	10
Doi Saket	91,000	9	2,270	8
Doi Tao	45,648	5	0	0
Fang	166,911	16	3,363	12
Hang Dong	111,346	11	6,311	23
Hot	113,254	11	0	0
K. Doi Lo	59,007	6	2,697	10
K. Mae On	98,620	10	1,499	5
Mae Ai	87,097	9	4,144	15
Mae Chaem	92,374	9	114	1
Mae Rim	62,432	6	3,855	14
Mae Taeng	126,067	12	3,070	11
Mae Wang	17,795	2	2,568	9
Muang Chiang Mai	41,832	4	272	1
Omkoi	45,765	5	413	2
Phrao	144,332	14	4,072	15
Samoeng	133,604	13	1,188	4
San Kamphaeng	122,634	12	4,080	15
San Pa Tong	315,820	31	30,273	23
San Sai	218,371	22	3,468	12
Saraphi	155,239	15	3,777	14
Wiang Haeng	38,724	4	240	1
Total	**2,587,833**	**256**	**83,395**	**215**

Note: Chicken_pop = Chicken Population
Chicken_size = Chicken Sample Size (in this study)
Duck_pop = Duck Population
Duck_size = Duck Sample Size (in this study)

Source: Thailand Department of Livestock Development (DLD, 2008). Available from http://www.dld.go.th/ict/yearly/yearly50/stock50.html

Table A-3: Backyard Chicken and Open Field-reared Duck Populations and Sample Sizes in Chiang Rai Classified by District

District	Chicken_pop	Chicken_size	Duck_pop	Duck_size
Chiang Khong	144,442	14	2,391	8
Chiang Saen	118,679	12	4,906	18
K. Doi Luang	58,954	6	326	1
K. Wiang Chiangrung	71,936	7	2,164	8
Khun Tan	120,675	12	734	3
Mae Chan	177,360	17	15,296	55
Mae Fa Luang	82,676	8	774	3
Mae Lao	57,378	6	1,621	6
Mae Sai	61,798	6	17,674	64
Mae Suai	161,880	16	2,167	8
Muang Chiang Rai	221,124	22	8,996	32
Pa Daet	83,267	8	1,964	7
Phan	341,883	34	8,035	29
Phaya Mengrai	86,812	9	3,329	12
Thoeng	218,740	22	5,149	18
Wiang Chai	75,148	7	2,115	7
Wiang Pa Pao	96,361	10	3,069	11
Wieng Kaen	44,208	4	1,997	7
Total	**2,223,321**	**220**	**82,707**	**297**

Note: Chicken_pop = Chicken Population
Chicken_size = Chicken Sample Size (in this study)
Duck_pop = Duck Population
Duck_size = Duck Sample Size (in this study)

Source: Thailand Department of Livestock Development (DLD, 2008). Available from http://www.dld.go.th/ict/yearly/yearly50/stock50.html

Table A-4: Backyard Chicken and Open Field-reared Duck Population and Sample Size in Lampang Classified by District

District	Chicken_pop	Chicken_size	Duck_pop	Duck_size
Chae Hom	132,873	13	262	1
Hang Chat	152,979	15	4,456	16
Ko Kha	99,245	10	792	3
Mae Mo	52,827	5	215	1
Mae Phrik	38,971	4	172	1
Mae Tha	119,817	12	218	1
Muang Lampang	202,933	20	14,663	52
Mueang Pan	77,665	8	1,327	5
Ngao	126,788	12	1,932	7
Soem Ngam	41,887	4	243	1
Sop Prap	18,164	2	49	0
Thoen	129,772	13	903	3
Wang Nua	111,083	11	1,826	6
Total	**1,305,004**	**129**	**27,058**	**97**

Note: Chicken_pop = Chicken Population
Chicken_size = Chicken Sample Size (in this study)
Duck_pop = Duck Population
Duck_size = Duck Sample Size (in this study)

Source: Thailand Department of Livestock Development (DLD, 2008). Available from http://www.dld.go.th/ict/yearly/yearly50/stock50.html

Table A-5: Backyard Chicken and Open Field-reared Duck Populations and Sample Sizes in Nan Classified by District

District	Chicken_pop	Chicken_size	Duck_pop	Duck_size
Ban Luang	32,783	3	356	1
Bo Klue	15,616	1	627	2
Chalermphrakiet	39,127	4	832	3
Chiang Klang	59,697	6	4,323	15
K. Phu Pieng	102,460	10	2,627	10
Mae Charim	39,390	4	1,059	4
Muang Nan	79,630	8	3,432	12
Na Mun	49,447	5	502	2
Na Noi	74,457	7	1,807	6
Pua	147,629	15	2,957	11
Santi Suk	20,385	2	471	2
Song Kwae	10,169	1	175	1
Tha Wang Pha	98,512	10	4,483	16
Thung Chang	27,494	3	349	1
Wiang Sa	30,710	3	283	1
Total	**827,506**	**82**	**24,283**	**87**

Note: Chicken_pop = Chicken Population
Chicken_size = Chicken Sample Size (in this study)
Duck_pop = Duck Population
Duck_size = Duck Sample Size (in this study)

Source: Thailand Department of Livestock Development (DLD, 2008). Available from http://www.dld.go.th/ict/yearly/yearly50/stock50.html

Table A-6: Backyard Chicken and Open Field-reared Duck Populations and Sample Sizes in Phayao Classified by District

District	Chicken_pop	Chicken_size	Duck_pop	Duck_size
Chiang Kham	236,210	23	2,490	9
Chiang Muan	38,807	4	1,737	6
Chun	94,861	9	2,520	9
Dok Kham Tai	175,515	17	5,643	20
K. Phu Kam Yao	81,314	8	6,527	24
K. Phu Sang	67,084	7	1,651	6
Mae Chai	140,805	14	3,356	12
Muang Phayao	801,943	80	30,228	109
Pong	187,882	19	0	0
Total	**1,824,421**	**181**	**54,152**	**195**

Note: Chicken_pop = Chicken Population
Chicken_size = Chicken Sample Size (in this study)
Duck_pop = Duck Population
Duck_size = Duck Sample Size (in this study)

Source: Thailand Department of Livestock Development (DLD, 2008). Available from http://www.dld.go.th/ict/yearly/yearly50/stock50.html

Table A-7: Backyard Chicken and Open Field-reared Duck Populations and Sample Sizes in Phrae Classified by District

District	Chicken_pop	Chicken_size	Duck_pop	Duck_size
Den Chai	89,866	9	1,773	6
Long	30,087	3	2,686	10
Muang Phrae	275,683	27	9,024	32
Nong Muang Kai	52,012	5	1,508	6
Rong Kwang	209,088	21	3,656	13
Song	261,337	26	4,253	15
Sung Men	204,246	20	4,980	18
Wang Chin	207,533	21	2,508	9
Total	**1,329,852**	**132**	**30,388**	**109**

Note: Chicken_pop = Chicken Population
Chicken_size = Chicken Sample Size (in this study)
Duck_pop = Duck Population
Duck_size = Duck Sample Size (in this study)

Source: Thailand Department of Livestock Development (DLD, 2008). Available from http://www.dld.go.th/ict/yearly/yearly50/stock50.html

APPENDIX D

QUESTIONNAIRE

Epidemiological Study of Avian Influenza in Backyard Chickens and Open Field-reared Ducks in Northern Thailand

การศึกษาทางระบาดวิทยาของโรคไข้หวัดสัตว์ปีก ในไก่เลี้ยงหลังบ้าน และเป็ดเลี้ยงแบบปล่อย ในเขตภาคเหนือของประเทศไทย

Wasan Chantong, DVM, MSc VPH

วสันต์ จันทอง, สพ.บ., MSc VPH

Date/ วันที่ ……**Investigator/ ผู้เก็บข้อมูล** …….

