Epidemiology study and risk assessments of highly pathogenic avian influenza H5N1 in free flying birds in Thailand

by

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I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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Abstract

The highly pathogenic avian influenza virus H5N1 was the cause of a pandemic of avian influenza in poultry throughout many parts of the world. The role of wild birds in the transmission and cycling of this virus has been uncertain and the current study was designed to collect further data on the role of wild birds in the transmission of H5N1 in Thailand. The study site for the current study was located in Nakorn Pathom province, the central part of Thailand, where both backyard poultry and low biosecurity poultry farms are common and co-exist. The analysis of existing extensive data from the national wild bird surveillance program for HPAI H5N1 virus in Thailand, found that since 2004 the prevalence of infection with H5N1 in wild birds was low (1.0% 95%CI (0.7, 1.2). However, the annual prevalence varied considerably over this period with a peak of 2.7% (95%CI 1.4, 4.1) in 2004, which dropped to 0.5% (95%CI 0.3, 0.8) and 0.6% (95%CI 0.3, 1.0) in 2005 and 2006, respectively, and then rose again to 1.8% (95%CI 1.0, 2.6) in 2007. During this period, sixteen species of wild birds tested positive for H5N1 virus infection. All samples from juvenile birds were negative for H5N1 virus, whereas the virus prevalence in pooled samples from adult birds was 0.6% (95%CI 0.4, 0.9). The positive birds belonged to twelve species which were mainly resident species that are commensal with human activities. Infected wild bird samples were only found in provinces where poultry outbreaks had occurred. A risk factor study conducted in this project using a questionnaire for villagers on farm practices and wild birds observed in the area revealed that factors associated with disease included replacing poultry individually into households/farms, buying native chickens and/or fighting cocks from commercial hatcheries and the presence of lesser whistling ducks (*Dendrocygna javanica*) on farms. Selecting healthy poultry when purchasing replacement birds was identified as a protective factor in this study.

The longitudinal wild bird surveillance programs conducted in this study revealed that the serological and virological prevalence of H5N1 virus were low in the wild bird population. The seroprevalence as tested by the H5N1 serum neutralization test (NT) was 2.1% (95% CI 0.7, 3.5). Species that tested positive to NT were rock pigeon (*Columba livia*), Asian pied starling (*Gracupica contra*), spotted dove (*Streptopelia chinensis*), oriental magpie robin (*Copsychus saularis*), blue-tailed bee-eater (*Merops philippinus)*, myna (*Acridotheres spp.*), and pond heron (*Ardeola spp.*). The prevalence of H5N1 virus detection was 0.5% (95% CI 0.0, 1.1); the two H5N1 virus -positive samples were from Asian pied starling (*Gracupica contra*) and white vented myna (*Acridotheres grandis*). Wild birds that tested positive to H5N1 virus were mostly common terrestrial birds, some of which showed no clinical signs of disease. Molecular epidemiology showed that the viruses isolated from the survey were most closely related to poultry viruses isolated in Thailand (A/chicken/Thailand/PC-168/2006, A/chicken/Phichit/NIAH606988 /2006, and A/quail/Thailand /CU-333/06). There was no evidence to support the presence of unique strains in wild birds in Thailand.

A wild bird observational study undertaken demonstrated that habitats which contain the potential for a high risk of interspecies transmission of HPAI H5N1 viruses were open system duck farms and household/backyard areas. In these areas wild birds were commonly observed feeding together and in close contact with domestic poultry and pigs. Common terrestrial birds considered as bridge species (e.g. pigeons, sparrows, mynas, starlings, and doves) were likely to be involved in the disease transmission. Moreover, a qualitative risk assessment conducted in this study showed that the risk of wild birds transmitting the disease to poultry was low with an overall risk ranking of "Medium severity". For quantitative risk assessment conducted, the risk of an infected lesser whistling duck defaecating an infectious dose of HPAI H5N1 virus close to a domestic duck in an open system duck farm was 5.8×10^{-6}. This risk increased to 2.5×10^{-1} when all ducks visiting an open system duck farm were considered in a year.

In conclusion, wild birds can help maintain the virus in wild and domestic bird populations through spill back and spill over. However, risk of wild birds transmitting HPAI H5N1 virus to poultry in the current study was considered to be low. Monitoring of the disease in wild birds and poultry should be performed in Thailand, and the biosecurity of small and backyard poultry farms should be improved.

Publications

Jarunee Siengsanan, Rattapan Pattanarangsan, Witthawat Wiriyarat, Kridsada Chaichoen, Sarin Suwanpukdee, Parntep Ratanakorn. Surveillance of Avian Influenza H5N1 in wild birds in Thailand. Wildlife Disease Association-Australasia 2007 conference; 22-28 September 2007 at Dryandra woodland, Western Australia.

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Chapter 1

INTRODUCTION AND BACKGROUND

1.1 Introduction

The panzootic of highly pathogenic H5N1 avian influenza that commenced in poultry in South East and East Asia in late 2003 caused exceptionally high mortality in waterbirds. In mid 2005 a variant of the virus appeared in Qinghai Lake in north-west China and killed large numbers of wild water birds and then spread rapidly over long distances causing outbreaks in Kazakhstan, Siberia, Tibet and Mongolia (2005). Subsequently infection in wild birds and/or poultry was detected in parts of Europe (Burgos and Burgos, 2008), the Middle East, Africa and South Asia over a relatively short time period (Chen et al., 2005; Ellis et al., 2004; Olsen et al., 2006; Pothieng and Jamjomroon, 2006). Some experts believe that HPAI H5N1 virus spread simply by movement of domestic poultry and contamination of fomites, however wild birds, especially migratory wild birds, may have carried the disease over long distances (Normile 2005). The virus also can be transmitted via a contaminated environment (Stallknecht et al., 1990), especially water sources (Hinshaw and Webster, 1982). Places where birds congregate together such as species-preferred stopover sites may be important for the natural transmission of the virus between bird species. These findings led to the formulation of the hypothesis that the virus was being seeded into new habitats by migratory birds and then by interaction with local and nomadic water birds was being spread to farm and village poultry by direct or indirect means.

Currently there is limited information on which species of wild birds have the potential to be persistently infected with H5N1 viruses without causing disease; what potential there is for

H5N1 virus to be transmitted between wild birds and farm or village poultry in endemic areas; and on the interactions between local, nomadic and migratory birds in habitats where H5N1 infections have been detected and whether any species of migratory birds are involved.

An opportunity arose to collaborate with the existing wild bird H5N1 virus surveillance program in Thailand run by the Monitoring and Surveillance Centre for Zoonotic Diseases in Wildlife and Exotic animals (MoZWE) at the Faculty of Veterinary Sciences, Mahidol University (VSMU). Collaboration in terms of epidemiological skills was provided to the team at Mahidol University by colleagues from the School of Veterinary and Biomedical Sciences at Murdoch University to analyse existing wild bird H5N1 surveillance data from Thailand. Gaps in the wild bird surveillance data were identified and targeted surveillance of significant wild bird species was planned and undertaken in Thailand. The goal was to identify wild bird species that have the potential to be significant carriers and transmitters of H5N1 viruses to other wild birds, as well as to farm and village poultry. Additionally, study sites were established to conduct in-depth investigations of transmission pathways of H5N1 between selected infected wild bird species and farm and village poultry that share the same habitat in Thailand.

1.2 Virology

1.2.1 Nature of the virus and its replication

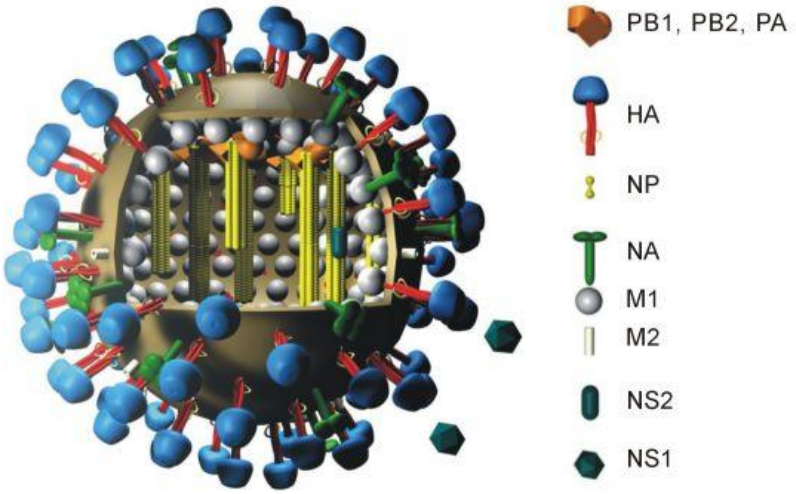
Influenza viruses are negative sense single strand RNA viruses that belong to the genus influenza virus in the family Orthomyxoviridae. The viruses are classified into three types; A, B, and C according to the genetic and antigenic characteristics of their nucleoprotein (NP) and matrix protein (M). Influenza viruses that cause diseases in animals belong to type A,

however disease in humans can be caused by types A, B and C. The type A viruses are classified into subtypes based on their surface glycoprotein antigens including haemagglutinin (HA) and neuraminidase (NA) (Swayne, 2000). The HA is categorised into sixteen subtypes (H1, H2… H16) while NA is categorised into nine subtypes (N1, N2 … N9). In humans type A influenza viruses commonly cause annual seasonal outbreaks of influenza as well as occasional influenza pandemics, type B influenza can cause less frequent seasonal influenza cases and although type C can infect humans it rarely causes serious disease (Stephenson and Zambon, 2002).

The virus particle is spherical in shape with a diameter of 80-120 nm, however sometimes it takes a filamentous or pleomorphic form (Figure 1.1) (Suarez, 2008). The influenza A virion surface consists of a lipid bilayer envelope containing large surface glycoprotein spikes (peplomers) that have HA or NA activities surrounding and closely associated with an inner layer composed of Matrix (M1) proteins which in turn surrounds eight helically symmetrical nucleocapsid segments of different sizes (Potter, 2004). The nucleocapsid segments consist of genome segments associated with an RNA polymerase complex consisting of three polymerase proteins (PA, PB1, PB2) and enclosed within a capsid of helically arranged nucleoprotein (NP) (Padtarakoson, 2006)). The HA and NA are located as spikes which radiate out from the surface of the lipid envelope of the virus and another matrix protein (M2) is arranged as tetramers to form an ion channel which passes through the envelope (Padtarakoson 2006). Haemagglutinin antigen on the surface of the virus particle is a glycoprotein which exists in precursor form that has to be cleaved by proteases into HA1 and HA2 subunits for infection to proceed (Potter 2004). The HA1 is a receptor binding subunit and HA2 has a cell fusion function. The function of NA is as a receptor destroying enzyme to enable release of mature progeny virions from the infected cell. All type A influenza viruses have 8 genome segments that express 10 viral proteins namely PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, and NS2 (Suarez 2008).

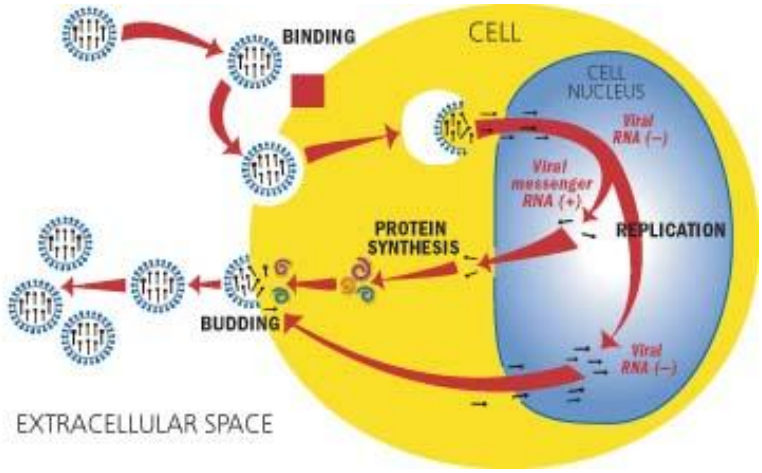
Replication of influenza virus begins with the attachment of the HA spike to specific sialic acid residue receptors located on the surface of the target host cells (Figure1.2; Suarez 2008). The NA can reverse this interaction if the viruses bind with inappropriate host cells (Suarez 2008). After cleavage of the HA by host proteases the virus can fuse with the host cell membrane and be incorporated into an endosome by receptor mediated endocytosis. The endosome fuses with cellular lysosomes, and with the lowered pH in the endosome H+ ions pass via the M2 ion channels into the virion to release the nucleocapsids (Perdue, 2008; Suarez, 2008). The RNA and polymerase complex then migrates into the nucleus of the host cells through pores in the nucleus. Inside the nucleus, transcriptase enzyme transcribes RNAs to positive strand RNAs which can serve as messenger RNAs (mRNA) or can be replicated to negative sense virion RNAs to be incorporated into progeny virions (Potter 2004). The mRNAs move from the nucleus and are translated into viral proteins using ribosomes in the endoplasmic reticulum of the host cell cytosol. The viral envelope proteins HA, NA, and M2 undergo glycosylation in the Golgi apparatus and are then transported and inserted into the host cell membranes. The internal proteins and viral RNA segments are formed into nucleocapsids in the cytosol and associate with the M1 protein in proximity to the cell membrane. Virions are formed by budding of nucleocapsids and M1 protein through cell membrane containing the inserted viral glycoproteins. Release of budded virions from the cell is mediated by the action of the viral neuraminidase (Potter 2004, Suarez 2008).

Figure 1.1; Structure of influenza virus type A



Source: (Eickmann, 2005)

Figure 1.2; Steps of the viral replication in a host cell



Source:http://www.news.cornell.edu/stories/Oct05/avianflu.thevirus.ws.html

1.2.2 Evolution of the avian influenza (H5N1) virus

Influenza viruses have previously caused serious outbreaks of disease in both humans and animals. Evolution of the viruses is driven by both mutation of individual viral genes (antigenic drift) and reassortment of gene segments from different influenza viruses into a new virus (antigenic shift) (Padtarakoson, 2006). Antigenic drift occurs when minor mutations occur due to proof-reading errors in the viral RNA replication process and result in insertion of different amino acids in viral proteins which can alter antigenicity. Antigenic shift involves major gene changes resulting from reassortment of the 8 viral genes from each of two influenza viruses during replication in the same cell and results in the emergence of a genetically different virus from the progenitor viruses. A high mutation rate is an important characteristic of RNA viruses resulting in the emergence of new strains, adaptation to a range of hosts, and development of different forms of pathology/clinical disease. Influenza viruses are believed to have caused pandemics since AD1590 (Potter, 2001). However, the influenza virus was first isolated only in 1932 (Potter 2001). The emergence of H1N1 (Spanish Flu) in 1918 was one of the most widely reported pandemics which spread worldwide resulting in the death of up to 60 million people (Cox and Subbarao, 2000; Johnson and Mueller, 2002). In 1957, the H2N2 (Asian flu) outbreak occurred, followed by H3N2 (Hong Kong Flu) in 1968 and H1N1 (Russian flu) in 1977 (Horimoto and Kawaoka, 2005).

In contrast with human influenza viruses, certain avian influenza viruses have been shown to exist as low pathogenic (LPAI) or high pathogenic (HPAI) biotypes based on their ability to cause severe disease in domestic galliforme birds (OIE, 2005). To date the avian influenza A viruses that have shown the HPAI biotype in domestic poultry are predominantly in the H5 and H7 subtype, although two H10 viruses have been reported (OIE, 2005). Outbreaks of HPAI caused by H5 and H7 avian influenza viruses have been reported sporadically since

1959 but not all H5 and H7 viruses have the HPAI biotype (Swayne and Suarez, 2000). Occasionally zoonotic spread of H5 and H7 HPAI viruses has resulted in human infections and deaths, but there have also been human infections with LPAI viruses such as H9N2 viruses (Webster, 2005). One influenza virus subtype H5 (H5N3) isolated from a disease outbreak in common terns (*Sterna hirundo*) in South Africa in 1961 caused a high level of mortality and was the first report of significant deaths of avian influenza in a wild bird species. (Becker, 1966). More recent outbreaks of disease in galliforme poultry caused by H5 HPAI viruses have included the outbreaks caused by H5N2 in Mexico in 1994 (García et al., 1997), H5N1 in Hong Kong in 1997 (Shortridge 1999) and H5N2 in Italy in 1997-98 (Capua et al., 1999). The Hong Kong H5N1 HPAI outbreak was preceded in 1996 by a disease outbreak in geese in Guangdong province, China caused by A/goose/Guangdong/1/96 (H5N1) virus (GsGd) (WHO 2008; Yee, Carpenter, and Cardona 2009).

In 1997 during the outbreaks of H5N1 HPAI in galliforme poultry in Hong Kong, the virus spread to humans resulting in 18 cases of which 6 died (Shortridge, 1999). Once the zoonotic spread was confirmed the decision was made to depopulate the entire poultry population and more than 1.5 million chickens and other poultry were culled (Auewarakul, 2006; Chan, 2002). Although avian influenza virus subtype H5 is commonly isolated and usually does not cause disease in waterfowl species, strains of H5N1 HPAI viruses isolated since late 2002 have caused severe disease and sudden death in wild waterfowl and other wild bird species (Ellis et al. 2004; Webster 2005). The first evidence of H5N1 infection in wild birds was reported in Hong Kong in 2002 where the virus killed a variety of wild waterfowl (Ellis et al., 2004; Sturm-Ramirez et al., 2004). The H5N1 HPAI virus that evolved and resulted in the massive epizootic from 2003 to the present, not only resulted in fatalities in both wild and domesticated birds, but also caused disease with a high mortality rate in humans and other mammals (Peiris et al., 2007).