Farmer/ ชื่อเจ้าของสัตว์……

Address/ ที่อยู่……

---

1. Backyard Animals/ ข้อมูลสัตว์เลี้ยงหลังบ้าน

A. Species Available/ ชนิดสัตว์ที่เลี้ยง

□ Chickens/ไก่ □ Ducks/เป็ด □ Pigs/สุกร

□ Geese/ห่าน □ Ostriches/นกกระจอกเทศ □ Quails/นกกระทา

□ Other (indicate)/อื่นๆ (ระบุ).....

B. Number of Poultry per Household/ จำนวนสัตว์ปีก ต่อ ครัวเรือน (ตัว)

□ < 10 □ 10 – 50 □ 51 – 100

□ 101 – 200 □ 201 – 500 □ 501 –1000

□ > 1000

C. Type of Chickens and/or Ducks / ชนิดของเป็ด และ/หรือไก่

- Chickens/ไก่

□ Natives/พื้นเมือง □ Broilers/ไก่เนื้อ

□ Layer/ไก่ไข่ □ Fighting cocks/ไก่ชน

- Ducks/เป็ด

□ Native-, layers (Mullard)/พื้นเมือง (เป็ดไข่) □ Meat (Muscovy)/เป็ดเนื้อ (เป็ดเทศ)

□ Other /อื่นๆ (ระบุ) ……

DLD Certification/การรับรองจากกรมปศุสัตว์

□ Certified/รับรอง □ Applying/กำลังขอรับรอง □ Non-certified/ไม่ได้รับรอง

D. Vaccination Given/วัคซีนที่ใช้ในสัตว์ปีก

□ No/ไม่ได้ฉีด □ Pox/ฝีดาษ □ ND/นิวคาสเซิล

□ Gumboro/กัมโบโร่ □ Cholera/อหิวาห์ □ Other/อื่นๆ ……

E. Current Health Problem (s)/ปัญหาสุขภาพสัตว์ที่พบในปัจจุบัน

□ No/ไม่มีปัญหา □ Pox/ฝีดาษ □ ND/นิวคาสเซิล

□ Gumboro/กัมโบโร่ □ Cholera/อหิวาห์ □ Other/อื่นๆ ……

2. Previous Bird flu Infection/ประวัติการติดเชื้อไข้หวัดนกของสัตว์ในครัวเรือน

□ Yes/ มี □ No (please skip to # 4.)/ไม่มี (กรุณาข้ามไปข้อ 4.)

3. History of the Outbreak(s)/ประวัติการระบาดของโรคไข้หวัดนก

- Year of Outbreak (s)/ปีที่เกิดการระบาด

□ 2004/2547 □ 2005/2548 □ 2006/2549 □ 2007/2550

- Type of Infected Animals/ชนิดสัตว์ที่ติดเชื้อ

□ Chickens/ไก่ □ Ducks/เป็ด □ Pigs/สุกร

□ Geese/ห่าน □ Ostriches/นกกระจอกเทศ □ Quails/นกกระทา

□ Other (indicate)/อื่นๆ (ระบุ).....

- Number of Infected Animals/จำนวนสัตว์ที่ติดเชื้อ

Sick/ป่วย……Died/ตาย……Destroyed/ทำลาย……

- Human Infection /การติดเชื้อในคน

□ No/ไม่มี □ Yes/ มี Number of /จำนวน Sick/ป่วย .....Died/ตาย.....

- Compensation /การจ่ายเงินทดแทน

□ No/ไม่มี □ Yes/มี Amount of money/จำนวนเงิน ……..Baht/บาท

- Prevention and Control/การป้องกันและควบคุมโรค

□ No/ไม่มี □ Yes/มี

- Method(s)/วิธีการที่ใช้

- □ Culling/ทำลาย □ Netting/ล้อมตาข่าย □ Movement restriction/ จำกัดการเคลื่อนย้าย
- □ Clean and disinfection/ ฆ่าเชื้อ ทำความสะอาด □ Kept in closed house/ เลี้ยงสัตว์ในกรง
- □ Stop raising/เลิกเลี้ยงสัตว์ □ Other/อื่นๆ.....

- Time of Outbreak (from start to stop)/เวลาจากเริ่มต้นถึงหยุดระบาด .....Days/วัน

4. History of Bird flu Outbreak (s) in Neighboring Areas/ประวัติการระบาดในพื้นที่ใกล้เคียง

□ Within 1-km radius/ภายในรัศมี 1 กม.

Place/ สถานที่ .....Time (year)/ปี.....

□ Within 5-km radius/ภายในรัศมี 5 กม.

Place/ สถานที่ .....Time (year)/ปี.....

□ Within 10-km radius/ภายในรัศมี 10 กม.

Place/ สถานที่ .....Time (year)/ปี.....

□ Within 20-km radius/ภายในรัศมี 20 กม.

Place/ สถานที่ .....Time (year)/ปี.....

5. Farming Information/ ข้อมูลฟาร์ม

A. Poultry's House/ โรงเรือนสัตว์ปีก

□ No/ ไม่มี Place to stay overnight/ที่สัตว์นอน.....

□ Yes/ มีโรงเรือน Under human's house/ ใต้ถุนบ้าน หรือ Outside/ นอกตัวบ้าน

B. Raising System/ ระบบการเลี้ยง

□ Confinement/ จำกัดบริเวณในบ้าน □ Free roaming/ ปล่อยอิสระ

C. Feed and Feeding/ อาหารและการให้อาหาร

□ By owner only/ เจ้าของให้เท่านั้น

□ By owner & nature/ เจ้าของให้และอาหารตามธรรมชาติ

□ Natural feeds only/ อาหารตามธรรมชาติเท่านั้น

Note: Natural feeds, e.g. grass, grains, seeds, fish, snails and/or human leftovers.
หมายเหตุ: อาหารตามธรรมชาติ เช่น เศษหญ้า เมล็ดพืช ปลา หอย หรือ ของกินเหลือจากมนุษย์

6. Land use Information/ ข้อมูลการใช้ที่ดินในพื้นที่ □ Only Human's Houses/ที่อยู่อาศัย

A. Rice Paddy Fields/ พื้นที่นาข้าว

□ Within 500-m radius/ภายในรัศมี 500 ม. Area/ พื้นที่ …….Rai/ไร่

□ Within 1-km radius/ภายในรัศมี 1 กม. Area/ พื้นที่ ……...Rai/ไร่

B. Seed- or Grain Production/การเพาะปลูกพืชมีเมล็ด

□ Within 500-m radius/ภายในรัศมี 500 ม. Area/ พื้นที่ …….Rai/ไร่

□ Within 1-km radius/ภายในรัศมี 1 กม. Area/ พื้นที่ ……..Rai/ไร่

7. Wetland Information/ ข้อมูลแหล่งน้ำ □ No/ ไม่มี

A. Reservoirs/สระ ฝาย หนอง บึง เขื่อน

The nearest reservoir; Name/ชื่อแหล่งน้ำที่ใกล้ที่สุด .....