All subtypes of avian influenza, including combinations of H1-H16 and N1-N9, have been isolated from avian species (Alexander, 2007; Webster, 1998). Wild waterfowl are considered to be the natural reservoirs as many subtypes of influenza viruses can be isolated from these species without evidence of clinical disease (Webster et al., 1992). However, HPAI viruses are rarely isolated from wild birds and usually emerge by mutation from LPAI after being introduced to domesticated poultry (Alexander, 2000a). Surveillance programs have demonstrated that LPAI viruses can be isolated from up to 15% of ducks and geese and up to 2% of other species of wild birds (Alexander, 2000a). In 1998 a phylogenetic study of nucleoproteins demonstrated that all mammalian influenza viruses were probably derived from an avian influenza reservoir (Webster, 1998). That study also revealed that influenza viruses in some host-specific lineages had evolved from avian influenza viruses and viruses from humans and pigs also showed evidence of evolution from the same origin. Moreover, sub-lineages of avian influenza viruses tend to show limited variation in a geographical region and are considered to be in evolutionary stasis (Webster 1998). The water bird avian influenza (AI) viruses have been separated into two superfamilies; American and Eurasian clades (Schäffr et al., 1993; Webster et al., 2007b). Comparative studies of the frequency and extent of amino acid changes in individual viral proteins have shown that mammalian influenza viruses have a higher evolutionary rate than avian influenza viruses (Webster et al. 2007).

The occurrence of genetic re-assortment in influenza A viruses is generally related to the frequency of mixed infections with these viruses in nature (Horimoto and Kawaoka 2005). Pigs are well known as intermediate hosts serving as mixing vessels for re-assortment of influenza virus as they can be readily infected by both avian and human influenza A viruses (Webster, 1998). However, with the numbers of human H5N1 cases, humans should now also be considered as potential mixing vessels, particularly with the increased chance of co-

infection with human seasonal influenza strains (Yuen and Wong, 2005). It is considered that the 1997 HPAI H5N1 was a triple re-assortment involving viruses from multiple avian species including geese, chickens, ducks, and quail and this virus was transmitted directly from avian species to humans (Wilschut and McElhaney, 2005). So far only rare cases of human to human transmission of HPAI H5N1 have been reported, including a family cluster in Thailand (Ungchusak et al., 2005), an Indonesian family which had seven members infected by HPAI H5N1 with six fatalities, and a Vietnamese nurse who was infected after nursing a patient infected with HPAI H5N1 (Black and Armstrong, 2006).

Data obtained from surveillance of wild birds (Guan et al., 2004) demonstrated that H5N1 was widespread in outbreak regions as seen in Hong Kong and that re-assortment occurred through interspecies transmission which may have involved aquatic and terrestrial wild birds, poultry and indirectly human activity. After introduction into new hosts recent H5N1 HPAI viruses have shown periods of rapid evolution with multiple changes in the amino acid sequences in multiple viral proteins, although the HA and some internal genes of human strains have been relatively conserved. Hiromoto and Kawaoka (2005) noted that six internal genes (PB1, PB2, PA, NP, M proteins, and NS proteins) of human H5N1 viruses showed variability in amino acid substitutions, even though the viruses were isolated in the same year and from the same geographical location. Thus, these amino acid sequences that were specific to human variants may play a role in the disease transmission directly from poultry to humans (Zhou et al., 1999). It must also be considered that mutations may also occur at any time which could result in a human to human transmissible strain developing (Cinatl et al., 2007). Emergence of a new highly pathogenic H5N1 HPAI strain that was capable of human to human transmission would have the potential to cause a very serious pandemic in the human population (Alexander, 2000b).

1.3 Epidemiology of HPAI H5N1 virus

1.3.1 Mode of transmission

Avian influenza is transmitted via the faecal-oral route and this could be via direct contact with infected birds or indirectly via contamination of the environment including water and feed (Garamszegi and Møller, 2007; Webster, 1998). In areas of high poultry density, HPAI viruses can also be transmitted through the nasal and oral routes (Horimoto and Kawaoka 2005). Transmission of avian influenza virus to mammals, especially humans, can occur through direct exposure with infected poultry (Cinatl et al., 2007; Dwyer, 2008). Humans can be infected by HPAI H5N1 directly from sick poultry that excrete viruses in their faeces or through exposure to secretions through handling, slaughtering, preparing, and/or consuming uncooked contaminated products (Peiris et al., 2007). Bridges et al. (2002) revealed that the risk of infection in humans increased in occupations with intensive exposure to poultry such as butchers. Other mammals including tigers (Keawcharoen et al., 2004), a dog (Songserm et al., 2006a, and a cat (Songserm et al., 2006b; Weber et al., 2007) (Songserm et al., 2006a; Weber et al., 2007) have become infected after being fed infected poultry carcasses.

1.3.2 The spread of the disease and its molecular epidemiology

The NA, HA, and internal genes of A/goose/Guangdong/1/96 (Gs/Gd/96; H5N1) virus are believed to be descended from H1N1 virus (A/Duck/Hokkaido/55/96), H5N3-like viruses (A/Swan/Hokkaido/51/96), H3N8 (A/Duck/Nanchang/1681/92) and H7N1 (A/Duck/Nanchang/1904/92) (Mukhtar et al., 2007). The emergence of the HPAI virus subtype H5N1 in Hong Kong in 1997 was caused by a triple reassortant virus with the HA gene being contributed by Gs/Gd/96 virus (Cauthen et al., 2000; Webster et al., 2005; Xu et

al., 1999), internal genes coming from an A/Quail/Hong Kong/G1/97 (H9N2)-like virus (Guan et al., 1999; Guo et al., 2000; Webster et al., 2005) and the NA gene from A/Teal/Hong Kong/W312/97 (H6N1)-like virus (Hoffmann et al., 2000; Webster, 2005). Human and chicken H5N1 viruses found in Hong Kong in 1997 contained an avian-like receptor binding to SA alpha 2,3 Gal-containing receptors only (Matrosovich et al., 1999), which is a specific characteristic of the HAs of avian viruses (Connor et al., 1994).

In Hong Kong since 1997 similar viruses have continued to circulate in the region after the depopulation of poultry at that time (Webster et al., 2005). A phylogenetic study revealed that H5N1 viruses isolated from terrestrial and aquatic birds in Hong Kong in 2000 had HA, NA and some internal genes (like Gs/Gd/96 virus) that were related to other viruses isolated from aquatic birds (Guan et al., 2002). Re-assortment of the Gs/Gd/96-like viruses with other avian viruses resulted in the appearance of multiple genotypes of H5N1 viruses over a short time period (Gaun et al. 2002). The H5N1 viruses isolated from ducks in the southern part of mainland China during 1999-2002 were also closely related to Gs/Gd/96 (Chen et al., 2004). Kou et al. (2005) reported a new genotype of the H5N1 virus (A/Tree sparrow/Henan/1/04 to A/Tree sparrow/Henan/4/04) in tree sparrows (*Passer montanus*) in China in 2004. This virus contained HA and NA genes from Gs/Gd/96-like viruses, nuclear protein genes from the 2001 genotype A H5N1 viruses, and other internal genes from an unknown influenza virus (Kou et al., 2005).

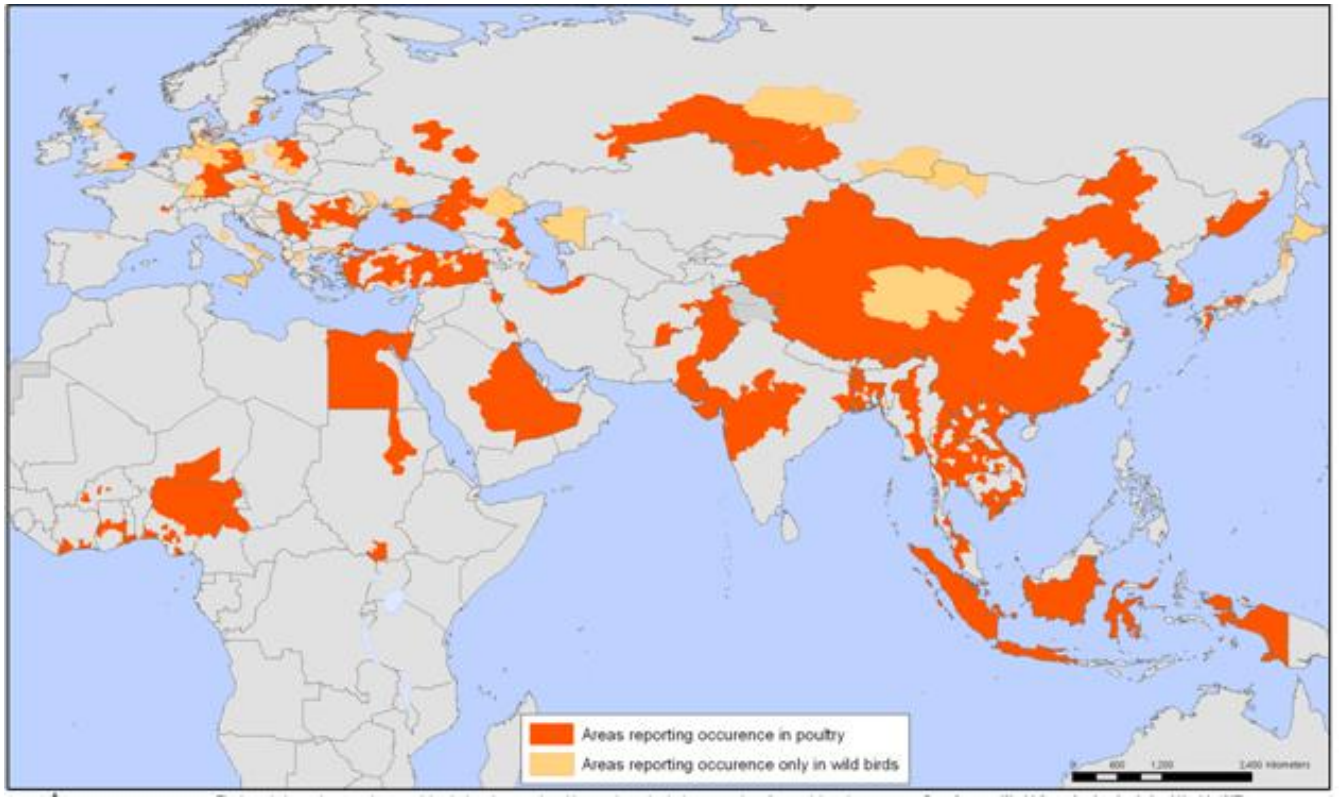
The H5N1 HPAI viruses isolated from live poultry markets in Hong Kong in 2001 were classified into five genotypes (A, B, C, D, and E) (Guan et al., 2002) and from live poultry markets and farms in Hong Kong and mainland China in 2002 into eight genotypes (V, W, X1, X2, X3, Y, Z, and Z+) (Li et al. 2004). In 2003-2004, HPAI H5N1 outbreaks with viruses of the same HA lineage as Gs/Gd/96 virus were again reported across East and South East Asian countries including Cambodia, China, Indonesia, Japan, Laos, Malaysia,

South Korea, Thailand, and Vietnam, and then subsequently from Europe, the Middle East, and Africa (Figure1.3; - (WHO, 2008c)). The HPAI H5N1 viruses isolated from outbreaks in Thailand, Vietnam, Cambodia, Indonesia, and Southern China in 2003 -2004 were of the Z genotype, while viruses from Japan and South Korea in 2004 were of the V genotype (Peiris et al., 2007). In 2004 the genotype Z viruses were further classified into two clades; clade 1 and clade 2.1 based on the closeness of the genetic relationship of their HA genes (Peiris et al., 2007). Later in 2004 and 2005, three main HPAI H5N1 clades were identified: clade 1 included isolates from humans and birds in Vietnam, Thailand, and Cambodia and from birds in Laos and Malaysia; clade 2 included isolates from birds in China, Indonesia, Japan, and South Korea; and clades 1 and 3 both included viruses from birds and humans from Hong Kong (The World Health Organization Global Influenza Program Surveillance network 2005). After the rapid westward spread of Z genotype viruses from the H5N1 HPAI outbreak at Qinghai Lake in China in 2005, further evolution of the HA gene has occurred and H5N1 viruses have now been classified into 9 clades and clade 2 subdivided into a further 10 subclades (OIE-FAO network of expertise on avian influenza (OFFLU; www.offlu.net (accessed 10 January 2008)). Recently, Nguyen et al. (2008) reported there were changes in the geographical distribution of H5N1 isolates found in the Northern (where clade 1 was overtaken by clades 2.3.2 and 2.3.4) and Southern (clade 1) provinces of Vietnam.

A molecular study in Thailand of five human H5N1 isolates and a chicken H5N1 isolate from 2004 (A/Thailand/1(KAN-1)/04, A/Thailand/2(SP-33)/04, A/Thailand/3(SP-83)/04, A/Thailand/4(SP-528)/04, A/Thailand/5(KK-494)/04, and A/Chicken/Thailand /CH-2)/04) reported that the isolates had a cleavage site in the HA gene similar to A/Hong Kong/156/97 virus and genetically were related to genotype Z H5N1 viruses (Puthavathana et al., 2005). A molecular study revealed that an isolate,

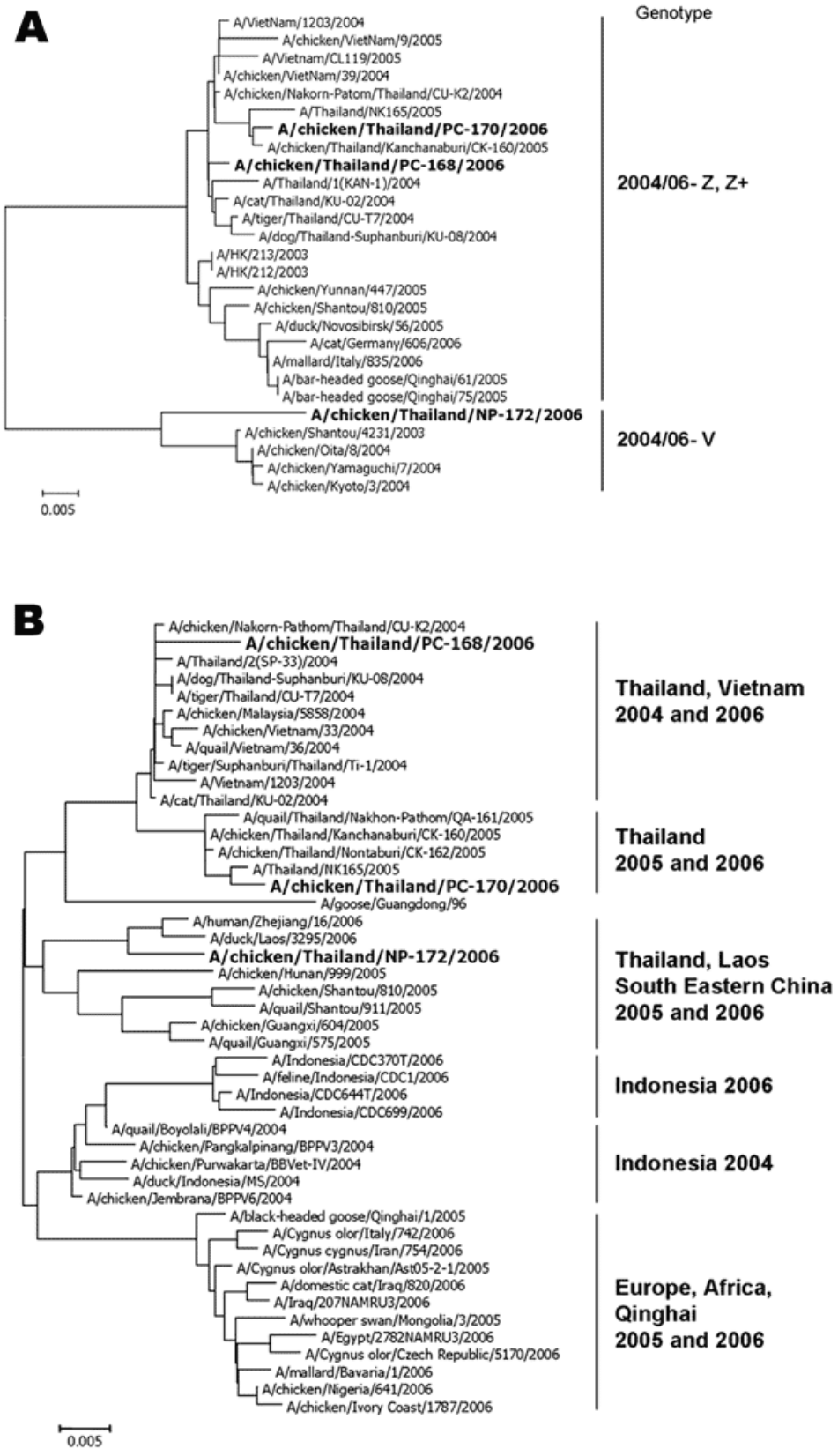
A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04, from poultry showed a high degree of similarity to human isolates during the same epidemic in early 2004 (Viseshakul et al., 2004). Characterization of the Thai H5N1 viruses isolated from a variety of species, including wild birds, cats, and tigers, from 2004 to 2006 showed they were also genotype Z viruses (Buranathai et al., 2006). The Thai viruses were members of the same AI virus lineage and were closely related to influenza A/Duck/China/E319.2/03 (Tiensin et al., 2005; Viseshakul et al., 2004). A study revealed that the Thai isolates of HPAI H5N1 showed only minor changes in their HA, NA, M, NS, and PB2 genes and that there was no evidence of human to human transmission or oseltamivir resistance (Buranathai et al., 2006). However, in 2006 outbreaks of HPAI H5N1 in poultry occurred in Pichit province (Dudley, 2006) and Nakorn Phanom province in Thailand (Marshall, 2006). Isolates from Pichit province were genotype Z while an isolate from Nakorn Pranom province was classified in genotype V (Figure 1.4) (Chutinimitkul et al., 2007).

Figure 1.3; Map showing areas reporting confirmed avian influenza H5N1 cases in poultry and wild birds since 2003



Source : WHO (2008b)

Figure 1.4; Phylogenetic tree of avian influenza H5N1 viruses isolated between 2004 and 2006; A) polymerase acid protein and B) Haemagglutinin gene (HA)



Source: Chutinimitkul et al.(2007)

1.3.2 Host range in wild birds

Over 105 species of wild birds belonging to 26 families, especially wild waterfowls (Anseriformes and Charadriiformes), have been infected with a range of LPAI viruses with various HA/NA combinations (Olsen et al., 2006). Webster (1998) stated that not only are aquatic birds natural reservoirs for avian influenza A viruses particularly LPAI, but their migratory routes match the geographical distribution of the viruses. Wild terrestrial birds may contribute in the interspecies transmission and spread of H5N1 viruses due to their ecology, habitat, and interspecies interactions (Boon et al., 2007). A variety of terrestrial wild birds that have died in Hong Kong have been shown to be infected with HPAI H5N1 (Ellis et al., 2009). Since outbreaks of HPAI H5N1 occurred in many countries in 2004, research has been undertaken into the epidemiology of the disease in an attempt to learn more about the pathways of disease transmission and to help develop better control and prevention plans. Interactions between the host, agent, and environment are important aspects of the epidemiology of wild bird avian influenza (Stallknecht and Brown, 2007). The susceptibility to HPAI H5N1 infection varies in different species of wild birds (Brown et al., 2008). Once infection enters into wild bird populations, these birds may play a role in the ecology and epidemiology of the virus and can be involved in the introduction of the virus into other populations and its subsequent secondary spread (Cattoli and Capua, 2007).

1.3.3 Persistence of the virus in the environment

Persistence of H5N1 viruses in the environment, especially in water bodies, has been investigated in a range of studies. Studies by Brown et al. (2007) demonstrated that two Asian HPAI H5N1 viruses persisted in water for moderate periods of time. The viruses in their trial persisted in water with salinities of 0, 15, and 30 ppt (parts per thousand) at 17°C for up to 26, 30, and 19 days respectively and at 28°C for up to 5, 5, and 3 days respectively (Brown et

al. 2007). Influenza viruses can remain infectious in lake water for up to 30 days at 0°C and 4 days at 22°C (Fouchier et al., 2007; Olsen et al., 2006). It has been demonstrated that some LPAI viruses can remain infective in water for up to 102 and 207 days at 28°C and 17°C, respectively (Stallknecht et al., 1990). In Thailand, a study on the persistence of H5N1 by Songserm et al. (2005) revealed that the virus in chicken faeces was killed within 30 minutes of being placed in sunlight at 32-35°C. However, the virus could survive in chicken faeces for up to 4 days in the shade at a temperature of 25-32°C, as well as in paddy fields for up to 3 days.

1.3.4 The role of wild birds in the persistence or transmission of H5N1

There is a lack of scientific data on the role played by wild birds in the persistence or transmission of H5N1 in infected regions. This partly relates to the limited knowledge on the ecological and behavioural pattern of both terrestrial and aquatic wild birds in much of the infected regions so that the epidemiology and transmission remains unclear in wild bird species (YasuÉ et al., 2006). Further studies on the ecology and behaviour of wild birds, including interspecies interactions, are needed to fill the missing gaps in the understanding of the transmission of the virus and will be a focus of this thesis.

1.4 Clinical Findings and Pathology of H5N1 HPAI disease

The pathology associated with infection with HPAI H5N1 in animals appears to depend upon the host and the infecting virus strains. In chickens and other galliforme poultry, HPAI viruses replicate widely in endothelial cells throughout the body resulting in oedema and cyanosis of the head and comb, haemorrhages of the feet, leg shanks and visceral organs, and lesions of multiple organ failure resulting necrosis of the endothelium of blood vessels in heart muscle, brain, adrenal gland and pancreas (Swayne, 2000). Historically, HPAI viruses

caused no clinical signs and limited pathology in domestic ducks but recently some H5N1 viruses have induced severe HPAI in domestic ducks (Webster et al., 2007a). In wild birds, LPAI viruses, which normally cause no disease, preferentially replicate in the intestine and are then shed in the faeces of infected birds (Fouchier et al., 2007; Webster et al., 1978). Infection with avian influenza viruses appears to be species and age susceptible (Pantin-Jackwood et al., 2007; Stallknecht and Shane, 1988). Captive birds (including greater flamingo (*Phoenicopterus ruber*), little egret (*Egretta garzetta*), rosybill pochard (*Netta peposaca*), red-crested pochard (*Netta rufina*), coscoroba swan (*Coscoroba coscoroba*), chestnut breasted teal (*Anas castanea*), white faced whistling duck (*Dendrocygna viduata*), Hawaiian Goose (*Nesochen sandvicensi*)) and wild birds including grey heron (*Ardea cinerea*) and black headed gull (*Larus ridibundus*)) have died as a result of infection with HPAI H5N1 and have shown gross pathological signs of lung oedema and/or congestion and on histopathology there has been evidence of necrosis in multiple organs (Ellis et al. 2004).

From reports on the initial human cases, H5N1 infected patients showed high fever, cough, shortness of breath, diarrhoea, and pneumonia (Beigel et al., 2005; Chotpitayasunondh et al., 2005; Tran et al., 2004). Subsequently, some human patients develop an Acute Respiratory Distress Syndrome (ARDS) and renal failure (Peiris et al., 2007; Subbarao et al., 1998; Yuen et al., 1998). Pigs experimentally infected with HPAI H5N1 viruses from Thailand and Vietnam developed mild clinical signs, but there was no evidence of transmission to in-contact pigs (Choi et al., 2005). Reports in Thailand of infection in domestic dogs and cats after the consumption of H5N1 infected chicken carcasses indicate that they undergo systemic infection and die shortly after infection. They display clinical signs of high fever, panting, and depression, and there is evidence of multiple organ inflammation and necrosis post mortem (Songserm et al., 2006a; Songserm et al., 2006b). Keawcharoen *et al.* (2004) reported that infected tigers and leopards in a Thai zoo displayed respiratory and neurological signs prior to death. Ferrets challenged with H5N1 virus developed clinical

signs including high fever, anorexia, diarrhoea and neurological signs followed by death associated with histopathological changes of the brain, lung, and liver including necrosis, degeneration, and/or inflammatory cell infiltrates (Govorkova et al., 2005).

Brown, Stallknecht, and Swayne (2008) reported that in their experimental studies, swans including black swan (*Cygnus atratus*), trumpeter swan (*Cygnus buccinator*), whooper swan (*Cygnus cygnus*), and mute swan (*Cygnus olor*)) were more susceptible to infection with HPAI H5N1 than were geese (cacking goose (*Branta hutchinsii*) and bar-headed geese (*Anser indicus*)). Their study also revealed that all of these swans and geese developed clinical signs including listlessness and neurological dysfunction consisting of seizures with multiple organ necroses and inflammation on microscopy. However some geese (1 out of 4 cacking geese and 3 out of 5 bar-headed geese) recovered from their milder clinical signs and had no evidence of neurological dysfunction. Previous experiments have revealed that some species of wild birds are more susceptible to HPAI H5N1 than are others. Mute and whooper swans were highly susceptible to natural infection with HPAI H5N1 during an outbreak in Germany (Teifke et al., 2007). Another study indicated that mute swans were highly susceptible to HPAI H5N1 viruses (Kalthoff et al., 2008a). In that experiment, immunologically naïve mute swans were inoculated with HPAI H5N1. Most showed inconspicuous clinical signs, however some deaths occurred. There was viral shedding for up to six days and gross pathological lesions included the presence of widespread haemorrhages.

1.5 Impacts of HPAI outbreaks

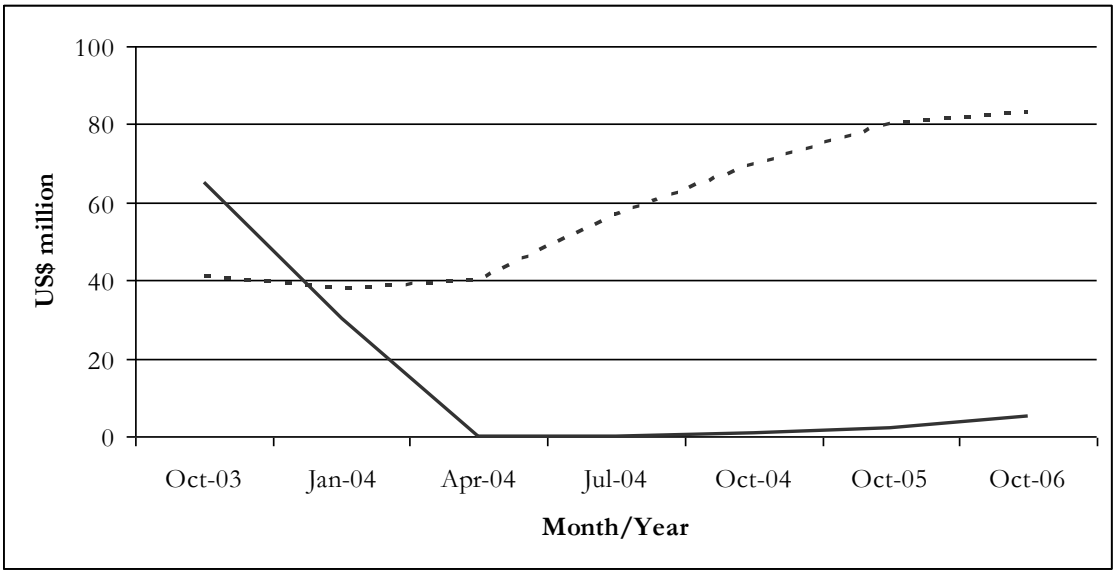
Impacts of outbreaks of influenza in humans can involve both social and economic aspects. Social impacts generally include illness and death. Each year, an estimated 3 to 5 million people suffer from influenza (Wilschut and McElhaney, 2005). The morbidity rate for symptomatic infection is 5-20% and deaths are estimated to be up to half a million worldwide (Black and Armstrong, 2006). The economic impact can be calculated in terms of direct and indirect effects. Direct impacts would be the cost of medication and/or hospitalisation for symptomatic infections. This can be even more severe in patients infected with highly virulent influenza viruses like HPAI H5N1 with signs including high fever, lower respiratory symptoms, diarrhoea, vomiting, abdominal pain, pleuritis, pain, and occasionally bleeding from the nose and gums. Mortality rates in this group have been up to 89% for patients younger than 15 years of age (Beigel et al., 2005) and these patients require hospitalisation with intensive medical care. The major indirect impact of human influenza infections is widespread disruption to the workforce and reduction of work productivity (Dile, 1999). As well, economic loss can include a fall in tourism in affected countries and loss of business confidence due to the fear of a human pandemic (Elçi, 2006).

The cost of containing HPAI outbreaks can have a major impact on agricultural industries in both direct and indirect costs including disruption and loss of food resources. In order to control avian influenza in Asia, many Asian countries culled millions of chickens (Karesh et al., 2005) at a cost of at least US$10 billion (Melville and Shortridge, 2006). This directly affected the trade and economy of these countries. Karesh et al. (2005) stated that outbreaks of avian influenza in the future may create an impact on global food supply. With the massive costs involved in control and prevention programs such as quarantine, depopulation, vaccination and even use of therapeutic treatment for humans (such as antiviral drugs), it is

essential that their cost-effectiveness is evaluated. For example, Meltzer, Cox, and Fukuda (1999) suggested that vaccination programs for disease control and prevention are cost-effective for livestock industries. However, Oshitani (2006) argued that vaccination programs and the use of antiviral drugs in humans was feasible or sufficient to control a severe global pandemic of influenza. In contrast, Jennings and Peiris (2006) suggested that in the face of a pandemic of influenza the use of vaccination and antiviral drugs and non-therapeutic public health measures could reduce the impact of the disease. If a global influenza pandemic occurs it is predicted that the reproductive rate (the average number of infections an infectious individual can generate in a fully susceptible population) will be as high as 1.9 but with current resources only 20% of the world population will be readily treatable with antiviral drugs and 30-50% of the world population will be infected but not treated (Colizza et al. (2007). With limited resources available it will be essential to apply risk assessment techniques in order to develop cost-effective control and prevention policies for this disease.

The poultry industry is one of the most important industries in Thailand. It is estimated to generate approximately 90% of Thailand's export livestock income (Rushton et al., 2005). Thailand produces 800 million chickens per year and employs more than 400,000 workers within the industry (Simmerman et al., 2004). Outbreaks of HPAI H5N1 in Thailand during 2004 to 2005 resulted in 25.9 million birds being culled to control outbreaks (Simmerman et al., 2004). A report by Burgos and Burgos (2007) showed how the Thai poultry exporting trade was affected by HPAI H5N1 outbreaks (Figure 1.4).

Figure 1.4; Effect of HPAI H5N1 on Thai poultry exporting trade



Source: Burgos and Burgos (2007)

Solid line = export value of uncooked poultry, dashed line = export value of cooked poultry

1.6 Outbreaks of HPAI H5N1 in Thailand

In late 2003, large scale die-offs of all poultry types in Central and Northern parts of Thailand were reported (Tiensin et al., 2005). In early January 2004, the first HPAI H5N1 human case was reported in Thailand (Chotpitayasunondh et al., 2005). There were a total of 25 human cases with 17 deaths in Thailand during the first outbreak in 2004 (WHO, 2008a). Human surveillance for AIV was applied to monitor the disease situation and prevent possible human to human transmission (Pawitan, 2006). In mid January 2004, poultry surveillance was conducted for the first time. The first officially confirmed HPAI H5N1 case occurred in a layer chicken farm located in Supanburi province, central Thailand, and was reported by the National Institute of Animal Health (NIAH) on the January 23rd 2004 (OIE, 2004c).

During 2004 to 2006 five H5N1 HPAI epidemics were reported by the Department of Livestock Development (DLD), Royal Thai Government; see Figure 1.5 (Thanapongtharm

and Noimoh, 2006). At this time the policy adopted to control the disease included stamping out, quarantine, controlling poultry movement inside the country, zoning and intensive surveillance for the rapid detection of the disease. However the use of vaccine to control the disease was prohibited (OIE, 2009). Since 2004 the DLD has performed intensive poultry surveillance for HPAI H5N1 (Simmerman et al., 2004). The outbreaks have affected more than 60 of the 73 provinces resulting in the culling of over 62 million chickens (Tiensin et al., 2005).

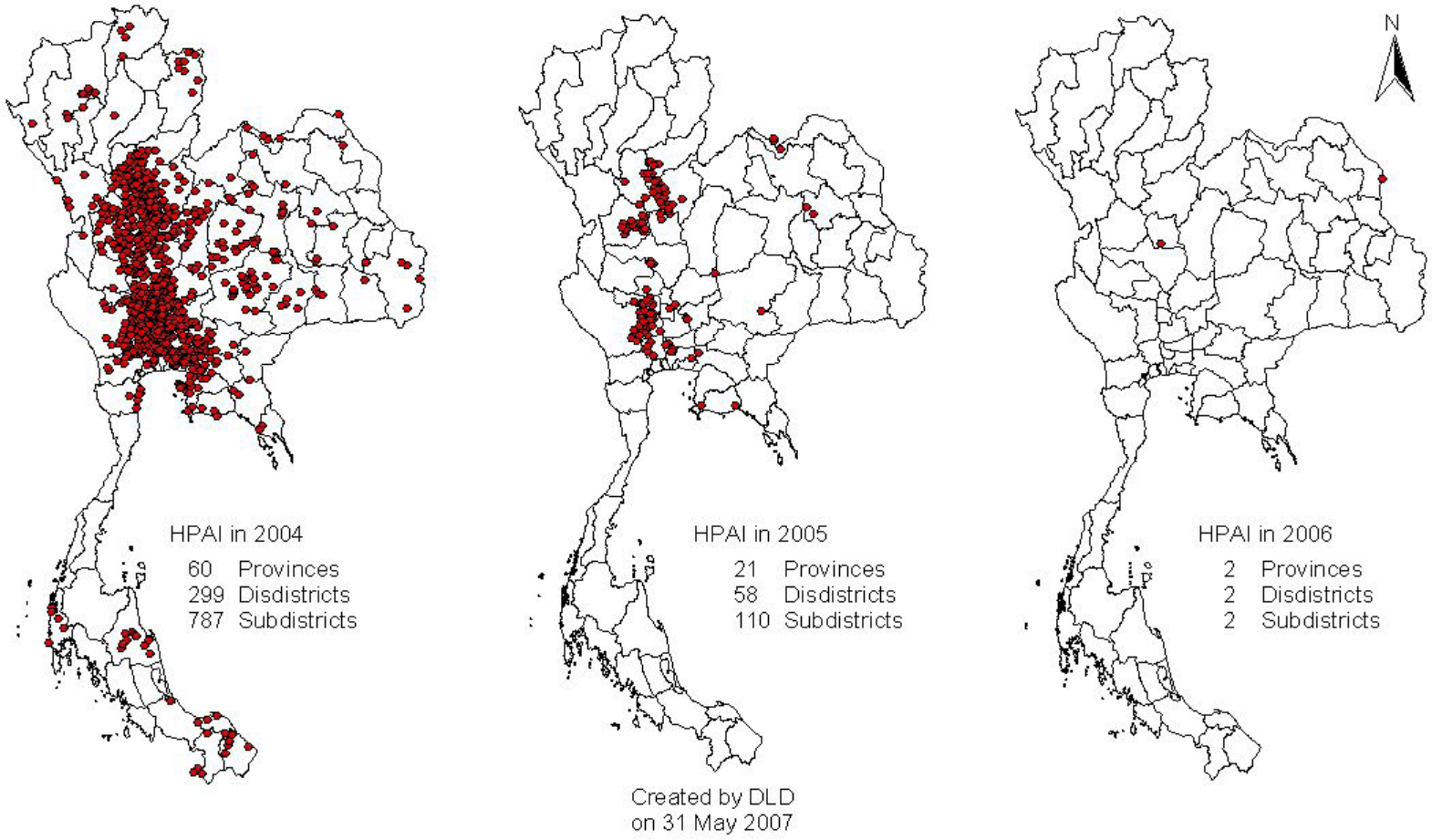
As outbreaks of HPAI H5N1 occurred in numerous countries across several continents within a short period of time, wild birds were blamed as the mode for transmitting the virus (Feare, 2007). Consultation with various expert groups led to recommendations of enhanced surveillance and wild bird surveillance programs for avian influenza H5N1 were established in many countries, including Thailand. These programs had the objectives of early detection of HPAI H5N1 viruses in wild birds and to determine the role of wild birds in disease transmission. National avian influenza surveillance of wild birds in Thailand has been conducted since 2004, under the authority of the Department of National Parks, Wildlife, and Plant Conservation (DNWPC), Royal Government of Thailand (Pothieng and Jamjomroon, 2006).