Distance /ระยะทาง.....meters/เมตร

Numbers of reservoirs in the area /จำนวนแหล่งน้ำ.....แห่ง

B. Rivers/Streams /แม่น้ำ ลำคลอง

The nearest river/stream; Name/ ชื่อแม่น้ำ ลำคลองที่ใกล้ที่สุด……

Distance/ระยะทาง .....meters/เมตร

Numbers of rivers/streams in the area/ จำนวนแม่น้ำ ลำคลอง.....สาย

8. Places and Activities Related to Live Poultry/ สถานที่และกิจกรรมที่เกี่ยวข้องกับสัตว์ปีก

A. Fighting Cock Arena (s)/ บ่อนไก่ชน

□ No/ ไม่มี

□ Yes/ มี Within 500-m radius/ภายในรัศมี 500 ม.;

Number/จำนวน .....แห่ง

Within 1-km radius/ภายในรัศมี 1 กม.;

Number/ จำนวน.....แห่ง

B. Poultry Live Markets/ ตลาดไก่มีชีวิต

□ No/ ไม่มี

□ Yes/ มี Within 500-m radius/ภายในรัศมี 500 ม.;

Number/จำนวน .....แห่ง

Within 1-km radius/ภายในรัศมี 1 กม.;

Number/ จำนวน.....แห่ง

C. Movement of Poultry/ การเคลื่อนย้าย ลำเลียงสัตว์ปีก

□ No/ ไม่มี

□ Yes/ มี Within 500-m radius/ภายในรัศมี 500 เมตร;

- Number of roads /จำนวนถนน .....สาย

Within 1-km radius/ภายในรัศมี 1 กม. ;

- Number of roads / จำนวน.....สาย

APPENDIX E

LABORATORY PROCEDURES CONDUCTED AT NVSL

1. IDEXX AVIAN INFLUENZA VIRUS ANTIBODY TEST KIT: ELISA

The IDEXX FlockCheck® MultiS-Screen Avian Influenza Virus Antibody Test Kit is an enzyme-linked immunosorbent assay (ELISA) designed to detect avian influenza (AI) type A antibodies in serum from multiple avian species, including chicken, turkey, duck, goose and others (the test sensitivity is 95.4% and 99.7% specificity). The antibodies against the influenza A viruses can be detected by 13 days post-infection (IDEXX Laboratories, Inc., 2009).

❖ DESCRIPTIONS AND PRINCIPLES

The relative level of antibody to avian influenza in chicken and duck serum can be measured by this test assay, which is performed on 96-well plates that have been coated with avian influenza A viral antigen. Upon incubation of the test sample in the coated wells, AI specific antibody forms a complex with the coated antigen. After washing away unbound material, an ant-AI monoclonal antibody enzyme conjugate is added to the wells. In the absence of AI antibodies in the test sample, the conjugate is free to bind to the AI antigen on the plate. Conversely, if there are antibodies to avian influenza present in the sample, the anti-AI conjugate is blocked from binding to the antigen. Unbound conjugate is washed away and enzyme substrate is added. Subsequent color development is inversely proportional to the amount of anti-AI antibodies in the test sample.

❖ MATERIALS REQUIRED

Precision pipettes and multiple-delivery pipetting device with disposable tips, 96-well plate reader, and tubes for diluting samples, distilled or deionized water and device for the delivery and aspiration of wash solution.

❖ **PREPARATION OF SAMPLES**

Dilute test samples tenfold (1/10) with sample diluents prior to being assayed (e.g., by diluting 15 µl of sample with 135 µl of sample diluents). Be sure to change tips for each sample. Samples must be thoroughly mixed prior to dispensing into the coated plate.

❖ **PREPARATION OF WASH SOLUTIONS**

The 10X Wash Concentrate should be brought to room temperature, 18°C - 25°C and mixed to ensure dissolution of any precipitated salts. The Wash Concentrate must be diluted 1/10 with distilled/deionized water before use (e.g., 30 ml of concentrated plus 270 ml of water per plate to be assayed).

❖ **TEST PROCEDURE**

Reagent should be allowed to come to room temperature at 18°C - 25°C, and then mixed by inverting and swirling.

2. Obtain antigen-coated plate(s) and record the sample position on a FlockCheck worksheet.
3. Dispense 100 µl of UNDILUTED Negative Control into wells A1 and A2.
4. Dispense 100 µl of UNDILUTED Positive Control into wells A3 and A4.
5. Dispense 100 µl of diluted sample into appropriate wells.
6. Incubate for 60 minutes (±5 minutes) at room temperature 18°C - 25°C.
7. Wash each well with approximately 350 µl of diluted 1X wash solution 3-5 times.
8. Dispense 100 µl of Anti-AI: Horseradish Peroxidase Conjugate into each well.
9. Incubate for 30 minutes (±2 minutes) at room temperature 18°C - 25°C.
10. Repeat step 6.

11. Dispense 100 µl of TMB substrate solution into each well.
12. Incubate for 15 minutes (±2 minutes) at room temperature 18°C - 25°C.
13. Dispense 100 µl of Stop Solution into each well to stop the reaction.\
14. Blank reader with air.
15. Measure and record absorbance values at A(650).

❖ RESULTS

For the assay to be valid, the Negative Control mean absorbance A(650) should be greater than or equal to 0.600 and the Positive Control mean S/N must be less than 0.50. For invalid tests, technique may be suspected and the assay should be repeated. The presence or absence of antibody to AI is determined by the sample to negative (S/N) ratio for each sample.

❖ CALCULATION

1. Negative Control mean (NC X-bar) = $\frac{\text{Well A1 A(650) + Well A2 A(650)}}{2}$
2. Positive Control mean (PC X-bar) = $\frac{\text{Well A3 A(650) + Well A4 A(650)}}{2}$
3. S/N Ratio = $\frac{\text{Sample X-bar A(650)}}{\text{NC X-bar}}$

❖ INTERPRETATION OF RESULTS

Samples with an S/N ratio $\geq$ 0.50 are considered negative for the presence of AI antibodies. Sample with S/N values < 0.50 should be considered AI antibody positive. Heat inactivation of samples may adversely affect results and should be avoided.

2. HEMAGGLUTINATION-INHIBITION (HI) ASSAY

Hemagglutination-inhibition assay is used to detect and quantitate subtype-specific antibodies in poultry serum following infection with influenza A virus. The procedures were conducted at the USDA National Veterinary Services Laboratories (NVSL) in Ames, Iowa, followed the USDA NVSL Testing Protocol for Hemagglutination-Inhibition Test for Subtype Identification of Influenza A Virus Antibody (AVPRO0806.04; NVSL, 2005).

❖ PRINCIPLE

The hemagglutination-inhibition (HI) test is used to detect and quantitate subtype-specific antibodies in serum, plasma, and yolk following infection with influenza A virus. The basis of the HI test is (1) hemagglutination (HA) occurs when hemagglutinins on the virus envelope interact with receptors on the surface of erythrocytes, and (2) inhibition of HA occurs in the presence of subtype-specific antibodies in serum, plasma, and yolk.

In the current nomenclature for influenza A viruses, 16 hemagglutinin (H) subtypes (Hl - H16) have been described. Any 1 of the 16 subtypes is present in influenza A viruses isolated from avian species. It is recommended, when performing HI tests with AIV, to use antigens and antiserums negative for homologous neuraminadase (a second glycoprotein on the surface of influenza virus) to avoid problems with steric inhibition (inhibition caused by the interaction of homologous neuraminidase (NA) antigen on the virus surface and antibodies specific to that neuraminidase (N-subtype).

❖ MATERIALS

➢ Equipment & Instrumentation

1. Single and multichannel micro pipettes
2. Microtiter plate shaker
3. Freezer (-20^{o}C [-15^{o}C or colder], -70^{o}C [-65^{o}C or colder])
4. Refrigerator (4^{o}C [± 2^{o}C])
5. Centrifuge (low speed, refrigerated)
6. Various sizes of test tube racks
7. Vacuum source for aspirating liquids (vacuum pump with sidearm flask or Chapman-Type filter pump attached to a water line)
8. Microplate centrifuge carriers for 96-well plates

➢ Reagents & Supplies

1. U-bottom microtiter plates (96 well) and plate covers
2. Glassware/plastic ware
    - Pipettes (serologic, 1 ml, 5 ml, 10 ml)
    - Micro pipette tips (200µl)
    - Graduated cylinder (100 ml)
    - 17x100-mm snap-cap tubes
    - 15x150-mm stoppered tubes
    - 250-ml Erlenmeyer flask
    - 50-ml conical centrifuge tubes
3. Microtiter plate sealing tape
4. Phosphate-buffered saline (PBS), 0.01 M, pH 7.2 (± 0.1)

5. Bovine serum albumin (BSA), fraction V (Intergen Co., 3305-01)
6. Common laboratory chemicals
7. Sodium azide, Practical (Mallinckrodt, 1953-57)
8. Alsever's solution
9. Rooster (male chicken) blood
10. Antibody-positive and antibody-negative serum against each H subtype used in the test
11. Inactivated viral antigen for each H subtype used in the test
12. Beta-propriolactone (β-PL) (Sigma, P5648)
13. Disodium phosphate solution (DSP), 0.5 M

❖ PREPARATION OF THE TEST

➢ Personnel qualifications/training

- Personnel must be familiar with proper handling, diluting, pipetting, storing, and disposal of test reagents and biological materials.
- Personnel must be familiar with calibration, maintenance, and use of instruments included in this protocol.

➢ Preparation of equipment/instrumentation

Refrigerators, freezers, centrifuges, and micro pipettes are calibrated and certified according to respective National Veterinary Services Laboratories (NVSL) standard operating procedure (SOP) numbers.

- **Preparation of reagents/control procedures**

Washing erythrocytes:

Dispense 10-20 ml rooster blood, preserved in Alsever's solution into a 50 ml centrifuge tube and fill the tube with PBS. Gently invert the tube several times to wash the erythrocytes. Centrifuge at 800 x g (1,800 rpm in a Beckman J6-B centrifuge with JS 4.2 rotor) for 10 minutes in a refrigerated centrifuge. Aspirate PBS and buffy coat (bone-colored layer of cells on top of red blood cells) from the tube. Refill the tube with fresh PBS. Repeat the washing and centrifugation cycle 2 additional times. Washed erythrocytes can be stored at 4^{o}C for up to 1 week.

- **Preparation of 0.5% erythrocyte suspension:**

Dispense 199 ml PBS into a 250-ml Erlenmeyer flask. Add 1 ml washed, packed erythrocytes to the PBS, rinsing the pipette thoroughly to remove all erythrocytes. Erythrocytes can be stored for up to 1 wk at 4^{o}C. Discard erythrocytes if hemolysis is observed.

- **Preparation of 10% erythrocyte suspension**:

Dispense 9 ml PBS into an appropriate vessel. Add 1 ml washed, packed erythrocytes to the PBS, rinsing the pipette thoroughly to remove all erythrocytes. Erythrocytes can be stored for up to 1 week at 4^{o}C. Discard erythrocyte suspension if hemolysis is observed.

- **0.4% bovine serum albumin (BSA)-phosphate buffered saline (PBS)-sodium azide (SA) (0.4% BSA-PBS-SA):**

Add 10 ml 4% BSA to 89 ml PBS. Add 1 ml 10% sodium azide solution to the BSA-PBS.

- **Preparation of reference HA antigens**:

Dilute HA antigens with 0.4% BSA-PBS-SA to a concentration of 8 HA units (HAU) per 50 µl (4 HAU/25 µl).

- **Preparation of reference serums**:

Dilute reference serums to a titer between 1:16 and 1:64 with 0.4% BSA-PBS-SA. The titer is determined by HI test with 4 HAU of homologous antigen. When necessary, remove natural serum agglutinins from reference serums by treating diluted serum with 0.1 ml packed erythrocytes per 1 ml serum. Incubate for 30 min at room temperature with occasional mixing to keep erythrocytes suspended. Centrifuge treated serum at 800 x g (1,800 rpm in a Beckman J6-B centrifuge with JS 4.2 rotor) for 10 minutes and retain serum. Diluted serum can be stored for several weeks at 4^{o}C. For long-term storage: freeze at -20^{o}C.

- **Preparation of the sample**

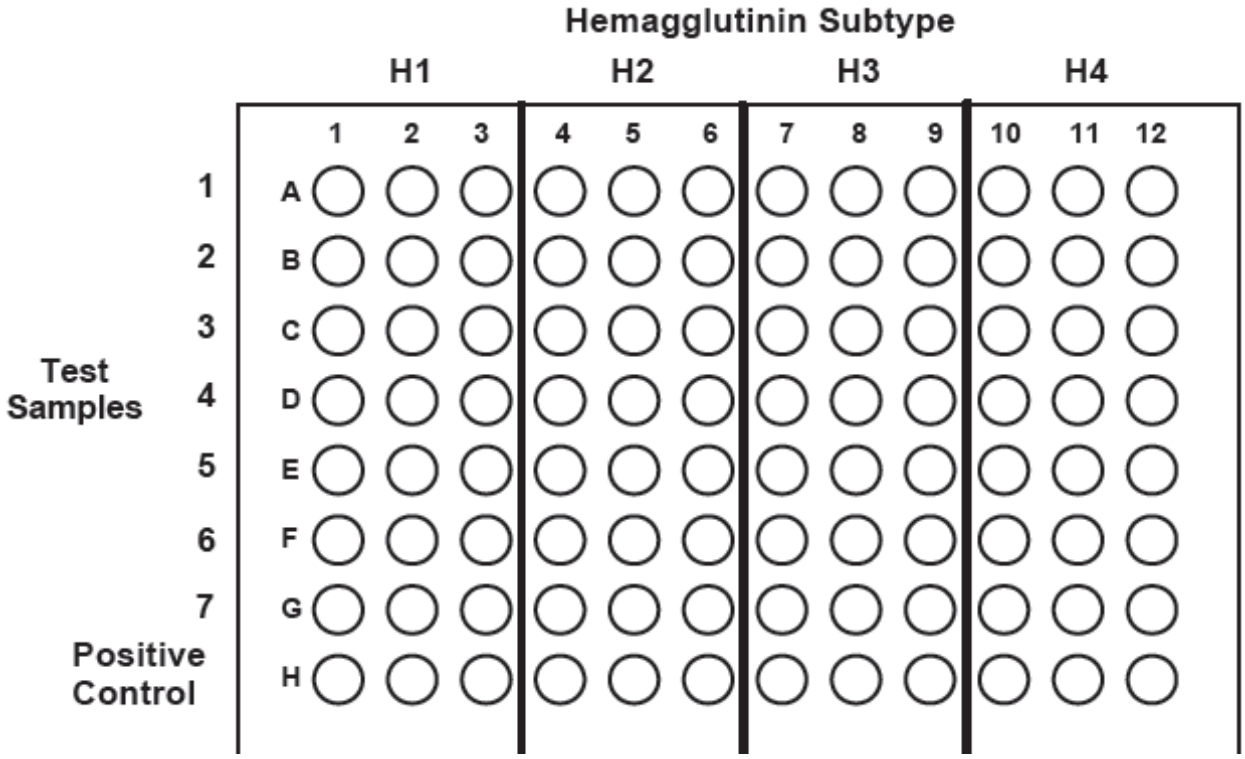
1. Serum is the preferred sample for the HI test. Plasma may also be used; however, under certain conditions plasma samples may coagulate, rendering the sample unusable. Blood samples must be of good quality and free of bacterial contamination and hemolysis. Since antibodies against H-subtype of influenza A viruses could be produced and contained in egg yolk and poultry blood, the test samples extracted from egg yolk or dried blood on filter paper strips can also be used in the HI test. Procedures for the use of these alternate samples can be found in the current version of AVSOP2220 (procedure for extracting egg yolk antibodies) and AVSOP0800 (collection and processing avian blood samples on filter paper).

2. When necessary (if auto agglutination is observed in serum control with serum and RBCs only), treat serum, plasma, and yolk samples to remove natural serum agglutinins by placing the tube of serum sample into 56 ^{o}C water bath for 30 minutes.