A study revealed that in Thailand, associations between the HPAI H5N1 outbreaks and agricultural practices like free-grazing ducks, native chickens, fighting cocks, and rice production were strong (Gilbert et al., 2006). Paddling or free-grazing ducks are common in Thailand and are important for increasing rice production as they are used to clean up snails and insects in newly harvested rice paddy fields (Gilbert et al., 2007). Paddling ducks generally travel from one area to another for up to three years before slaughter. This practice has benefited both rice and duck farmers for many centuries. However ducks can be infected by HPAI H5N1 virus without displaying clinical signs, can shed virus for up to 17 days and

potentially can act as reservoirs of disease (Hulse-Post et al., 2005). A spatial study showed that duck abundance and density of paddy fields were associated with the occurrence of the virus (Gilbert et al., 2008). Based on this the Thai Government created a policy to limit the movement of paddling ducks and encouraged closed system duck farms in order to control the outbreaks (Tiensin et al., 2007).

In early 2007, several cases were reported in a layer duck farm in the Phitsanulok province, (central Thailand) and a layer chicken farm in Nong Khai province (north-east Thailand) (OIE, 2004c). Recently on January 22nd 2008, there was a new outbreak in broiler chickens in the Nakhon Sawan province and in native chickens in the Pichit province (central Thailand) (OIE, 2008).

Figure 1.5; Distribution of HPAI H5N1 outbreak cases (red dots) in poultry in Thailand (2004 – 2006)



Source: Thanapongtharm and Noimoh 2006

1.7 Surveillance program for HPAI H5N1 virus in wild birds in Thailand

The national surveillance program for HPAI H5N1 virus in wild birds has been active since 2004 in order to determine the prevalence of infection and possible transmission pathways of the disease in wild bird populations in Thailand. It is also used to determine possible relationships between HPAI H5N1 outbreaks in domestic poultry and evidence of infection in wild birds, as well as to identify gaps in the current surveillance programs. Since 2004 more than 30,000 wild birds have been sampled throughout the country (Photeing and Jaimjomroon 2006).

The procedure for field sampling involves the trapping of wild birds by DNWPC staff using a range of techniques depending upon the location and skill of the staff involved in the sampling. The majority of samples collected have been cloacal swabs, however some tracheal or throat swabs, blood samples, and carcasses have also been collected. After collection field samples were submitted to laboratories including the NIAH laboratory, regional DLD laboratories and laboratories in the Faculties of Veterinary Science at Chulalongkorn, Khon Kaen, Kasesart, Chaingmai, and Mahidol Universities (Pothieng and Jamjomroon, 2006).

According to the report of the national surveillance program by the DNWPC (Photeing and Jaimjomroon 2006), the wild birds that tested positive to HPAI H5N1 virus during 2004-2005 were red-whiskered bulbul (*Pycnonotus jocosus*), Asian open bill stork (*Anastomus oscitans*), little cormorant (*Phalacrocorax niger*), scaly breasted munia (*Lonchura punctulata*), black collared starling (*Sturnus nigricollis*), Eurasian tree sparrow (*Passer montanus*), lesser whistling duck (*Netta rufina*), wood sandpiper (*Tringa glareola*), red collared dove (*Streptopelia tranquebarica*), zebra dove (*Geopelia striata*), black drongo (*Dicrurus macrocercus*), rock pigeon (*Columba livia*), common myna (*Acridotheres tristis*), white vented myna (*Acridotheres Javanicus*), and cattle egret (*Bubulcus ibis*).

1.8 Background and development of the current project

Understanding the epidemiology of HPAI H5N1 in wild birds and undertaking a risk assessment for the transmission of the virus to other birds and poultry are important when developing disease control and prevention programs. The Food and Agricultural Organization (FAO) stated that effective surveillance and diagnosis are important as they provide information on the ecology and behaviour of the virus (FAO, 2004). However, involvement of wild birds in the disease's transmission has not been clearly understood due to a lack of information about the behaviour and ecology of wild birds. The research described in this thesis was undertaken to: help address this knowledge gap; determine the prevalence of HPAI H5N1 in wild bird populations; study the behaviour and ecology of the wild bird species commonly found in Thailand; understand the molecular epidemiology of the virus; and evaluate the potential for disease transmission in wild birds through performing a risk assessment.

Outbreaks of HPAI H5N1 in poultry in Thailand are most likely to occur in the central area of the country where the majority of land use is involved with rice production and its associated paddling ducks (Gilbert et al 2006; Thanapongtharm and Noimoh 2006). Rice paddy fields are common in Thailand as rice is one of the main agricultural products of the country. Moreover, paddy fields are known as habitats for some domestic poultry like backyard chickens and free grazing ducks, as well as a wide range of migratory and non-migratory species of wild birds. Common habitats of wild birds also include open system poultry farms, backyards, and natural wetlands and ponds where wild and domestic birds can come into contact. Asian open bill storks have been reported as one of the major wild species affected by the virus (Uchida et al., 2008). It is known that the storks generally feed on snails clustering in rice paddy fields, which may facilitate close contact and disease transmission

between storks and domestic poultry. However, the role of the storks in disease transmission remains questionable.

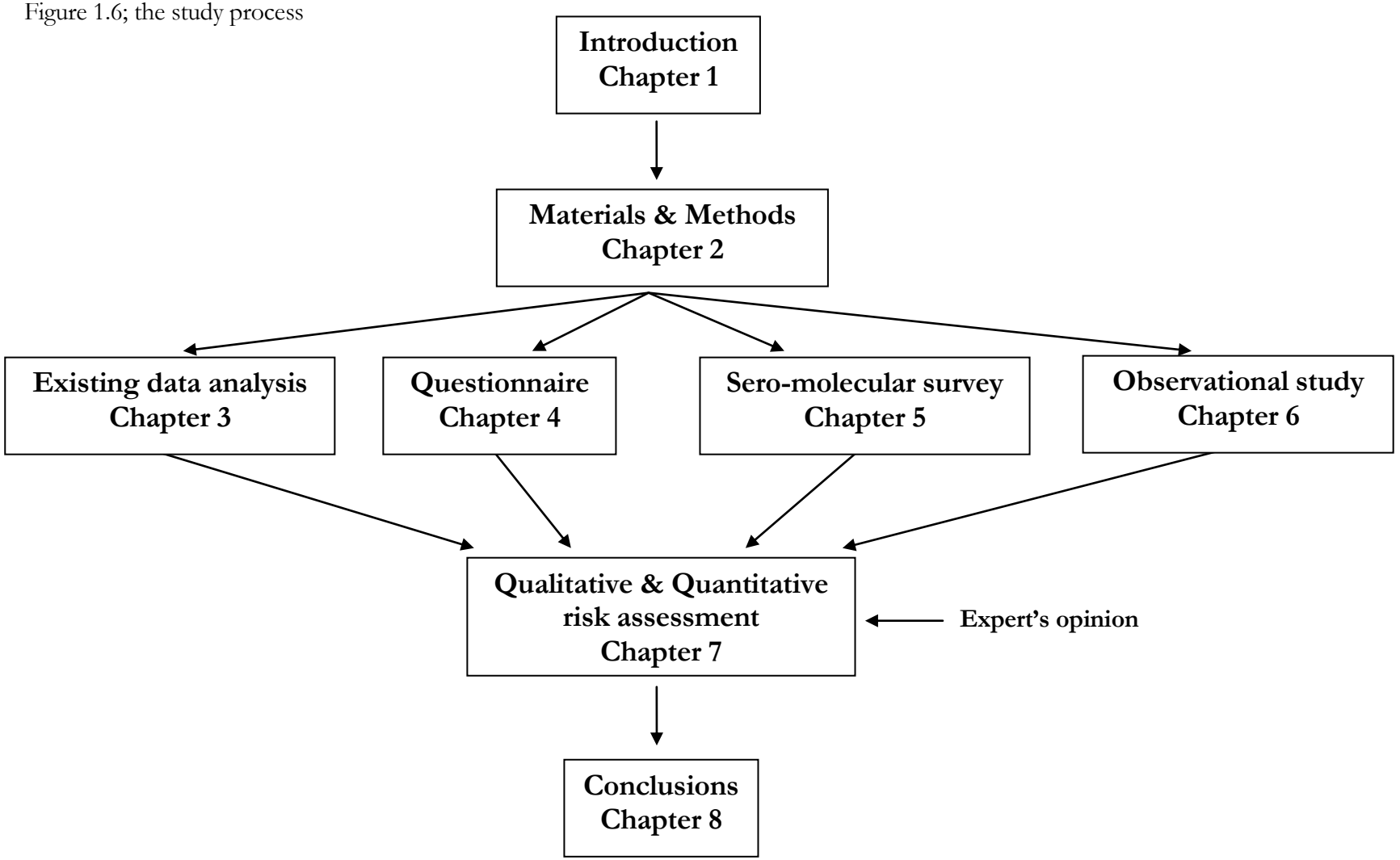
Infected wild birds have mostly been detected in the central part of Thailand (Photeing and Jaimjomroon 2006). Wild birds that live in the central part of Thailand are both terrestrial and aquatic species and include some migratory species. According to a previous report from the DNWPC (Photeing and Jaimjomroon 2006), wild birds that are affected by HPAI H5N1 virus are mainly terrestrial. Free living birds (feral or common terrestrial birds) are believed to be involved in the spread of HPAI H5N1 virus (Gauthier-Clerc et al., 2007). Direct contact with feral birds can cause primary infection in poultry (Alexander, 2007). As there is a wide range of species of wild birds living in an area, species that had previously been recognised as being infected (from previous reports or existing data) were targeted for sampling in this study. Also some wild bird species that either shared habitats or had a close relationship with domesticated poultry were considered to be potentially higher risk species. Thus, a targeted surveillance program in a variety of wild terrestrial birds was applied in this study.

In order to gain more information about the circulation of virus in the wild bird population, it was decided that it would be advantageous to take bird behaviour and ecology into account when designing a study plan. As there is little ecological or behavioural data about native wild birds, observations were included to gain more information about these aspects. Risk assessments were included in the project to estimate the risk of a HPAI H5N1 infected wild bird shedding an infectious dose of virus in a poultry keeping area in the Central part of Thailand.

The project was a combination of retrospective and prospective studies (Figure 1.6). Retrospective studies included analysis of existing data from a wild bird surveillance program for HPAI H5N1 virus, and data collected by questionnaire from villagers. In addition,

existing data used in this study was a part of the National surveillance program for HPAI H5N1 virus in wild birds under the cooperation between the DNWPC and MoZWE, VSMU. The prospective studies included a virological and serological surveillance program for HPAI H5N1 virus in wild birds in a study site in central Thailand, molecular epidemiology studies of isolated viruses, observational studies of wild bird-poultry interactions in selected study sites and qualitative and quantitative risk assessments using data from the virological, observational and questionnaire studies. Surveillance programs in wild birds have been used as a tool to gather more information on the disease's prevalence and distribution (Smith et al., 2009).

Figure 1.6; the study process



1.8.1 Objectives

The objectives of the work reported in this thesis were:

1. To use existing surveillance data to identify disease patterns and trends of previous outbreaks in wild bird in Thailand
2. To identify high risk species involved in the disease transmission from an outbreak area to another area.
3. To understand the transmission pathways of avian influenza H5N1 between wild birds and domesticated poultry.
4. To undertake a risk assessment of the virus transmission between wild and domestic species in the central part of Thailand.

1.8.2 Hypotheses

Hypotheses tested in this thesis were:

1. The prevalence of highly pathogenic avian influenza H5N1 is low in the wild bird population in Thailand
2. Wild birds with close contact with domestic poultry have a higher risk of infection with HPAI
3. Wild birds play no significant role in the transmission of H5N1 (spill back)

Chapter 2

MATERIALS AND METHODS

2.1 Study design

Both retrospective and prospective studies were conducted to obtain data for this project. These data were then used to undertake qualitative and quantitative risk assessments to examine the role of wild birds in disease transmission. Before the project commenced, collaborative agreements were established that covered applications to use data, planning the data collection and analysis, sample collection procedures, and the laboratory testing. Permission for collection of samples from wild birds and administration of questionnaires were obtained from the DNWPC and Department of Livestock Development (DLD) respectively, as well as from the Human and Animal Ethics Committees at both Mahidol and Murdoch Universities. After preliminary planning a study site was selected and surveyed.

Retrospective studies included analysis of existing data and analysis of data collected from the questionnaires administered to villagers. Prospective studies included virological and serological surveillance for HPAI H5N1 in the study sites; performing molecular epidemiological studies on isolates found in the surveillance study; performing a wild bird observational study; and performing a qualitative and quantitative risk assessment. In this chapter the general materials and methods used in the project are described, including the processes for the prospective and retrospective studies, the selection of the study sites, procedures for collection of field samples, and laboratory testing protocols [Viral isolation (MDCK cell culture), Haemagglutination test (HA), Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), RNA sequencing and Microneutralisation test (NT)]. More specific

methodologies of the prospective and retrospective studies are described in the relevant chapters.

2.2 Study site selection

The study sites were selected to include various land types where wild birds and domestic poultry are commonly found together which may facilitate transmission of the virus to be studied. The main criteria used when selecting the study sites included:

- Mixed habitat types for wild birds and domestic poultry where interaction between wild birds themselves and wild birds and domestic poultry may occur
- Sites where interactions between wild birds and poultry can be observed and recorded several times over a 12 month period
- Sites in Central Thailand with a history of previous outbreaks of HPAI H5N1 in poultry.

2.2.1 Mixed habitat types for wild birds and domestic poultry

The study sites covered common habitat types in the central part of Thailand including wild bird roosting and/ or feeding grounds, rice paddy fields, villages, and poultry farms where contact and/or interaction between wild birds and poultry was likely to occur.

Wild bird roosting and/or feeding sites, where substantial numbers of resident or transient wild birds are present, were selected for inclusion in the study sites. These included winter roosting sites of the Asian open bill stork and covered nesting areas of rock pigeons.

Feeding grounds where wild birds are commonly seen during daytime are in rice paddy fields and natural wetlands or ponds. For example, common wild birds known to feed or live in rice

paddy fields include rock pigeons, white vented myna (*Acidotheres grandis)*, Asian pied starling (*Gracupica contra*) common myna, great egret (*Ardea alba*), intermediate egret (*Egretta intermedia*), little egret, Chinese pond heron (*Ardeola bacchus*), Javan pond heron (*Ardeola speciosa*), cattle egret (*Bubulcus ibis*), and waterfowls such as cotton pygmy-geese (*Nettapus coromandelianus*) (Lekagul and Round, 1991). Water birds, such as Asian open bill storks (*Anastomus oscitans*) and lesser whistling duck (*Dendrocygna javanica*) (Lekagul and Round, 1991), may also occasionally stop over in agricultural areas during their migration (VanEerden et al., 2005) in Thailand.

Residential villages where backyard poultry [poultry production sector 4 (FAO, 2009)] are commonly kept are also a habitat for common terrestrial birds. Poultry feed is available in such areas and many terrestrial birds frequent these areas where they scavenge for food. Therefore villages containing backyard poultry were included as study sites to observe and record interaction between wild birds and poultry.

Poultry farms, especially open system farms [poultry production sector 3 (FAO, 2009)], were located within the study areas. Low bio-security poultry farms were also targeted as study sites to observe and record wild bird and poultry interactions.

2.2.2 The study area and its history of H5N1 HPAI poultry outbreaks

The study area chosen was located in the Banglane District in Nakhon Pathom province where HPAI H5N1 outbreaks had previously been reported in poultry during the first and second outbreaks in 2004 and early 2005 (DLD, 2005; OIE, 2008). Also a report from the DNWPC on surveillance of wild birds had recorded two H5N1 HPAI positive samples from a duck/goose (the Thai common names for these two words are identical and it is not known if the positive bird was a duck or a goose) and a rock pigeon (collected between January and

April 2005). The study sites in the area were located in Bangpasri, Banglane, Klongnokkatong, and Bangsripa subdistricts in Banglane district, Nakhon Pathom province. The study area was approximately 25 kilometres in diameter. In Figures 2.1 and 2.2, the location of Banglane district and land use in one study site is displayed.