3. The serum is treated at the 1:4 dilution (1 part sample, 2 parts PBS, and 1 part 10% erythrocytes). When equal volumes of treated serum and antigen are combined in the HI test the initial serum dilution is a 1:8 dilution. Sufficient serum should be treated to test each serum against the antigens selected for the test. For example, if the sample will be tested against all 16 H subtypes, approximately 0.4 ml of treated serum will be needed. If a 96-well microtiter plate is used to treat serum, several wells may be required for each serum (place serums in the plate using the same format as for the HI test [Figure A-1] so that multiple serums can be transferred simultaneously to the HI plates). The following method for treating serum in microtiter plates results in a 1:4 dilution of serum.

    - Dispense 100 µl PBS to 3 wells for each serum to be treated.
    - Add 50 µl serum/plasma/yolk extract to each well
    - Add 50 µl 10% erythrocyte suspension to each well
    - Mix thoroughly on microtiter plate shaker and incubate at room temperature for 30 minutes, mixing every 10 minutes or so to keep erythrocytes suspended.
    - Centrifuge plates at 200 x g (1,000 rpm in Beckman J-6B centrifuge with JS 4.2 rotor) to pellet erythrocytes.

Figure A-1: Example of Hemagglutination-inhibition (HI) test plate



Source: NVSL Testing Protocol (NVSL USDA-APHIS, Ames, IA, 2005).

❖ **PERFORMANCE OF THE TEST**

➢ **Hemagglutination (HA) Test**

The HA test is used to standardize H subtype antigens for the HI test.

1. Dispense 50 µl PBS into a row of 12 wells in a microtiter plate for each H subtype antigen used in the test. One additional row of wells should be included for a positive HA control.
2. Add 50 µl undiluted antigen to the 1st well of each corresponding row.
3. Serially dilute the antigen (first through 11th well) with a multichannel micro pipette set to deliver 50 µl. The resulting dilutions will range from 1:2 in the first well, to

1:2048 in the 11th well; the 12th row, containing only PBS, will serve as a cell control.

4. Add 50 µl 0.5% erythrocyte suspension to each well and shake/agitate the plate to thoroughly mix reactants.

Note: Keep erythrocytes thoroughly suspended during the dispensing process.

5. Cover the plate with microtiter plate sealing tape and incubate at room temperature until a distinct button has formed in the cell control well (usually takes 20 to 30 minutes).
6. Record results as follows: Wells with complete hemagglutination are recorded as "+" (positive HA); wells with a distinct button formation are recorded as "-" (negative HA); wells with partial button formation (fuzzy margins, or donut-like appearance) are recorded as "I" (incomplete HA). When interpretation between complete and incomplete inhibition is doubtful tilt the microtiter plate at about a 45 degree angle for 20-30 seconds and look for a "tear drop" appearance of erythrocytes in the wells with complete inhibition; wells with partial inhibition will not "tear drop".
7. The endpoint of the titration is the highest dilution of antigen causing complete hemagglutination. The endpoint dilution is considered 1 HA unit (HAU); 8 HA units in 50 µl (4 HAU in 25 µl) are used in the HI test. The dilution containing 8 HA units is determined by dividing the endpoint by 8 (the desired number of HAUs), e.g. if the HA endpoint titer was 1:256, the dilution which would contain 8 HA units in 50 µl would be 1:32 (256 ÷ 8).
8. Make appropriate quantities of 8-HAU antigen by diluting the antigen with 0.4% BSA-PBS.

- **Hemagglutination-Inhibition (HI) test procedure**

Note: The procedure described here is to test serum/plasma/yolk extract samples against each of the 16 H subtypes of influenza A virus. To conserve time and resources, three (3) serial twofold dilutions (1:8, 1:16, and 1:32) of each sample are tested against each antigen. Variations of this format can be used if, for example, endpoints are needed.

1. Mark a microtiter plate(s) as in Figure 4-9, or in another manner to test each sample. Include 1 additional row for each subtype to serve as a positive serum control. Additional plates are labeled in a similar fashion for the remaining H subtypes. A serum control (25 µl test serum + 25 µl PBS) for each serum and a erythrocyte control (50 µl PBS) should also be included.

Note: Only 1 positive control per subtype is needed per test.

2. Dispense 25 µl standardized H antigen (8 HAU/50 µl) into the corresponding series of 3 wells for each H subtype as shown in Figure 4-9. In addition, a back titration (HA performed with the antigen dilution used in the test) is conducted for all H subtype antigens used to assure that correct HAUs are present. Back titrations are performed as described in Hemagglutination (HA) section except that 6 well dilutions are used instead of 11.

Note: Special attention should be given to the selection of antigens used in the HI test to avoid homologous neuraminidase with the test samples. False-positive reactions, caused by steric inhibition (inhibition caused by the interaction of homologous neuraminidase antigen and antibodies) may result when the test sample contains the same neuraminidase subtype as the antigen.

3. Add 25 µl of erythrocyte-treated serum(s) with a multichannel micro pipette to the first well.
4. Serially-dilute the serum(s) beginning with the first well (1:8 serum dilution), through the 3rd well (1:32 serum dilution) with a multichannel pipette set to deliver 25 µl.

   Note: Serial dilutions for each H subtype series should be performed as soon as possible after addition of erythrocyte-treated samples to antigen.
5. Repeat steps 4.2.3, and 4.2.4 for each H subtype.
6. Cover plate(s) and incubate for 30 min at room temperature.
7. Add 50 µl 0.5% erythrocyte suspension to each well and shake/agitate the plate to thoroughly mix.

   Note: Keep erythrocytes thoroughly suspended during the dispensing process.
8. Cover the plate with microtiter plate sealing tape and incubate at room temperature until a distinct button has formed in the positive control well (usually takes 20 to 30 minutes).
9. Record results: Wells with complete hemagglutination are recorded as "+" (positive HA); wells with a distinct button formation are recorded as "-" (negative HA); wells with partial button formation (fuzzy margins, or donut-like appearance) are recorded as "I" (incomplete HA). When interpretation between complete and incomplete inhibition is doubtful, tilt the microtiter plate at about a 45 degree angle for 20- 30 sec and look for "tear drop" appearance of erythrocytes in the wells with complete inhibition; wells with partial inhibition will not form "tear drop."

   Note: wells with complete inhibition should form a "tear drop" at the same rate as the positive control wells. Wells with complete or incomplete inhibition that show a delayed "tear drop" as compared to the positive control should not be interpreted as inhibition.

❖ INTERPRETATION OF THE TEST RESULTS

- Serum/plasma/yolk extract samples are considered positive (indication of exposure) if inhibition of hemagglutination is observed at the 1:8 dilution or higher. Endpoints are reported as the highest serum dilution causing complete inhibition of hemagglutination.
- A test is considered valid if (1) the correct number of HAUs (8/50 µl) for each H antigen subtype is present, as determined by the back titration, (2) the serum/plasma/yolk extract sample inhibiting hemagglutination has a different neuraminidase subtype than the antigen used in the test, (3) the serum control (serum + PBS) does not show hemagglutination, and (4) expected HI titer is observed with homologous antigen and antiserum. If these conditions are not met the test should be repeated.
- If erythrocytes in the cell control wells do not settle into a well-defined button, check the following as possible causes:
  - PBS, incorrect formulation
  - Excessive evaporation from plates during the test
  - Erythrocytes to old
  - Incorrect concentration of erythrocytes

❖ REPORT OF THE TEST RESULTS

Record results, in ink, on the HI Test Worksheet, and transfer endpoint results to the Avian Influenza Serology Summary Worksheet. When all subtyping is completed, give case report (APHIS form 10-4 or 8004) and Summary Worksheet to Head of Avian Viruses Section for reporting, and place a copy in the Case Summary Workbook.

REFERENCES

REFERENCES

Alexander, D.J. (1982). Ecological aspects of influenza viruses in animals and their relationship to human influenza: a review. *Journal of Royal Society of Medicine, 75*, 799-811.