Prospective and Retrospective studies in this thesis were performed within this study site except for the analysis of existing data on an Avian Influenza (H5N1) surveillance program in wild birds in Thailand (see section 2.3.1).

Figure 2.1; Location of Banglane district in Thailand

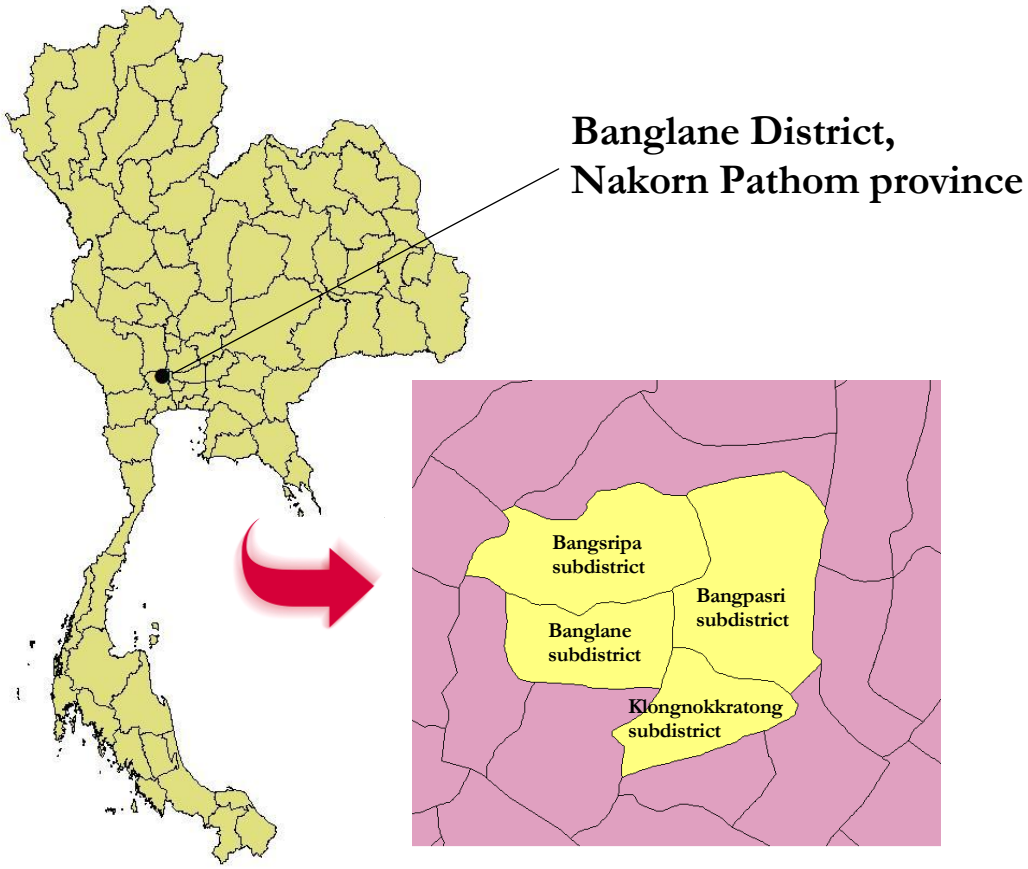
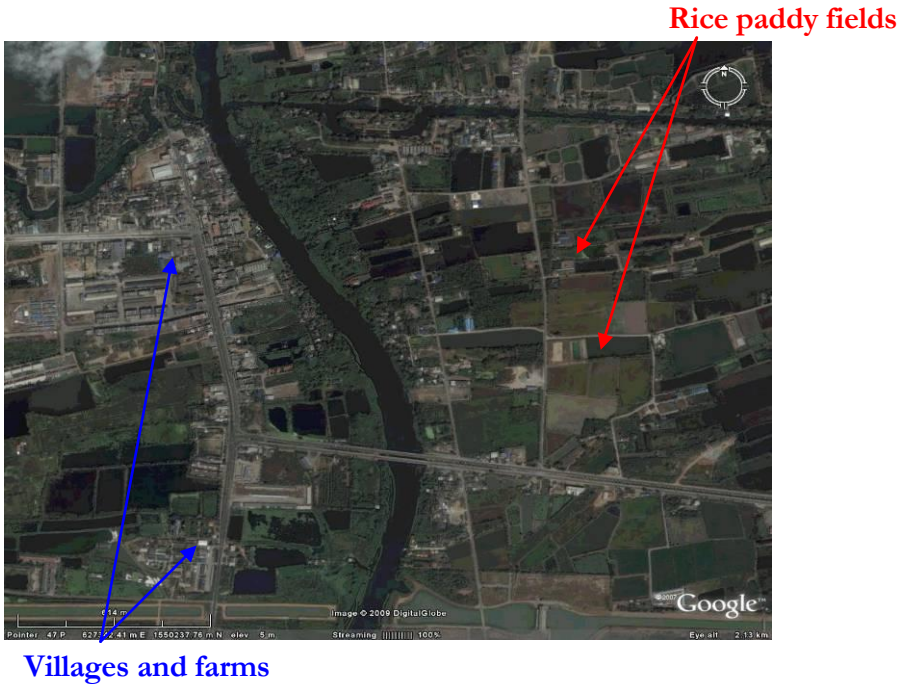


Figure 2.2; Satellite image of the study site in Banglane subdistrict, Banglane district shows rice paddy fields and villages



Source: Google Earth (2008)

2.3 Retrospective studies

2.3.1 Existing analysis of data on an Avian Influenza (H5N1) surveillance program in wild birds in Thailand (2004-2007)

Existing surveillance data was obtained from the MoZWE, VSMU for further analysis. The wild bird surveillance program was launched by DNWPC, Royal Thai Government. Under the collaboration between VSMU and the School of Veterinary and Biomedical Sciences, Murdoch University, existing data on the VSMU's wild bird surveillance was analysed in order to determine trends and identify risk factors, such as species, ages, health status, sample types, and location, for disease outbreaks in wild birds. These data are a part of the national surveillance program where wild bird samples are collected from a variety of locations throughout Thailand.

A database (Microsoft Excel spreadsheet version 2003; (Microsoft®)) of the wild bird surveillance program for H5N1 virus was generated from data held by the MoZWE. The statistical program SPSS (version 17.0) was used to analyse the data. Analysis and outcomes of the H5N1 surveillance program in wild birds in Thailand (2004-2007) will be discussed in subsequent chapters. The results obtained from this retrospective study were used to design a survey of H5N1 infection in wild birds and to determine the risk of transmission of the virus between wild birds and poultry in Thailand. This is described in detail in subsequent chapters.

2.3.2 Questionnaire study

Questionnaires were administered to villagers within the study site to gather information about the history of disease outbreaks in poultry in their villages, information on their farm practises and interaction between wild birds and poultry in these locations. Details of the development and pilot testing of the questionnaires are provided in Section 4.2.1. The questionnaires were administered to villagers living in 30 villages. These villages were selected based on the presence of multiple types of poultry practises and were located in four subdistricts of the Banglane District, Nakhon Pathom province which had outbreaks of H5N1 HPAI in previous years. Local DLD staff and village volunteers helped introduce the interviewers to villagers. After receiving permission to interview villagers, they were interviewed by trained interviewers. The interviewers were staff from the Faculty of Veterinary Science, Mahidol University who had background knowledge about H5N1 HPAI in Thailand and who had been through specific training in conducting the questionnaire by the project leader.

Statistical analyses used included descriptive statistics, univariable statistics including Fisher's exact or Pearson's Chi square test for independence and ANOVA and the multivariable

logistic regression. Details of the findings from the questionnaire study and its statistical analysis are provided in Chapter 4.

2.4 Prospective studies

2.4.1 Serological and virological surveillance program for HPAI H5N1 virus and molecular study in wild birds (2007 and 2008)

The prospective surveillance program for HPAI H5N1 virus in this study was designed to identify the prevalence of the infection in wild birds within the study site and to detect evidence of virus circulation in the wild population. This surveillance program combined testing by serology, virus isolation, and molecular characterisation of any H5N1 viruses detected. The survey was conducted within the Banglane district, Nakhon Pathom province. Wild birds in the area were caught and sampled every two months from February 2007 to October 2008 with support by the DNWPC. If H5N1 virus was isolated at any sampling time re-sampling of the site was undertaken two weeks after the positive sample was collected. Wild bird samples collected from this surveillance program were tested by the MoZWE, VSMU. Field sample collection and laboratory test procedures are described below.

Field data and laboratory results were entered into Microsoft Excel version 2003 and analysed with SPSS. The prevalence and 95% confidence intervals (normal approximation methods) were calculated for all species as well as individual wild bird species. Associations between infection and factors listed in the field sample collection data sheet were determined using a Chi-square test for independence and odds ratios and their 95% confidence intervals. The variables used for analysis included date or month/season or time of collection, location, sample type, wild bird species, and age and health status of the sampled birds. Statistical analysis of wild bird surveillance data was performed separately but comparisons between the

results of the surveillance programs in wild birds and data from the concurrent surveillance program for H5N1 HPAI in domestic poultry in the study area were undertaken to identify relationships between outbreaks in domestic poultry and wild birds. Data on poultry outbreaks was obtained from the DLD and OIE websites.

The molecular epidemiology of HPAI H5N1 viruses isolated from the wild birds collected during the survey was conducted in collaboration with the Department of Microbiology & HKU-Pasteur Research Centre, University of Hong Kong. The viruses detected during the surveillance study were isolated and sequenced by MoZWE, VSMU. Genetic sequences of the HA and NA genes were determined and the sequence data was used to conduct phylogenetic analysis. The HA and NA sequences from H5N1 viruses isolated from wild birds in this study were compared with an epidemiologically appropriate range of reference H5N1 avian influenza virus sequences that had been submitted to the Genbank database (http://www.ncbi.nlm.nih.gov/Genbank/) as shown in Chapter 5 (Figures 5.3 and 5.4). A phylogenetic tree showing the relationships between the H5N1 viruses isolated from wild birds in Thailand from 2004 to 2008 and other H5N1 viruses from the region was constructed. Details of these findings and discussion of the epidemiological significance is provided in Chapter 5.

2.4.2 Observational study

Eight observation sites in the Banglane district were selected representing the four main habitat types (wild bird roosting sites, natural wild bird feeding grounds, backyard areas, and open system poultry farms). Two sites were selected for each habitat type. The sites were described and GPS points were marked. An observational data recording form was generated with the aims of recording types of birds present, their general behaviour and any interactions which may be involved in the spread of disease. Each site was observed twice a month for

half an hour between March 2008 and February 2009. Thus, each site was observed for a total of 720 minutes over the study. Common names of wild birds seen in the sites, with their interactions and behaviours, were recorded on the form. Data were entered into a Microsoft Excel spreadsheet and analysed with SPSS. Details of the observational study analysis are given in Chapter 6.

2.4.3 Risk assessments

Qualitative and quantitative risk assessments were applied in this study in order to estimate the probability of transmission of H5N1 viruses between wild birds and domestic poultry in relatively close proximity to each other in the central part of Thailand. The risk assessment process followed was based on OIE guidelines; Handbook on Import Risk Analysis for Animals and Animal Products (Volume 1; Qualitative risk assessment (OIE, 2004a) and Volume 2; Quantitative risk assessment (OIE, 2004b)). The risk assessment is described and discussed in detail in Chapter 7.

2.5 Collection of field samples

2.5.1 Selection of wild bird species and surveillance within the study site

Surveillance was undertaken in the study area every two months between 2006 and 2007. In each survey trip, at least 30 individual wild birds were sampled. However, this survey was not a random survey due to the difficulty in trapping birds and the uncertainty of the size of the wild bird population. Wild birds were shot for sample collection by a DNWPC sample collection team. These birds were mainly collected from along the roads within the study site. Areas where wild birds were sampled were selected to cover the four main habitat types (wild bird roosting sites, rice paddock/ agricultural fields, farms, backyard/ residential areas).

Consequently, a targeted convenience-sampling regime was performed. Common residential wild bird species that have a higher chance of contact and/or interaction with poultry were targeted to approximate this study to a cross-sectional study (Dohoo et al., 2003).

2.5.2 Sample techniques and procedures; collection of swabs and blood

Samples including tracheal (or choanal or oropharyngeal) and cloacal swabs, blood, and carcasses were collected. Swabs (one swab per tube) were kept in 1.0 ml of viral transport medium (VTM) which contained 0.5% (w/v) Bovine plasma albumin, Penicillin G ($2x10^6$ U/litre), Steptomycin (200 mg/litre), Gentamicin (250 mg/litre), Nystatin ($0.5x10^6$ U/litre), Polymyxin B ($2x10^6$ U/litre), Ofloxacin (60 mg/litre) and Sulfamethoxazole (0.2 g/litre). Techniques for sample collection included:

Oropharyngeal swab:

A dry swab (Thai gauze®) was placed into the mouth swabbing against the wall of the oropharynx and the choanal opening and then placed into VTM.

Tracheal swab:

This technique was commonly used in dead birds or live large birds (such as storks). A dry swab was inserted through the tracheal opening and the tracheal wall was gently swabbed and then the swab was placed into VTM.

Cloacal swab:

A dry cotton swab was inserted into the birds vent and the cloacal wall was swabbed thoroughly. The swab, often with faecal material attached, was then were placed into tubes containing VTM.

Blood samples:

In live birds, blood samples were collected from the wing (ulnar vein), median metatarsus vein or jugular vein by using a 24-26G needle and a 1 ml syringe. Between 0.5 and 1 ml of blood was collected from live birds, depending upon the size of the bird. For dead birds, blood was collected by cardiac puncture using an 18G needle and 3 ml syringe and a volume of up to 2 ml was collected. Blood samples were transferred to Eppendorf® tubes. After swabs and blood were collected, carcasses were placed into individual plastic bags.

All samples and specimens were stored in eskies that contained ice packs or in mobile refrigerators (approximately 4°C). Samples and specimens were then transported to the MoZWE, VSMU laboratory within 48 hours of collection.

2.5.3 Recording of field sample data

All data and information of each field sample collection were recorded into the field sample collection data sheet designed by the MoZWE (Appendix I). Data included sampling date, species of bird, age and health status of sampled animal, type of sample, and location of sampling. The age of sampled birds was classified as juvenile, adult, or unknown (if not stated on the submission sheet), and their health status at the sampling time was recorded as healthy (no clinical signs), sick (clinical signs), dead (opportunistically found dead), or unknown (where the status was not recorded on the submission sheet).

2.5.4 Safety procedure

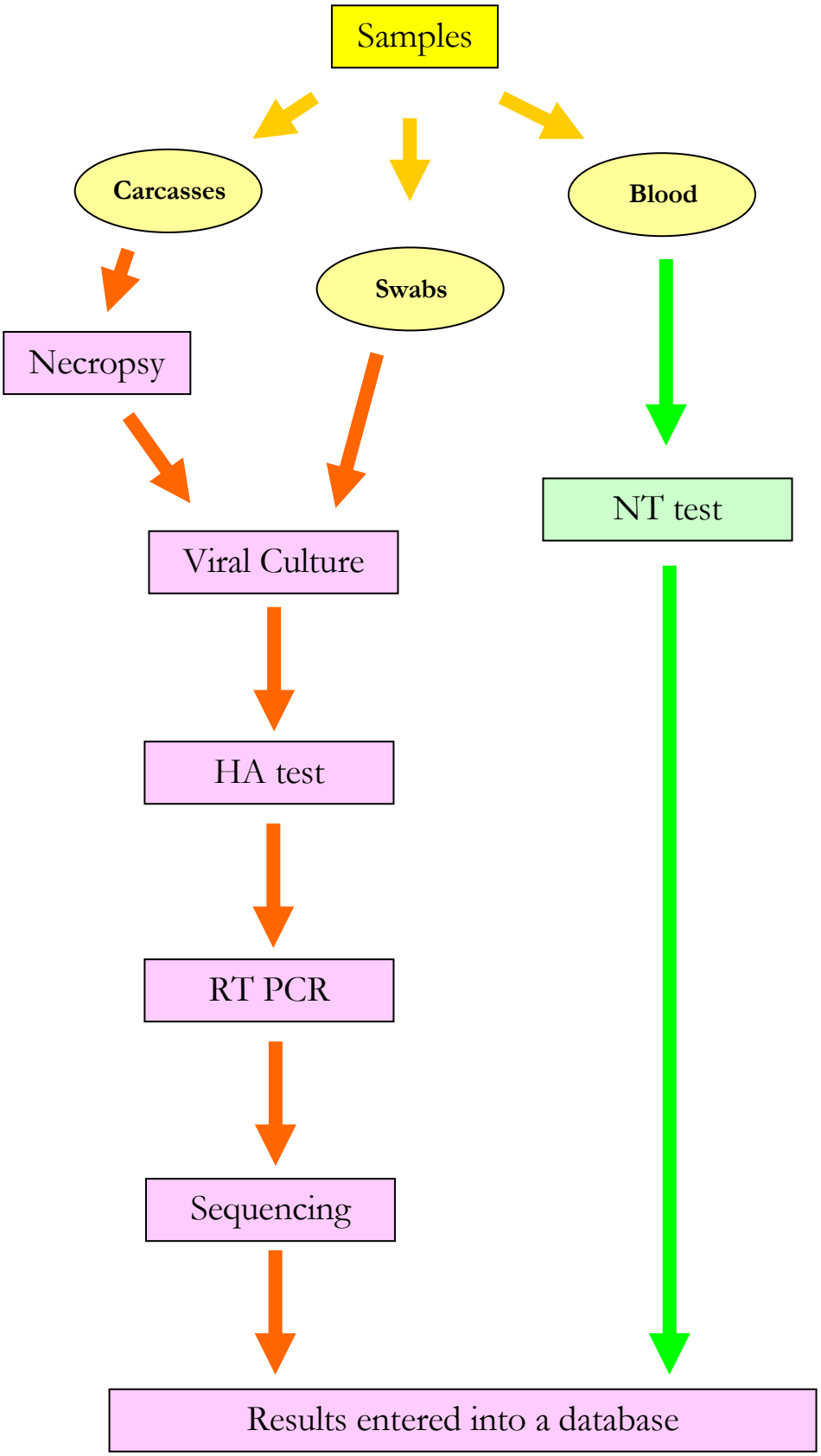
For safety purposes, activities that involved trapping and/or collection of samples from wild birds were operated under a strict safety protocol as the area was a previous HPAI H5N1 outbreak area. Wild birds were trapped by authorized DNWPC staff and samples were collected and processed by trained veterinarians. During the field trip, staff were required to

wear full personal protective equipment (PPE) including white protective gowns, caps, double gloves, N95 mask and rubber boots. After the completion of sample collection the PPE was spayed with 70% alcohol or Virkon® (Antec International Limited). After the end of each field trip the PPE, except for the rubber boots, were autoclaved and then burnt. The rubber boots were rinsed and left overnight soaking in Virkon® before air drying. The transport vehicle and any containers used were washed with a soap solution and/or disinfected with a 70% alcohol spray.

2.6 Laboratory diagnosis

The standard test protocols for the diagnosis of HPAI H5N1 virus infection used in the MoZWE, VSMU laboratory are based on those outlined in the WHO manual for diagnosis of HPAI (2002). This included viral isolation by MDCK cell culture and haemagglutinin testing (HA); viral gene detection by reverse transcriptase PCR (RT PCR) using primers specific to H5, N1 and M genes; and antibody detection by microneutralisation assay (NT). A flow diagram for the testing protocol is shown in Figure 2.3. All specimens were submitted to the virology laboratory at the MoZWE, VSMU. If specimens were not processed within 24 hours they were stored at -80°C.

Figure 2.3; Flow chart of laboratory process for avian influenza H5N1 viral detection



2.6.1 Necropsy procedure

If carcasses were submitted to VSMU, they were taken to the secure necropsy unit and post-mortem examination was conducted by trained veterinary staff from the MoZWE in order to observe gross lesions and to collect tissue samples for further pathology and molecular studies. The following procedure was used: Wild bird carcasses were stored at -80°C if they were not processed immediately. Operators were required to wear full PPE and necropsies were performed in a biosafety cabinet. All gross lesions found on necropsy were recorded on the necropsy sheet. Tissue samples, including trachea, lungs, brain, liver, spleen and intestines, were collected and then placed in labelled Petri dishes and kept in an esky packed with ice (approximately 4°C). The esky was submitted immediately to the cell culture laboratory. The necropsy room and equipment were cleaned and disinfected with 70% alcohol or Virkon® as appropriate.

2.6.2 Viral culture

The FAO (2006) reported that the H5N1 virus grows equally well in eggs as in Madin-Darby Canine Kidney (MDCK) cells. It was logistically difficult to do large scale viral isolation in eggs at the facility. Thus, viral culture in this project was done in MDCK cells.

The procedure was based on laboratory procedures as described by Bird and Forrester (1981) and Lennette and Schmidt (1979). The tissues were homogenized in a sterile, chilled mortar and pestle with added VTM to make a 10% w/v suspension. The suspensions were clarified by centrifugation at 2,500g at 4ºC for 15 minutes and the supernatants collected. All specimens, including swabs and supernatants (from tissue samples), were filtered by using 0.22 micron filters and tested for the presence of avian influenza viruses using the cell culture technique. Filtrated tracheal and cloacal swabs or tissue sample supernatants were inoculated

in 500 µl aliquots into the cell cultures showing approximately 80-90% confluence in 25-cm^2 tissue culture flasks and incubated at 37°C for 2 hours. The supernatant was discarded, 5 ml of TPCK-trypsin medium was added (500 g/ml of trypsin in MEM), and then flasks were incubated at 37°C in a 5% CO_2 incubator. Inoculated flasks were observed daily for 6-7 days for the presence of a cytopathic effect (CPE). Supernatants were collected if a 3+ or 4+ stage of CPE was observed, or on day 6 or 7 if there was no CPE. Cultures showing no CPE on first passage were subjected to a second passage as above. The remainder of each specimen was stored at -80 °C.

2.6.3 Haemagglutination test (HA)

The haemagglutination assay is a test for detecting haemagglutinating viruses such as the influenza virus. The HA technique used in this project was based on WHO methodology (WHO, 2002) as described briefly below.

Serial two-fold dilutions of specimens were made in 50 µl of phosphate-buffered saline (PBS) in 96-well U-bottom plates (Nunc Brand Products). To each well, 50 µl of 0.5% (v/v) chicken erythrocytes in PBS was then added. The plates were kept at 4°C for 1 h, after which the haemagglutination patterns were read and HA titres were determined from the last dilution showing complete haemagglutination. For reading the HA activity the plates were tilted at an angle of approximately 45° and observed for tear-shaped streaming of the RBCs (OIE, 2005). Cultures positive to HA were further processed to detect the presence of avian influenza virus and H5 and N1 specific genes as described below.

2.6.4 Multiplex Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The multiplex RT-PCR was employed to identify the type and sub-type of any haemagglutinating viruses isolated from inoculated MDCK cell cultures. Using the nucleotide sequence available in the GenBank database, multiple sequence alignment of H5, N1 and M genes were performed using the CLUSTALX program (version 1.8 from ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX). H5 and N1 primers were selected from conserved regions of 50 known sequences specific for H5N1 influenza A viruses. M primers were also selected from conserved regions of at least 50 known sequences from influenza A viruses. The influenza virus (type A) was identified by using primers specific for the M gene; H5 and N1 subtypes were identified by H5 and N1 specific primers (Lee et al., 2001). The procedure used for the multiplex PCR was based on the WHO recommendations and laboratory procedures for detection of avian influenza A (H5N1) virus (WHO, 2007) and is described below.

The RT-PCR was performed using a One-Step RT-PCR kit (Qiagen®, Valencia, CA., USA) containing primer mix. The 5 µl of reaction mixture contained denatured RNA, 10 µl of 5x OneStep RT-PCR buffer (Qiagen®), OneStep RT-PCR enzyme, 10 µl of 10 mM dNTP mix (Qiagen®), and 6 µl of primer mix (1.25 µmol each). RNase-free water was added to a total volume of 50 µl. Amplification of c-DNA from viral RNA was carried out at 50°C for 30 min for reverse transcription and the PCR procedure commenced with an initial denaturation of 95°C for 15 min followed by 35 cycles of denaturing at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 72 °C for 1 min. The PCR ended with a final extension step at 72°C for 10 min. The multiplex RT-PCR products were visualized by gel electrophoresis. The reference strain of influenza H5N1 virus (A/chicken/Thailand/vsmu-3-CBI/2005) was used as a positive control in the multiplex RT-PCR assays. Size-specific PCR products (335 bp for M, 544 bp for H5 and 274 bp for N1), that were obtained from the multiplex PCR in several field experiments, were sequenced to evaluate the specificity of the

assay. The analytical sensitivity of the test was evaluated by testing serial 10-fold dilutions of H5N1 viral RNA containing between 10 and 10^6 copies/μl of A/chicken/Thailand/vsmu-3-BKK/2004 (H5N1) virus. This virus stock defined titre, expressed as $TCID_{50}$ /ml, had been tested by Taqman real-time RT-PCR according to standard methods (Ng et al., 2005; WHO, 2008d). With the RNA standards the detection limit for this multiplex PCR test was 10^3 copies/ μl.

Samples that were HA positive but RT-PCR negative for all 3 target genes (M, H5, and N1) were also tested for Newcastle disease viruses by using specific primers in RT-PCR. For samples that were positive for the M gene only, the amplicon was sequenced and the sequence was compared with other influenza A viruses in the NCBI database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These M positive samples also were subtyped by using H1-H15 specific primers for RT-PCR reaction (Lee et al., 2001).

2.6.5 Gene sequencing:

The procedure for gene sequencing is described in Chapter 5 (Section 5.2).

2.6.6 Microneutralisation test (NT test)

Haemagglutinating Inhibition (HI) has been validated as a test for poultry but its cut-off values, sensitivity, and specificity, including the effects of non-specific inhibitors of haemagglutination, has not been determined for HI serology in wild birds. The microneutralisation test is a sensitive and specific test used to detect specific antibodies against influenza virus (WHO, 2008d). For logistical reasons and because of the higher specificity of the NT, the laboratory preferred to conduct serology in wild birds with the NT.

A serum sample was mixed with a known titre (usually 100 $TCID_{50}$) of the reference virus. Neutralizing antibodies in a serum sample, if present, inhibit the infection of MDCK cells by the influenza virus which inhibits the CPE of the virus in these cells (WHO, 2008d). The NT technique combines two main steps which include viral neutralisation by specific antibody and detection of the remaining virus. The procedure was adapted from Rowe and others (1999). A titre of ≥1:80 was considered positive for antibodies specific against H5N1 (Kalthoff et al., 2008b). This method was only operated in the Biosafety level 3 laboratory.

2.6.6.1 Serum treatment

Serum (100 µl) was treated by heat inactivation at 56°C for 30 minutes in a water bath. The inactivated serum was diluted 1:10 by Earle's minimal essential medium (EMEM) 1X (900 µl). 120 µl of the serum was transferred to the first well of two columns in a microtitre plate. Earle's minimal essential medium 1X (60 µl) was added into wells of the next two columns. 60 µl of serum were then transferred from the first column wells to the second column wells in the same row (Figure 2.4). Then 60 µl of the serum from the second column wells were transferred to the next row of wells. Serum was diluted (1:1:2) serially until the last column, where 60 µl from the last well was discarded. Thus, each well contained 60 µl with different two folded dilutions (1:10, 1:20 to 1:2560).

2.6.6.2 Virus antibody reaction

60 µl of virus suspension (A/chicken/Thailand/vsmu-3-BKK/2004; H5N1) (at a concentration of 200 $TCID_{50}$/100µl) was added to every well. The plate was then incubated at 37°C for 2 hours.

2.6.6.3 MDCK cells culture

The cells were grown in growth media in a 96 well culture plate overnight. The media was then discarded. The EMEM was used to twice wash the monolayer of MDCK cells (200

μl/well/each time). Maintenance media with trypsin TPCK (100 μl; Appendix II) was added. The virus-antibody mixture solutions (100 μl) were added to corresponding wells containing MDCK cells and the cell cultures were incubated for 2-3 days at 37°C in a 5% CO_2 incubator.

2.6.6.4 Back titration

Back titration of the virus suspension was conducted with each test to ensure a concentration of the challenge virus was 100 $TCID_{50}$ /0.1 ml (within the range 30-300 $TCID_{50}$ /0.1 ml). Dilutions of stock virus in maintenance media with trypsin TPCK expected to contain concentrations of 100, 10, 1, and 0.1 $TCID_{50}$/200 μl were prepared. The maintenance media on MDCK cells was discarded and replaced by 200 μl of the above virus titrations in duplicate and the cell cultures were incubated at 37°C in a 5% CO_2 atmosphere.

2.6.6.5 Control set

The set of controls in all tests, including the virus back titration, cell controls, and positive serum control wells, were duplicated and laid out as displayed in Figure 2.4.

2.6.6.6 End point determination; CPE based NT assay

The test plate was examined each day with a microscope for the presence of CPE and when the CPE was at its endpoint in the virus controls, the test was read. The CPE was rated from 0 to 4 based on the extent of the cell monolayer damage as shown in Table 2.1. The antibody titre of the serum was considered as the highest dilution where ≥50% of the cell monolayer showed absence of CPE.

Figure 2.4; Diagram of NT test plate

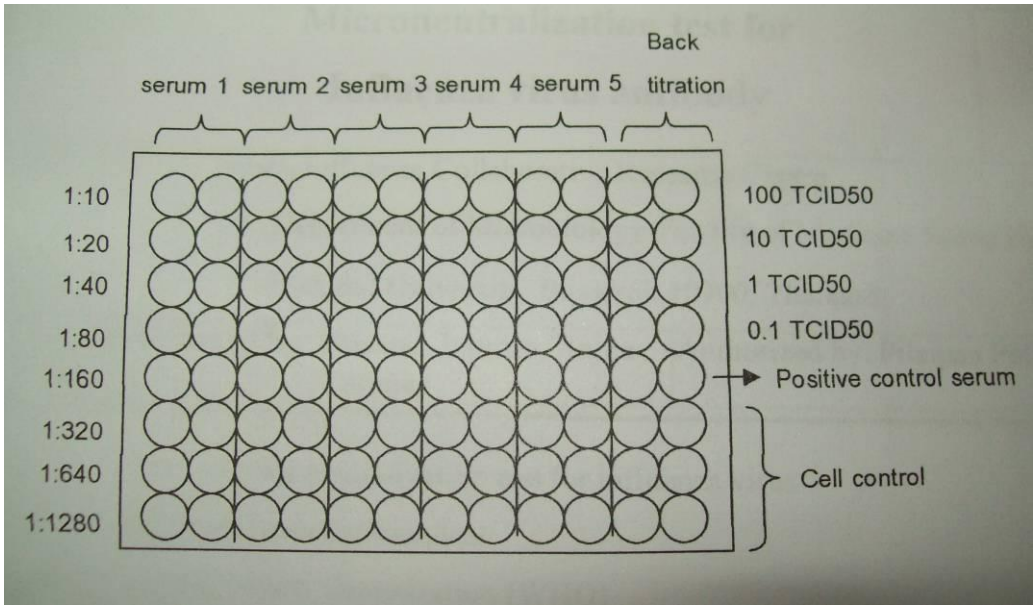


Table 2.1; Description of CPE scoring

Level of CPE	**Description**
4+	100% CPE
3+	70-80% CPE
2+	50%CPE
1+	<50% CPE;
0	No CPE

Chapter 3

COMPARISON OF OUTBREAKS OF H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA IN WILD BIRDS AND POULTRY IN THAILAND

This chapter is a published paper: Siengsanan, J.,Chaichoune,1 Rassameepen Phonaknguen,1Ladawan Sariya,1 Phirom Prompiram,1 Waraporn Kocharin,1 Sririporn Tangsudjai,Sarin Suwanpukdee,1 Witthawat Wiriyarat,1 Rattapan Pattanarangsan,1 Ian Robertson,Stuart D. Blacksell,2,3,4 and Parntep Ratanakorn (2009) Comparison of outbreaks of H5N1 highly pathogenic avian influenza in wild birds and poultry in Thailand. *Journal of Wildlife Diseases*, **45**(3).740–747.

3.1 Introduction

Highly pathogenic avian influenza (HPAI) H5N1 virus causes severe disease and sudden death in avian species. In Thailand, HPAI H5N1 outbreak was first reported during 2004 followed by five subsequent waves of HPAI H5N1 outbreaks in poultry as reported by the Department of Livestock Development (DLD), Government of Thailand (Thanapongtharm and Noimoh, 2006). These outbreaks affected more than 60 of 73 provinces resulting in the culling of over 62 million chickens (Tiensin et al., 2005). On 22 January 2008, a new outbreak in poultry was reported in a single province in Thailand (OIE, 2008).

Because outbreaks of HPAI H5N1 occurred in numerous countries across several continents within a short period, wild birds often were suggested as a source (FAO, 2008; Feare, 2007).

Recently, wild bird surveillance programs for HPAI H5N1 have been established in many countries, including Thailand, with the objectives of early detection of HPAI H5N1 viruses in wild bird populations and determining the role of wild birds in transmission. National avian influenza surveillance of wild birds in Thailand has been conducted since 2004, under the authority of the Department of National Parks, Wildlife, and Plant Conservation (DNWPC), Government of Thailand (Pothieng and Jamjomroon, 2006). In this study we report changes in HPAI H5N1 virus prevalence in wild birds compared to patterns of H5N1 HPAI outbreaks in poultry over the collection period 2004-2007.

3.2 Material and Methods

3.2.1 Collection of field samples

Wild bird samples were collected through collaboration between the DNWPC and the Monitoring and Surveillance center for Zoonotic diseases in Wildlife and Exotic animals (MoZWE), Faculty of Veterinary Science, Mahidol University, Nakhon Pratom, Thailand. Wild birds were caught using baited traps, hand nets or mist nets, or they were shot by DNWPC staff. Between 2004 -2005, various wild bird species were caught in different types of habitats in provinces where poultry were or were not affected. During 2006 and 2007, the survey program was targeted to particular areas where poultry outbreaks had occurred either recently or in the past. After live-capture, tracheal (or cloanal) and cloacal swabs were collected; for birds that were shot, tracheal and cloacal swabs were collected and in some cases carcasses also were submitted. Carcasses of birds found dead were submitted by the public via the government veterinary sectors. Individual or pooled (one to four birds from the same species and collected in same time and place) swabs were kept in viral transport media (VTM), which contained 0.5% (w/v) bovine plasma albumin, penicillin G ($2x10^6$ U/L), streptomycin (200 mg/L), gentamicin (250 mg/L), nystatin ($0.5x10^6$ U/L, polymyxin B

($2x10^6$ U/L), ofloxacin (60 mg/L) and sulfamethoxazole (0.2 g/L). All specimens were transported, chilled (at approximately 4° C) using ice boxes and/or mobile refrigerators, and delivered to the MoZWE laboratory within 48 hr.