Alexander, D.J. (2000, May 22). A review of avian influenza in different bird species. *Veterinary Microbiology: An International Journal, 74(1-2),* 3-13.

Alexander, D.J. (2007, July 26). An overview of the epidemiology of avian influenza. *Vaccine Journal, 25(30),* 5637-44.

Amonsin, A., Choatrakol, C., Lapkuntod, J., Tantilertcharoen, R., Thanawongnuwech, R., Suradhat, S., et al. (2008, November): Influenza virus (H5N1) in live bird markets and food markets, Thailand. *Emerging Infectious Diseases Journal, 14(11),* 1739-42.

Beach, R.H., Poulos, C., & Pattanayak, S.K. (2007). Agricultural household response to avian influenza prevention and control policies. Presented at the Annual Meetings of the Southern Agricultural Economics Association, February 3-6, 2007, Mobile, Alabama, 2007

Baigent, S.J. & McCauley, J.W. (2003). Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. *BioEssays Journal, 25,* 657-71.

Becker, W.B. (1966). The isolation and classification of tern virus: influenza virus A/tern/South Africa/1961. *Journal of Hygiene, 64,* 309–320.

Brown, J.D., Luttrell, M.P., Berghaus, R.D., Kistler, W., Kleeler, S.P., Howey, A., et al. (2010). Prevalence of antibodies to type A influenza virus in wild avian species using two serologic assays. *Journal or Wildlife Diseases, 46(3),* 896-911.

Butler, D. (2006). Thai dogs carry bird-flu virus, but will they spread it? *Nature Journal, 439,* 773.

Causey, D. & Edwards, S.V. (2008). Ecology of avian influenza virus in birds. *Journal of Infectious Diseases, 197,* S29–33

Chen, H., Deng, G., Li, Z., Tian, G., Li, Y., Jiao, P., et al. (2004). The evolution of H5N1 influenza viruses in ducks in southern China. *Proceedings of the National Academy of Sciences of the United States of America, 101,* 10452–7.

Department of Livestock Development (DLD, 2004). Avian influenza situations and control measures: Available from URL: http://www.dld.go.th/home/bird_flu/history_47.html

Department of Livestock Development (DLD, 2006a). Avian Influenza: Daily Report on August 2, 2006. Available from URL: http://www.dld.go.th/home/bird_flu/AICOC/AICOC_06/2_8_06.jpg [Cited on 2006 August 02]

Department of Livestock Development, Bureau of Animal Disease Control, Prevention, and Treatment (DLD, 2006b). *The guideline for Avian Influenza control* [Thai version]. Bangkok: Agricultural Co-operative Club of Thailand.

Department of Livestock Development (DLD), Thailand. (2007). Avian influenza situations and control measures: Available from URL: http://www.dld.go.th/home/bird_flu/birdflu.html

Department of Livestock Development (DLD, 2007). Avian Influenza: Daily Report on March 15, 2007. Available from URL: http://www.dld.go.th/home/bird_flu/AICOC/AICOC_07/15_3_07.html [Cited on 2007 March 15]

Department of Livestock Development (DLD, 2008). Statistics of Livestock in Thailand 2007. Available from URL: http://www.dld.go.th/ict/yearly/yearly50/stock50.html [Cited on 2008 January 1]

Department of Livestock Development (DLD, 2009a). Avian Influenza Control Center: Daily Situation; January 1, 2009. Available from URL: http://www.dld.go.th/home/bird_flu/birdflu.html [Cited on 2009 January 01]

Department of Livestock Development (DLD, 2009b). Poultry export by destination, 2008. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=65:--2551&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2009c). Statistics of chicken in Thailand, 1994-2003. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=60:--2546&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2009d). Statistics of duck in Thailand, 1994-2003. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=60:--2546&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2009e). Statistics of chicken in Thailand, 1999-2008. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=65:--2551&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2009f). Statistics of duck in Thailand, 1999-2008. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=65:--2551&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 01]

Department of Livestock Development (DLD, 2009g). Number of Poultry in Thailand on 1 January 2007. Available from URL: http://www.dld.go.th/ict/th/index.php?option=com_content&view=article&id=64:-2550&catid=73:2009-11-01-07-42-46 [Cited on 2009 November 1]

Department of Livestock Development (DLD, 2010). Statistics of chicken and duck, classified by region (2010). Available from URL: http://www.dld.go.th/ict/th/images/stories/stat_web/yearly/2553/chickenduck_ket.pdf [Cited on 2010 October 27]

Environmental Systems Research Institute, Inc. (ESRI, 2010). ArcGIS Desktop 10. ESRI Headquarters 380 New York Street, Redlands, CA 92373-8100 USA

Food and Agricultural Organization of the United Nations (FAO, 2004). FAO Recommendations on the prevention, control and eradication of highly pathogenic avian influenza in Asia. *FAO Position Paper, September 2004*. FAO Rome Italy, 59 pages

Food and Agricultural Organization of the United Nations (FAO, 2006). CPF Compartmentalization (Broiler Integration): Boonpeng Santiwattanatam. In: *Report of the Twenty-ninth Session of the Animal Production and Health Commission for Asia and the Pacific (APHCA)*. Bangkok: 2006; 89-91.

Food and Agricultural Organization of the United Nations (FAO, 2010a). FAO Regional Strategy for Highly Pathogenic Avian Influenza and other Emerging Infectious Diseases of Animals in Asia and the Pacific, 2010–2015. Bangkok: 2010; p. 23.

Food and Agricultural Organization of the United Nations (FAO, 2010b). Avian Influenza: continued support is required. Available from URL: http://www.fao.org/avianflu/en/news/hpai_support.html [Cited on 2010 September 17]

Food and Agricultural Organization of the United Nations (FAO, 2010c). Key achievements and lessons learned from the HPAI program. FAO Regional Strategy for Highly Pathogenic Avian Influenza and other Emerging Infectious Diseases of Animals in Asia and the Pacific, 2010–2015. Available from http://www.fao.org/docrep/012/i1474e/i1474e00.htm [Cited on 2010 March]

Food market exchange.com. (2003a). World Broiler Meat Trade Forecast, Outlook and Situation for 2003. Available from URL: http://www.foodmarketexchange.com/datacenter/product/poultry/chicken/detail/dc_pi_pt_chicken0805_01.php [Cited on 2008 May 01]

Food market exchange.com (2003b). Broiler Trade, Leading markets for Thai frozen chicken exports in 2002**.** Available from URL: http://www.foodmarketexchange.com/datacenter/product/poultry/chicken/detail/dc_pi_pt_chicken0403_01.php [Cited on 2004 March 01]

Friend, M., Franson, J.C., & Ciganovich, E.A. (Eds.) (1999). Avian influenza. In *Field Manual of Wildlife Diseases: General Field Procedures and Diseases of Birds* (pp. 181-4). Washington, D.C.: Biological Resources Division, U.S. Geological Survey.

Gauthier-clerc, M., Lebarbenchon, C., & Thomas, F. (2007). Recent expansion of highly pathogenic avian influenza H5N1**:** a critical review. *Ibis: The International Journal of Avian Science, 149 (2),* 202–214.

Gilbert, M., Chaitaweesup, P., Parakamawongsa, S., T., Premashthira, Tiensin, T., Kalpravitdh, W., Wagner, H., et al. (2006, February). Free-grazing ducks and highly pathogenic avian influenza, Thailand. *Emerging Infectious Diseases Journal, 12(2),* 227-34.

Gilbert, M., Xio, X., Chaitaweesup, P., Kalpravitdh, W., Premashthira, S., Boles, S., et al. (2007). Avian influenza, domestic ducks and rice agriculture in Thailand. *Agriculture, Ecosystems & Environment Journal, 119*, 409-15.