In total, 6,263 pooled samples representing 15,660 individual wild birds were collected. In 2004, 552 (8.8% of total) samples were tested representing a combination of individual and pooled samples from 692 birds. In 2005, 2,620 (41.8% of total) samples representing 7,562 birds were tested. In 2006, 2,070 (33.1% of total) samples representing 5,441 birds were tested, and in 2007, 1,021 (16.3% of total) samples representing 1,965 birds were tested. The survey included 50 provinces and more than 223 species of birds. Data for each sample collected were recorded on a field data sheet (either DNWPC or MoZWE forms) and included sampling date, species, age (juvenile, adult or unknown), health status (no clinical signs, clinical signs, dead, unknown), type of sample, and location.

3.2.2 Virus isolation and identification

Specimens were submitted to the virology laboratory at the MOZWE, Faculty of Veterinary Science, Mahidol University. If specimens were not processed within 24 hr they were stored at -80°C. Submitted carcasses were necropsied and tissue samples, including trachea, lungs, brain, liver, spleen and intestines, collected. Tissues were homogenized in a sterile chilled mortar and pestle with added VTM. The specimens were clarified by centrifugation at 2,500 x G at 4°C for 15 min, and the supernatants were collected.

After filtration with a 0.22 -μm filter, supernatants from swab and tissue samples were inoculated into Madin-Darby canine kidney (MDCK) cells or 11-day embryonated eggs. For MDCK cultures, 500 μl of sample was inoculated directly onto cells in 25-cm^2 tissue culture flasks and incubated at 37°C for 2 hr, at which time the supernatant was discarded and 5 ml

of TPTK-trypsin medium added (500 g/ml of trypsin in minimal essential medium). Flasks were incubated at 37°C in a 5% CO_2 incubator, and assessed for the presence of cytopathic effect daily for 4 days. The remainder of each specimen was stored at -80°C. For virus isolation using embryonic eggs, 200 µl of each sample was injected into the allantoic cavity of 11-day old embryonated eggs in triplicate. Viability of embryos was monitored daily for 3 days. The infected eggs were chilled at 4°C overnight before allantoic fluids were collected.

Virus was initially identified by hemagglutination assay (HA) according to the World Health Organization (WHO) methodology (WHO, 2008). Briefly, serial twofold dilutions of tissue culture media or allantoic fluid were made in 50 µl of phosphate -buffered saline (PBS) on 96-well U-bottom plates. To each well, 50 µl of 0.5% (v/v) chicken erythrocytes in PBS was then added. The plates were kept at 4°C for 1 hr, after which the HA titers were determined based on the last dilution showing complete hemagglutination.

Viral RNA was extracted from cell-culture supernatants or allantoic fluid using a viral RNA extraction kit (Qiagen, Valencia, California., USA). The multiplex reverse transcription-polymerase chain reaction (RT-PCR) was used to identify type and subtype of viruses. Using the nucleotide sequence available in the GenBank database, multiple sequence alignment of H5, N1 and M gene were performed using the ClustalX, version 1.8 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX). The H5 and N1 primers were selected from conserved regions of 50 known sequences specific for H5N1 influenza A viruses. The M primers were also selected from conserved regions of at least 50 known sequences from influenza A viruses. Viruses were identified as type-A influenza viruses by using RT-PCR employed the M gene specific primer set (forward primer M-65F: 5´ CCGAGATCGCACAGAGACTTGAAGAT 3´, reverses primers M-400R: 5´ GGCAAGTGCACCAGCAGAATAACT 3´). Subtype was determined using the H5 specific primer set (forward primer H5-155F: 5´ ACACATGCYCARGACATACT 3´, reverse primer H5-699R: 5´CTYTGRTTYAGTGT

TGATGT 3´) and the N1 specific primers set (forward primer N1-1078F: 5´ ATGGTAATGGTGTTTGGATAGGAAG3´, reverse primers N1-1352R: 5´ AATGC TGCTCCCACTAGTCCAG 3´).

The RT-PCR was performed using a One-Step RT-PCR kit (Qiagen) containing the appropriate primer mix. The 5 µl of reaction mixture contained denatured RNA, 10 µl of 5x OneStep RT-PCR buffer (Qiagen), OneStep RT-PCR enzyme, 10 µl of 10 mM dNTP mix (Qiagen), and 6 µl of primer mix (1.25 µmol each). RNase-free water was added to a total volume of 50 µl. Amplification of DNA was carried out at 50°C for 30 min and 95°C for 15 min for reverse transcription followed by 35 cycles of denaturing at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 72°C for 1 min. The PCR ended with a final extension step at 72°C for 10 min. The reference strain of influenza H5N1 virus (A/chicken/Thailand/vsmu-3-CBI/2005) was used as a positive control in the multiplex RT-PCR assays.

Size-specific PCR products (335 base pairs [bp] for M, 544 bp for H5 and 274 bp for N1) that were obtained from the multiplex PCR in several field experiments were sequenced to evaluate the specificity of the assay. The known concentration RNA received from previous identified virus (A/chicken/Thailand/vsmu-3-BKK/2004) was prepared for sensitivity test. Copy number of virus RNAs were calculated by using median tissue culture infected dose values and measuring by *Taqman* real-time RT-PCR according previous methods (WHO, 2008; Ng et al, 2005). To perform sensitivity tests, the RNAs were serially diluted 10-fold, ranging from 10^6 to10 copies/µl. All HA positive samples were identified and subtyped by using multiplex RT-PCR. For samples that were HA positive, but RT-PCR negative of all three targets (M, H5 and N1), attempt were made to detect the Newcastle viruses by using specific primer to RT-PCR (data not shown). For samples that were positive for M gene only, their amplicons were sequenced and nucleotide blasted by using the basic alignment

sequence tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These M positive samples also were subtyped by using H1-H15 specific primers for RT-PCR reaction (Lee et al. 2001).

3.2.3 Statistical analysis

Both field data records and laboratory results were entered into a Microsoft Excel, version 2003 Microsoft, Redmond, Washington) worksheet and kept at MoZWE as the avian influenza wild bird surveillance database. The database was analyzed using SPSS version 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The prevalence of avian influenza H5N1 virus isolated from wild bird samples with 95% confidence intervals (CI) were determined, and Pearson's chi-square analysis was used to determine significantly different prevalence results in each field category. However, results from the different capture technique were amalgamated to determine the final result.

3.3 Results

Overall, 60 out of 6,263 pooled samples (1.0%, 95% CI: 0.7, 1.2) tested positive for H5N1 virus. The peak annual prevalence was found in the first year of the outbreak and the annual prevalence significantly decreased in the following years ($p<0.0001$). Between 2005 and 2006, the annual prevalence of the virus remained stable, but rose significantly in 2007 (chi-square, $p<0.005$). However, these overall annual prevalence contained variation in species. The positive pooled samples collected throughout this period were taken from 16 different wild bird species in 12 families (Table 3.1, including rock pigeon (*Columba livia*), tree sparrow (*Passer montanus*), common myna (*Acridotheres tristis*), Asian pied starling (*Sturnus contra*), common koel (*Eudynamys scolopacea*), black drongo (*Dicrurus macrocercus*), white-vented myna (*Acridotheres grandis*), scaly-breasted munia (*Lonchura punctulata*), plain backed sparrow (*Passer*

flaveolus), unidentified pond- heron species, unidentified heron species*, unidentified dove species*, (all residential species), the Kentish plover (*Charadrius alexandrinus*) and brown-headed gull (*Larus brunnicephalus*) and Asian open bill stork (*Anastomus oscitans*). (all winter visitors) and duck species* (both residential and winter visitors) (Lekagul and Round, 1991). Even though many studies stated that wild waterfowl play role as natural reservoirs of avian influenza viruses (Munster et al., 2007; Stallknecht and Shane, 1988; Webster et al., 2007a), there was no significant difference between H5N1 detection in waterfowl and non-waterfowl in this study.

Interestingly, there was no significant difference between prevalence of H5N1 detection in waterfowl and non-waterfowl groups in this survey. All 178 pooled samples from juvenile birds were negative for H5N1 virus, whereas 31 of 4,899 (0.6%) samples from adults were positive (95% CI: 0.4, 0.9). However, there were 1,186 samples with no record of age. Overall, 0.6% (95% CI: 0.4, 0.8) of apparently healthy birds (30/ 4,897 pooled samples) tested positive, compared with 4.1% for birds sampled that were found dead (19 of 462 pooled samples, 95%CI: 2.3, 5.9). Families of wild birds that tested positive with their recorded health status are shown in Table 3.1. However, there were 833 samples with unknown health status.

Analysis of the data revealed that samples collected from birds opportunistically found dead, were significantly more likely to test positive to H5N1, than samples collected from apparently healthy birds (chi-square, $P<0.0001$). Tissue samples from carcasses were significantly more likely to be positive for H5N1 (9.9%, 95%CI 5.9, 13.9), than swabs (0.6%, 95%CI 0.4, 0.8; $P<0.0001$). Positive samples were detected from specimens collected from wild birds in 12 of 50 (24%) provinces sampled including Bangkok, Nakhon Sawan, Phra Nakhon Si Ayutthaya, Kanchanaburi, Nakhon Pathom, Suphan Buri, Chanthaburi, Nakhon Phanom, Ratchaburi, Ang Thong, Samut Prakan, and Buri Ram. Analysis of data in

comparison with data on poultry outbreaks showed that, as with the poultry, H5N1 virus was first detected in wild birds in 2004, and that the peak prevalence of both poultry and wild bird outbreaks occurred during this year. Similarly with poultry outbreaks, the frequency of infected wild bird samples increased significantly during winter months ($P< 0.005$). However, positive wild bird cases were only found in the provinces where domestic poultry outbreaks were reported, and wild bird outbreaks apparently did not spread throughout the country at the rate found with outbreaks in poultry (Figure 3.1).

The multiplex RT-PCR products consisted of 335 bp for M gene, 544 bp for H5 gene and 274 bp for N1 gene were visualized by gel electrophoresis. Some positive specimens were subjected to nucleotide sequencing [GenBank accession number EF178520 and EU716171 (M gene); EF178517 and EF178528 (H5 gene); EF178519 and EF178529 (N1 gene) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search to confirm the M, H5, and N1 gene detection]. The sensitivity of the multiplex RT-PCR was determined using 10-fold serial dilutions of the in known concentration RNAs of H5N1 virus. The DNA bands were visible at RNA standard dilution as low as 10^3 copies/ μl.

Table 3.1; Positive wild bird families and their health status in the wild bird surveillance during 2004-2007

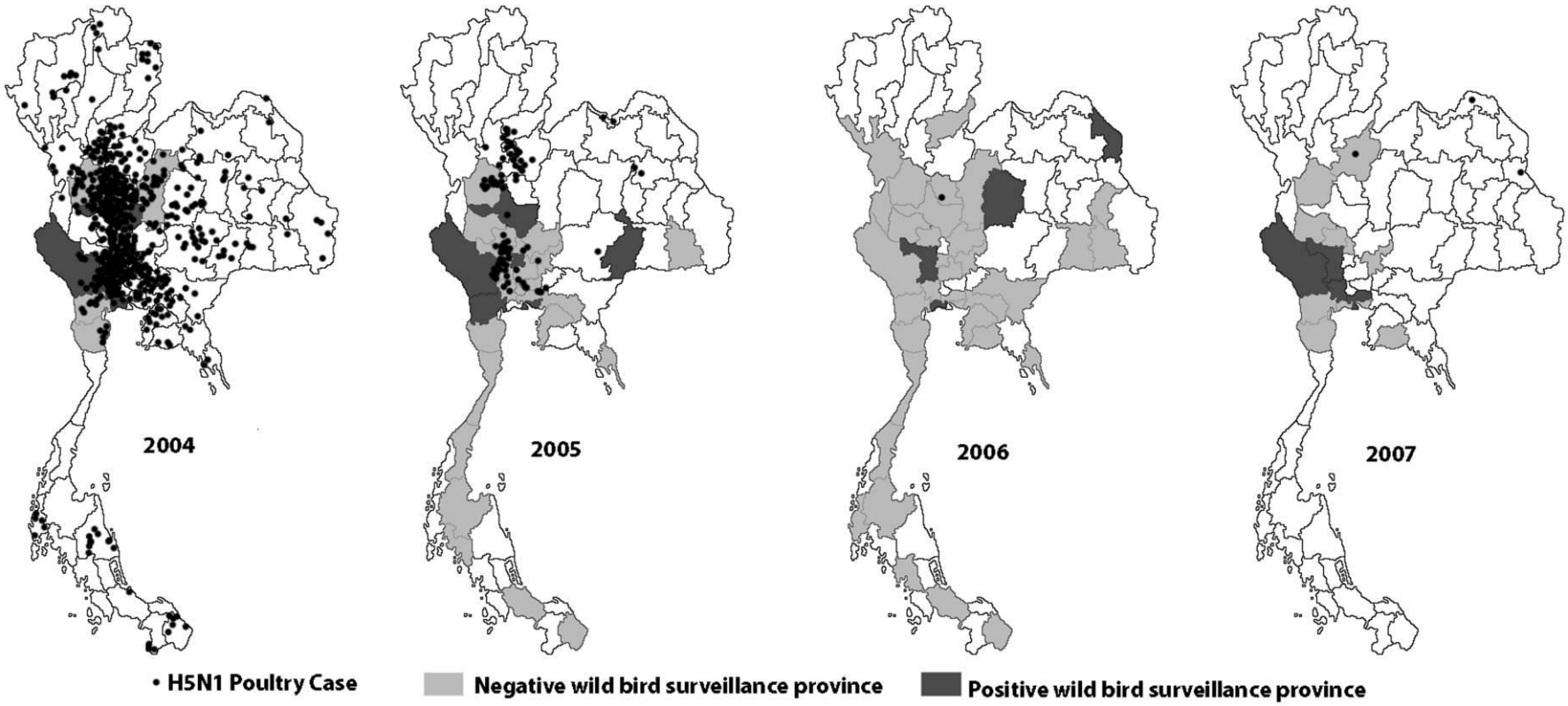
	2004				2005				2006				2007				
Family	H/ UI ^	sick / dead	Total sample	% positive (95% CI)	H/ UI ^	sick / dead	Total sample	% positive (95% CI)	H/ UI ^	sick / dead	Total sample	% positive (95% CI)	H/ UI ^	sick / dead	Total sample	% positive (95%CI)	**Total samples**
Anatidae	1*	0	14	7.14 (0.0, 20.6)	0	0	28	0 (0.0, 0.0)	0	0	22	0 (0.0, 0.0)	0	0	20	0 (0.0, 0.0)	85
Ardeidae	0	0	98	0 (0.0, 0.0)	0	0	236	0 (0.0, 0.0)	0	1	298	0.34 (0.0, 1.0)	2	0	109	1.83 (0.0, 4.4)	744
Charadriidae	0	0	5	0 (0.0, 0.0)	0	0	1	0 (0.0, 0.0)	1	0	41	2.44 (0.0, 7.2)	0	0	35	0 (0.0, 0.0)	83
Ciconiidae	7*	5	45	26.67 (13.7, 39.6)	5	0	547	0.91 (0.1, 1.7)	0	0	345	0 (0.0, 0.0)	0	0	82	0 (0.0, 0.0)	1,036
Columbidae	1*	1	148	1.4 (0.0, 3.2)	2	2	839	0.61 (0.0, 1.2)	3	5	250	3.2 (1.0, 5.4)	2	4	337	2.46 (0.5, 4.4)	1,594
Cuculidae	0	0	0	0 (0.0, 0.0)	0	0	0	0 (0.0, 0.0)	0	1	3	33.33 (0.0, 86.7)	0	0	4	0 (0.0, 0.0)	8
Dicruridae	0	0	1	0 (0.0, 0.0)	0	0	0	0 (0.0, 0.0)	1	0	9	11.11 (0.0, 31.6)	0	0	0	0 (0.0, 0.0)	11
Emberizidae	0	0	107	0 (0.0, 0.0)	3	0	566	0.53 (0.0, 1.1)	0	0	37	0 (0.0, 0.0)	3	0	97	3.09 (0.0, 6.5)	813
Estrildidae	0	0	0	0 (0.0, 0.0)	0	0	31	0 (0.0, 0.0)	0	0	40	0 (0.0, 0.0)	1	0	17	5.88 (0.0, 17.1)	89
Laridae	0	0	3	0 (0.0, 0.0)	1*	0	23	4.35 (0.0, 12.7)	0	0	28	0 (0.0, 0.0)	0	0	55	0 (0.0, 0.0)	110
Sturnidae	0	0	88	0 (0.0, 0.0)	1*	0	220	0.45 (0.0, 1.3)	1	0	118	0.85 (0.0, 2.5)	5	0	135	3.7 (0.5, 6.9)	568
Unidentified	0	0	0	0 (0.0, 0.0)	0	0	2	0 (0.0, 0.0)	0	0	17	0 (0.0, 0.0)	1	0	2	50 (0.0, 119.3)	22

	2004				2005				2006				2007				
Family	H/ UI ^	sick / dead	Total sample	% positive (95% CI)	H/ UI ^	sick / dead	Total sample	% positive (95% CI)	H/ UI ^	sick / dead	Total sample	% positive (95% CI)	H/ UI ^	sick / dead	Total sample	% positive (95%CI)	**Total samples**
Others	0	0	43	0 (0.0, 0.0)	0	0	127	0 (0.0, 0.0)	0	0	862	0 (0.0, 0.0)	0	0	128	0 (0.0, 0.0)	1,160
Total	**9**	**6**	**552**	2.72 (1.4, 4.1)	**12**	**2**	**2620**	0.53 (0.3, 0.8)	**6**	**7**	**2,070**	0.63 (0.3, 1.0)	**14**	**4**	**1,021**	1.76 (1.0, 2.6)	

^H/UI = Healthy appearance/ Unidentified health status

* number of pooled wild bird samples with unidentified health status

Figure 3.1; Thai provinces where domestic poultry outbreak cases were reported and wild bird surveillance conducted (modified map; Thanapongtharm and Noimoh, 2006)



3.4 Discussion

From the surveillance of wild birds in Thailand from 2004 to 2007, it is apparent that avian influenza H5N1 virus has been detected at a low level in wild bird populations since the first wild bird positive sample was found in February 2004. In this study, the annual prevalence in 2005 and 2006 significantly decreased compared to 2004, and then rose significantly in 2007.