Graham, J.P., Leibler, J.H., Price, L.B., Otte, J.M., Pfeiffer, D.U., Tiensin, T., et al. (2008, May-June). The animal-human interface and infectious disease in industrial food animal production: Rethinking biosecurity and biocontainment. *Public Health Reporters Journal, 123(3),* 282-99.

Heeney, J.L. (2006). Zoonotic viral diseases and the frontier of early diagnosis, control and prevention. *Internal Medicine Journal, 260,* 399–408.

Heinen, P. (2003). Swine influenza: a zoonosis. *Veterinary Science for Tomorrow.* Available from URL: http://www.vetscite.org/publish/articles/000041/print.html

Hulse-Post, D.J., Sturm-Ramirez, K.M., Humberd, J., Seiler, P., Govorkova, E.A., Krauss, S., et al. (2005): Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proceedings of the National Academy of Sciences of the United States of America, 102,* 10682–7.

Jacob, J.P., Butcher, G.D., Mather, F.B., & Miles, R.D. (2009). Avian influenza in poultry [Electronic version]. University of Florida, PS38, 1-4. Available from URL: http://doacs.state.fl.us/ai/aiindex.htm

Jeong, O., Kim, M., Kang, H., Ha, G., Oh, G., Yoo, J., et al. (2010). Validation of egg yolk antibody based C-ELISA for avian influenza surveillance in breeder duck. *Journal of Veterinary Microbiology, 144*, 287-92.

Jullabutradee, S. (2005). Limitations in the application of control measures during the first avian influenza outbreaks in Thailand [MSc Thesis]. Chiang Mai University, Chiang Mai, Thailand, 89 pages.

Killian, M.L. (2008). Avian influenza virus sample types, collection, and handling. In E. Spackman (Ed.), *Avian Influenza Virus Methods in Molecular Biology, 436,* (pp. 7-18). Totowa, NJ: Humana Press.

Killian, M.L. (2008): Hemagglutination assay for the avian influenza virus. In E. Spackman (Ed.), *Avian Influenza Virus Methods in Molecular Biology, 436,* (pp. 47 – 52). Totowa, NJ: Humana Press.

Lednicky, J.A., Hamilton, S.B., Tuttle, R.S., Sosna, W.A., Daniels, D.E., & Swayne, D.E. (2010): Ferrets develop fatal influenza after inhaling small particle aerosols of highly pathogenic avian influenza virus A/Vietnam/1203/2004 (H5N1) [Electronic version]. *Virology Journal, 7, 231*, 1-15. Available from URL: http://www.virologyj.com/content/7/1/231

Map of Thailand (2010): Available from URL: http://www.novabizz.com/Map/img/321.jpg

Michigan State University, Diagnostic Center for Population and Animal Health (DCPAH) (2008). The example figure of AGID test plate. Retrieved from Prof. Dr. Roger Maes in April, 2008.

Microsoft Corporation, Microsoft Office Excel (2007). Corporate Headquarters**,** Microsoft Corporation, One Microsoft Way, Redmond, WA 98052-6399 USA

Munster, V.J., Baas, C., Lexmond, P., Waldenstrom, J., Wallensten, A., Fransson, T., et al. (2007, May). Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog 3(5): e61. doi:10.1371/journal.ppat.0030061

NaRanong, V. (2008). Structural changes in Thailand's poultry sector: avian influenza and its aftermath. *TDRI Quarterly Review, 23*, 3-10.

Olsen, B., Munster, V.J., Wallensten, A., Waldenstrom, J., Osterhaus, A.D.M.E., & Fouchier, R.A.M. (2006, April 21). Global pattern of avian influenza A virus in wild birds. *Science, 312 (5772),* 384-8. doi: 10.1126/science.1122438

Pedersen, J.C. (2008): Hemagglutination-inhibition test for avian influenza virus subtype identification and the detection and quantification of serum antibodies to the avian influenza virus. In E. Spackman (Ed.), *Avian Influenza Virus Methods in Molecular Biology, 436,* (pp. 53 – 66). Totowa, NJ: Humana Press.

Roberston, S.I., Bell, D.J., Smith, G.J., Nicholls, J.M., Chan, K.H., Nguyen, D.T., et al. (2006): Avian influenza H5N1 in viverrids: implications for wildlife health and conservation. *Proceedings of Royal Society Biological Sciences 273*, 1729–32. doi:10.1098/rspb.2006.3549

Roth, W.G. (2005). A photo of 10-day-embryonating chicken egg. Available from URL: http://www.science-art.com/image/?id=2051 [Cited on 2008 April 22]

Rushton, J., Viscarra, R., Bleich, E.G., & McLeod, A. (2007): Impact of avian influenza outbreaks in the poultry sectors of five South East Asian countries (Cambodia, Indonesia, Lao PDR, Thailand, Viet Nam) outbreak costs, responses and potential long term control. *TCP/RAS/3010: FAO, Rome, Italy, 10*, 25 pages.

Saito, T., Watanabe, C., Takemae, N., Chaisingh, A., Uchida, Y., Buranathai, C., et al. (2009): Pathogenicity of highly pathogenic avian influenza viruses of H5N1 subtype isolated in Thailand for different poultry species. *Journal of Veterinary Microbiology, 2009 Jan 1, 133(1-2)*, 65-74.

SAS Institute Inc. (2010). SAS® 9.2. SAS Institute Inc. 100 SAS Campus Drive, Cary, NC 27513-2414 USA

Saurez, D.L. (2008). Influenza A virus. In D.E. Swayne (Ed.), *Avian Influenza* (pp. 3-22). Ames, IA: Blackwell.

Suarez, D.L. & Schultz-Cherry, S. (2000). Immunology of avian influenza virus: a review. *Developmental & Comparative Immunology Journal, 2000 Mar-Apr, 24(2-3)*, 269-83.

Simmerman, J.M., Thawatsupha, P., Kingnate, D., Fukada, K., Chaising, A., and Dowell, S.F. (2004): Influenza in Thailand: a case study for middle income countries. *Vaccine Journal, 23 (2004)*, 182–7.

Songserm ,T., Jam-on, R., Sae-Heng, N., Meemak, N., Hulse-Post, D.J., Sturm-Ramirez, K.M., et al. (2006a, April). Domestic ducks and H5N1 influenza epidemic, Thailand. *Emerging Infectious Diseases Journal, 12(4)*, 575-81.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Meemak, N., Pariyothorn, N., et al. (2006b, April). Avian influenza H5N1 in naturally infected domestic cat. *Emerging Infectious Diseases Journal. 12 (4)*, 681-3.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Pariyothorn, N., Payungporn, S., et al. (2006c, November). Fatal avian influenza A H5N1 in a dog. *Emerging Infectious Diseases Journal. 12 (11)*, 1744-7.

Spackman, E. & Suarez, D.L. (2008). Detection and identification of the H5 hemagglutinin subtype by real-time PCR. In: E. Spackman (Ed.), *Methods in Molecular Biology, Avian Influenza Virus* (pp. 27 – 33). Totowa, NJ: Humana Press.

Stallknecht, D.E. & Shane, S.M. (1988). Host range of avian influenza virus in free-living birds. *Veterinary Research Communications, 12*, 125-141.

Subbarao, K. & Joseph, T. (2007, April). Scientific barriers to developing vaccines against avian influenza viruses. *Nature Reviews Immunology 7*, 267-78. doi:10.1038/nri2054

Sullivan, H.J., Blitvich, B.J., VanDalen K., & Bentler, K.T. (2009). Evaluation of an epitope-blocking enzyme-linked immunosorbent assay for the detection of antibodies to influenza A virus in domestic and wild avian and mammalian species. *Journal of Virological Methods, 161*, 141-6.

Swayne, D.E. & Suarez, D.L., (2000). Highly pathogenic avian influenza. *Revue scientifique et technique, 19*, 463-82.

Swayne, D.E. & Halvorson, D.A. (2008). Influenza. In Y.M. Saif (Ed.), *Disease of poultry* (12th ed.) (pp. 153-84). Ames, IA: Blackwell.

Szretter, K.J. Balish, A.L., & Katz, J.M. (2006). Influenza: Propagation, Quantification, and Storage. *Current Protocols in Microbiology*. DOI: 10.1002/0471729256.mc15g01s3 [Cited in 2006 December]

Thanapongtham, W., Hongchumpol, N., & Phungjiem, P. (2007). The outbreaks of avian influenza in Thailand, 2004-2006 [Thai version]. *The Department of Livestock Development, Thailand*. Available from URL: www.dld.go.th/home/bird_flu/16-10-49KAN/file/3-1-50.doc

Thanawongnuwech, R., Amonsin, A., Tantilertcharoen, R., Damrongwatanapokin, S., Theamboonlers, A., Payungporn, S., et al. (2005). Probable tiger-to-tiger transmission of avian influenza H5N1. *Emerging Infectious Diseases Journal, 11,* 699-701.

Thailand.com. Overview of the Northeastern Region Available from URL: http://www.thailand.com/travel/overview/overview_northeast.htm [Cited on 2010 November 28]

Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., et. al. (2005, November). Highly pathogenic avian influenza H5N1, Thailand, 2004. *Emerging Infectious Diseases Journal, 11(11)*, 1664-72.

Tiensin, T., Nielen, M., Songserm, T., Kalpravidh, W., Chaitaweesub, P., Amonsin, A., et al. (2007). Geographic and temporal distribution of highly pathogenic avian influenza A virus (H5N1) in Thailand, 2004-2005: an overview. *Avian Diseases Journal, 51*, 182-8.

Toma, B., Vaillancourt, J.P., Dufour, B., Eloit, M., Moutou, F., March, W., et al. (Eds) (1999). *Dictionary of Veterinary Epidemiology*. Ames, IA: Iowa State Press, 284 pages.

University of Manibota, Department of Microbiology (2008). Hemagglutination test. Available from URL: http://www.umanitoba.ca/science/microbiology/staff/cameron/60_401.htm [Cited on 2008 April 23]

United States Department of Agriculture Foreign Agricultural Service (USDA, 2004). World Poultry Trade Overview - October 2004. Available from URL: http://www.thepoultrysite.com/articles/250/world-poultry-trade-overview-october-2004 [Cited on 2004 October 25]

United States Department of Agriculture, National Veterinary Services Laboratories Testing Protocol (USDA, 2005 June 1). *Hemagglutination-Inhibitionn Test for Subtype Identification of Influenza A Virus Antibody* (pp 1-17). Ames, IA: USDA Animal and Plant Health Inspection Service.

University of Edinburgh, Computer-aided Learning in Veterinary Education (2007). Win Episcope version 2.0. Available from URL: http://www.clive.ed.ac.uk/cliveCatalogueItem.asp?id=B6BC9009-C10F-4393-A22D-48F436516AC4

United States Department of Agriculture Foreign Agricultural Service (USDA, 2010). Thailand: Production, Supply and Demand for Broiler Meat In: *Thailand Poultry and Product; 2010 Annual, September 1, 2010*, 8 pages.

United States Department of Agriculture, National Veterinary Services Laboratories Testing Protocol (USDA, APHIS, 2005 June 1). *Hemagglutination-Inhibitionn Test for Subtype Identification of Influenza A Virus Antibody* (pp 1-17). Ames, IA: USDA Animal and Plant Health Inspection Service.

Woolcock, P.R. (2008): Avian influenza virus isolation and propagation in chicken eggs. In E. Spackman (Ed.), *Avian Influenza Virus (Methods in Molecular Biology, 436,* (pp. 35-46). Totowa, NJ: Humana Press.

World Health Organization (WHO) (2006). Avian influenza ("bird flu"). *WHO | Avian Influenza Fact sheet*. Available from URL: http://www.who.int/mediacentre/factsheets/avian_influenza/en/index.html

World Health Organization (WHO, 2007), Regional Office for South-East Asia. Role of village health volunteers in Avian Influenza surveillance in Thailand. New Delhi: 2007; 27 pages.

World Health Organization (WHO) (2008): Update on avian influenza A (H5N1) virus infection in humans. In: *The New England Journal of Medicine, 358,* 261-73.

World Health Organization (WHO) (2010a). Cumulative number of confirmed human cases of avian influenza A/(H5N1) Reported to WHO: 29 December 2010. *WHO | Avian Influenza Archives*. Available from URL: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_12_29/en/index.html

World Health Organization (WHO) (2010b). Areas with confirmed human cases of H5N1 Avian Influenza since 2003. *WHO | Avian Influenza Maps*. Available from URL: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_H5N1inHumanCUMULATIVE_FIMS_20100831.png

World Health Organization (WHO) (2010c). Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO. Available from URL: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_11_19/en/index.html [Cited on 2010 November 19]

World Health Organization (WHO) (2011). Situation up date archives. Available from URL: http://www.who.int/csr/don/2011_02_09/en/index.html [Cited on 2011 February 09]

World Organization for Animal Health (OIE, 2004). Avian Influenza: Disease information. Available from URL: http://www.oie.int/downld/AVIAN%20INFLUENZA/A2004_AI.php [Cited on 2004 January 23]

Word Organization for Animal Health (OIE). 2005. Chapter 2.7.12. Avian Influenza. Manual of Diagnostic Tests and Vaccines for Terrestrial Animal. Paris, France. Available from URL: http://www.oie.int/eng/normes/mmanual/A_00037.htm

World Organization for Animal Health (OIE, 2006). Avian Influenza: Disease information. Available from URL: http://www.oie.int/downld/avian%20influenza/A2006_AI.php [Cited on 2006 November 07]

World Organization for Animal Health (OIE) (2009 October).Highly pathogenic avian influenza: Aetiology; Epidemiology; Diagnosis; Prevention; and Control References. Available from URL: http://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/AVIAN_INFLUENZA_FINAL.pdf

World Organization for Animal Health (OIE) (2010a). 63 countries report H5N1 avian influenza in domestic poultry/wildlife, 2003-2010. *Avian Influenza*. Available from URL: http://www.oie.int/eng/info_ev/en_AI_factoids_2.htm[Cited on 2010 December 09]

World Organization for Animal Health (OIE, 2010b). Update on highly pathogenic avian influenza in animals (Type H5 and H7) 2007 and 2008. Available from URL: http://www.oie.int/downld/AVIAN%20INFLUENZA/A2008_AI.php [Cited on 2010 December 09]

World Organization for Animal Health (OIE) (2010c). Avian Influenza. OIE, Paris. Available from URL: http://www.fao.org/avianflu/en/clinical.html

World Organization for Animal Health (OIE) (2010d). - Terrestrial Animal Health Code: Chapter 10.4. Avian Influenza. OIE, Paris. Available from URL: http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_1.10.4.htm

World Organization for Animal Health (OIE, 2010e). Update on Highly Pathogenic Avian Influenza in Animals (Type H5 and H7) 2009 and 2010. Available from URL: http://www.oie.int/downld/AVIAN%20INFLUENZA/A2010_AI.php [Cited on 2010 December 09]

World Organization for Animal Health (OIE) (2011a). Avian Influenza-Disease Outbreak Map, 2005-2010. OIE, Paris.
Available from URL: http://www.oie.int/wahis/public.php?page=disease_outbreak_map

World Organization for Animal Health (OIE) (2011b). Update on highly pathogenic avian influenza in animals (Type H5 and H7). Available from URL: http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/2011/ [Cited on 2011 February 12]

Zhou, E.M., Chan, M., Heckert, R.A., Riva, J., & Cantin, M.F. (2003; Proceeding). A competitive Enzyme-linked Immunosorbent Assay for avian influenza serological surveillance. *Journal of Avian Diseases, 47 (Special Issue)*, 305-12.