The surveillance program operating during 2004-2006 was a more general survey with random surveillance over a wider area of the country. In 2007, the surveillance was targeted towards areas that had poultry outbreaks; this targeted approach may explain the increase in prevalence observed in 2007, but prevalence still was lower than observed in 2004. It should be noted that true prevalence estimates are based on the assumption that only one sample in the pool was positive; however, in this study prevalence (based on number of infected pools) may be overestimated because pooled samples containing between one and four individual bird samples. Our results suggest that spillover of HPAI H5N1 viruses from poultry to wild birds is an important factor. However, it is still not clear whether the virus persists in wild birds in the absence of detectable HPAI H5N1 in domestic birds. Other possible HPAI H5N1 virus sources would include contaminated environments from previous outbreaks and/or subclinical infected domestic poultry; surveillance for HPAI H5N1 in poultry in Thailand is mostly based on detection of clinical signs.

Previous studies have reported that avian influenza viruses are most often isolated from juvenile birds (Stallknecht and Shane 1988). Stallknecht and Brown (2007) reported that prevalence of avian influenza virus infection in juvenile ducks can exceed 30% in premigrating season. In contrast, in this study all 178 samples from juvenile birds were negative for H5N1, whereas prevalence of samples from adults was 0.6%; however age data were not available for 29 positive samples. Some factors that could have contributed to this

result include inaccurate age classification, insufficient samples of juveniles for specific species, insufficient age distribution at the point of sampling and location, and variations in age and species susceptibility (specifically related to population immunity). In addition, there may have been some bias in the current study, because sampling of wild species was mainly done at feeding areas where immature birds are less common. However, if species interaction is a factor in the transmission pathway for wild species, immature animals may have less time and spatial chance (in term of movement from their nesting sites) to be infected. In addition, immature birds are more susceptible to HPAI H5N1 (Pantin-Jackwood et al 2007), and may have been more likely to die after infection.

In our surveillance, 4.1% of 462 found dead birds were infected with H5N1 virus. However, wild bird carcasses are difficult to detect in the wild; Wobeser and Wobeser (1992)found that 70% of bird carcasses were removed by natural causes within 24 hr. In addition, Brown et al. (2008) stated that HPAI-infected wild birds can shed the virus before and after symptomatic onset. It is likely that different bird species have varying susceptibility to HPAI H5N1 infection and therefore some wild bird species could be expected to be more resistant to this disease (Boon et al., 2007). Some apparently healthy wild birds were also positive for H5N1 virus in this study. Overall, 50% (30/60) of the positive samples were collected from apparently healthy birds, 32% (19/60) from dead birds, and the health status of the remainder (11/60) was not reported.

One of main transmission pathways for waterfowl is the fecal-oral route via contaminated water (Brown et al 2007). It has been demonstrated that avian influenza viruses can persist in water and remain infective for extended durations at temperatures that are compatible with field conditions (28°C and 17°C; Stallknecht et al 1990). Thus, contamination and persistency of the viruses in environment may play an important role in the disease transmission. Additional studies on species susceptibility, virus persistence, and duration and level of virus

shedding are required to understand the pattern of H5N1 virus circulation in wild bird populations.

Existing surveillance data for avian influenza outbreaks in poultry in Thailand provided via a collaboration between MoZWE and DLD, the DLD website (http://www.dld.go.th/home/bird_flu/birdflu.html), and the OIE website (http://www.oie .int/downld/AVIAN%20INFLUENZA/A_AI-Asia.htm), were reviewed and results were compared to our wild bird surveillance data. Outbreaks of HPAI H5N1 in wild birds were first detected in 2004 as well as in domesticated poultry (Tiensin et al., 2007). In this survey, only 12 provinces out of 50 had positive wild birds found, whereas poultry outbreaks were found in 60 of the 73 provinces throughout Thailand (Tiensin et al., 2005). Thus, the outbreaks in the wild birds do not appear to have spread widely through out the country. Unlike the general pattern of outbreaks in poultry where the disease occurred with higher frequency in the central provinces due to the high density of rice fields and paddling ducks (Gilbert et al., 2006), outbreaks in wild birds were only found in those provinces where domestic poultry outbreaks were reported.

Poultry outbreaks increased significantly during winter (from November to February) compared with summer (from March to May) and the rainy (from June to October) seasons (Thanapongtharm and Noimoh, 2006); this temporal pattern also was similar to the seasonal frequency of positive wild bird samples detected in this study. Many factors may be involved in this spread, not only through the movement of wild bird species, but also through the movement of humans, domestic poultry, poultry products, farm waste and poultry feed. Understanding the interaction of all of these transmission pathways in the epidemiology of H5N1 avian influenza will contribute substantially to the long term control of H5N1virus.

In summary, outbreaks of HPAI in wild bird populations in Thailand occurred subsequent to outbreaks in domestic poultry. There was a decrease in the number of infected wild birds between 2004 and 2006; however, the prevalence increased in 2007 which may be associated with targeted surveillance. The infected wild bird species shared habitat and feeding areas with humans and/or domesticated poultry. Based on detection of virus in healthy birds it is possible that some wild bird species may be less susceptible to HPAI H5N1 viruses. In Thailand, the movement of wild bird species is considered to be of lower risk than movements of poultry in the spread of HPAI, but wild birds may play a role in the local persistence and transmission of the virus. Therefore, it is important to conduct additional studies to more fully understand the pattern of viral transmission in wild bird populations, contamination and persistence of the virus in environment, and the relationships between species and factors involved in the spread of HPAI H5N1.

Chapter 4

STUDY OF RISK FACTORS FOR HPAI H5N1 INFECTION IN SMALL POULTRY FARMS USING A QUESTIONNAIRE SURVEY

4.1 Introduction

Even though outbreaks of HPAI H5N1 have had a dramatic impact on the social and economic structure of Thailand, the spread and transmission pathways of the virus between poultry and wild birds remains unclear. As HPAI H5N1 viruses can transfer between infected poultry and wild birds (Lubroth, 2006), it is important to understand local wild bird ecology and behaviour and the interaction between wild birds and poultry. A questionnaire was designed to gain more information on the structure of villages and local farms, agricultural practices adopted, and attitudes, knowledge and awareness of villagers about AI. Outcomes of this study were used to identify risk factors for conducting the risk assessment outlined in Chapter 7.

4.2 Materials and Methods

4.2.1 Questionnaire design and trial

A questionnaire was designed to collect data about farm types and practices, number and type of poultry kept, history of HPAI outbreaks on the farm or in the district, wild birds commonly observed, location of wild bird roosting sites, and the knowledge and attitudes of

villagers to disease control of HPAI. Villagers were shown photographs of wild birds to reduce the misidentification of species. Both multiple choice and open ended questions were included in the questionnaire.

Once all questions had been formulated and compiled, the draft questionnaire was proof read and restructured by the project supervisors. A local ornithologist was asked to comment on the photo album of wild birds, resulting in only photos of common wild bird species being included in the album based on a bird guide for Thailand (Lekagul and Cronin, 1974).

After editing, a pilot version of the questionnaire was administered to five households in other villages within the same subdistricts but outside of the study site coverage. Questions that confused the respondents, led to misunderstandings, or were too difficult to recall appropriate answers were altered. Field veterinarians and scientists (n = 9) at the VSMU, who were going to be interviewers during the study, were asked to read though the questionnaire and comment on the questions. The English version of the final questionnaire used is attached in Appendix III. Permission was obtained from the Murdoch University Human Ethics Committee to administer the questionnaire to farmers in Thailand.

4.2.2 Study site design and plan

The study site area was visited prior to conducting the interviews. An official letter was sent to the local office of the DLD requesting permission to obtain information on the number of villagers, households, and domestic poultry present in the targeted villages and for their collaboration and field support. Thirty villages located in four sub-districts (Banglane, Bangpasri, Bangsripa, and Klongnokkatong), within the Banglane District, Nakhon Pathom province were chosen for inclusion in this study. Selection of villages was based on proximity to the wild bird surveillance area (detailed in Chapter 5). Once the villages were identified the

location and number of respondents (or households) owning poultry were obtained from the DLD. Respondents were selected based on two criteria: owning poultry and living within the thirty villages. Up to ten households were interviewed in each village.

The questionnaires were administered over a two month period on eight separate days with questionnaires administered to three to four villages each sampling day. The list of the villages sampled and their locations and the date of survey is outlined in Table 4.1. The survey team included one health service officer from the local DLD office, six field veterinarians and two field scientists from the MoZWE, VSMU.

4.2.3 Field questionnaire study

On each survey day, sets of questionnaire sheets with a wild bird photo album were given to interviewers. The survey team travelled into the study sites with local staff from the DLD to meet with the assigned DLD's local health service volunteers. The volunteers then took the team to their villages. Interviewers were dropped off at houses based on the list of households with poultry. Permission was received from each respondent (usually the house-owner) prior to administering the questionnaire. If the owner refused or was not available, the interviewer then moved to the next house. Each interview lasted between 30 and 45 minutes. All questions were read and answers transcribed by the interviewers. Permission was also asked to see the household's poultry rearing areas and/or farms. As HPAI H5N1 outbreaks had affected the areas previously, a number of villagers were no longer keeping poultry. Thus, some villages had less than ten households who were keeping poultry and in this situation all poultry-keeping-households were interviewed resulting in fewer interviews being conducted in these villages. Once the interview was finished, the interviewers moved to the next selected house or farm. At the end of each survey trip, the volunteers were paid and the questionnaires were collected from the interviewers.

4.2.4 Data analysis

Data from the questionnaires were entered into a Microsoft Excel spreadsheet and analysed with SPSS (Version 17.0) and/or Statistix 9 (Analytical Software, Tallahassee). Statistical analyses were separated into two parts: descriptive and inferential analyses. Descriptive analyses included frequency, percentiles, mean, and range. Households were categorised according to their answer to a question on a history of an outbreak of HPAI H5N1 in their poultry flock (outbreak and non-outbreak groups).

A range of putative risk factors for outbreaks were identified and the percentage of households with and without outbreaks and these factors and their 95% confidence intervals were calculated. Univariable analyses were then calculated using Pearson's Chi-square test for independence, Fisher's exact test and odds ratios and their 95% confidence intervals for categorical variables and an analysis of variance for continuous variables. Subsequently to performing univariable analyses a multivariable logistic regression model was generated (McQuiston et al., 2005). Variables that had a p value ≤ 0.25 on the univariable tests were offered for inclusion into the logistic regression model (Giuseppe et al., 2008; Kung et al., 2007) The model was built using a backward conditional method in SPSS. As well, a random effect and Hosmer Lemeshow statistic was calculated in the model. Odd ratios with 95% confidence intervals were calculated for the final model which included variables with significance ($p \leq 0.05$).

Table 4.1; Timetable of the field questionnaire study

Date	Village names, Sub district
22 January 2008	Tongkung village M2, Banglane Bangplaim village M3, Banglane Bangyung village M4, Banglane Bangyung village M4, Banglane
20 February 2008	Paikokwou village M11, Banglane Klongbanglane village M12, Banglane Klongbanglane village M1, Bangsripa Tontarn village M2, Bangsripa
27 February 2008	Thachang villages M3 and M10, Bangsripa Klongpitsamai village M4, Bangsripa
13 March 2008	Baankong village M5, Bangsripa Bangsomkling village M8, Bangsripa Bangpainard villages M6 and M7, Bangsripa
17 March 2008	Bangpasi village M1, Bangpasi Rangkumhyard villages M2 and M3, Bangpasi Rangnamsai village M4, Bangpasi
2 April 2008	Taladrangkratom villages M5 and M6, Bangpasi Klongmhomcham village M10, Bangpasi Klongsamiantra village M11, Bangsripa
3 April 2008	Baanaow village M7, Banglane Taladbanglane village M8, Banglane Klongsiriraj village M9, Banglane Klongsiriraj village M10, Banglane
9 April 2008	Klongrangkratom village M12, Bangpasi Klongpramorpisai village M13, Bangpasi Bangpasi village M10, Klongnokkratong

4.3 Results

The questionnaires were administered to 239 villagers (149 males and 88 females) originating from the 30 villages. The average age of the respondents was 49.2 years. The occupations of most respondents were farmers and/or local construction workers (Table 4.2). The majority of respondents (95.7%) had at least finished primary school education. The number of people in a household ranged from 1 to 40, with a mean of 5 people. A variety of poultry species were kept including native chickens or fighting cocks, layer and broiler chickens, layer and broiler ducks, geese and pet birds. The number of birds owned varied from 1 to 100,000 (mean = 1,853, median = 20). Other animals kept included dogs, cats, rabbits, hamsters, pigs, beef cattle, and fish/prawns (Table 4.3).

The average number of fighting cocks kept in a household (mean = 33) was much lower than for commercial poultry such as layer chickens (11,702), broiler ducks (2,052), and layer ducks (2,534). Approximately half (46.9%) of the villagers kept poultry for their own consumption, 31.8% kept them as pets, 29.5% kept them for local sale, 10.5% kept them as a business, 3.3% kept poultry for breeding and 21.3% kept fighting cocks for cock fighting competitions. Cock fighting competitions and training sessions were conducted daily. Some cock fighting competitions were located in other provinces including Ayutthaya, Rajchaburi, Supanburi, Nontaburi, Samutsakorn, and Pratumthani and were more formal and were held once a week. More than half of the fighting cock owners (56.4% out of 149) took their birds to fighting competitions.

Table 4.2; Summary of respondents' general information

Category	n	%
Gender		
Male	149	62.9
Female	88	37.1
Age		
<20	8	3.4
21-35	32	13.5
36-50	79	33.3
51-65	84	35.4
>65	34	14.3
Educational level completed		
No education	10	4.3
Primary school	176	74.9
Secondary school	21	8.9
High school	14	6.0
College/ University	14	6.0
Occupation		
Farmer	123	53.2
Business	5	2.2
Housewife/Retired	23	10.0
Seller	26	11.3
Student	5	2.2
Worker	49	21.2
Total	231	100.0

Table 4.3; Types and number of animals kept by villagers

Animals	Number of households owning these animals (%)	Mean	Range
Birds			
Native chickens/ fighting cock	182 (78.8)	32	1-200
Broiler chickens	1 (0.4)	7	7
Layer chickens	19 (8.2)	11,702	12-100,000
Chicken breeders	1 (0.4)	7	7
Broiler ducks	27(11.7)	2,052	1-45,000
Layer ducks	57 (24.7)	2,534	1-30,000
Muscovy ducks	1 (0.4)	30	30
Geese	4(1.7)	258	4-1,000
Pet birds	46 (19.9)	7	1-23
Other animals			
Dogs	169 (73.2)	3	1-13
Cats	91 (39.4)	2	1-20
Rabbits	3 (1.3)	3	2-6
Hamsters	1 (0.4)	3	3
Pigs	14 (6.1)	19	1-80
Beef cattle	20 (8.7)	49	1-750
Fish/prawns	17 (7.4)	*	*

* Could not be counted

Only 25.3% (of 194 chicken owners) kept their chickens housed in permanent constructed facilities, compared with 43.5% of 69 duck owners. The majority of chicken owners (39.7% of 194) kept their chickens in cages and/or coops while 35.1% let their chickens run freely in backyards. The majority of duck owners kept their ducks in housing (43.5%) while 27.5% left them free-roam in backyards. Nine duck owners grazed their ducks in paddy fields.

A variety of feedstuffs were given to poultry including commercial feed, self mixed feed (purchased ingredients which were subsequently mixed), kitchen leftovers/free ranging, and unmilled rice only. In Table 4.4 the rations fed to birds is outlined. Chickens were most commonly fed unmilled rice only (52.4%), compared with commercial food for pet birds and self-mixed food for ducks.

Most paddling ducks (66.7%; n=9) in this study travelled only within their sub-districts. Six flocks of paddling ducks were walked by their owners to paddy fields, while two flocks were transported by trucks to other paddy fields. One duck flock was either walked or transported to the fields. The owner of one flock said that his ducks had travelled to Kanchanaburi and Supanburi provinces by road. Five out of nine paddling duck owners said that ducks from other households or villages usually grazed in the same paddy area with their ducks– 'most of the time' 60% (3 out of 5 flocks), and 'sometimes' 40% (2 flocks).

Water given to poultry was sourced from various places depending upon the individual household, with the majority (52.5% of 326 respondents) using tap water (Table 4.5).

Table 4.4; Food given to poultry in the surveyed households

Food categories	N	%
Chickens		
Commercial feed	17	9.0
Self mixed feed	33	17.5
Kitchen leftovers/free ranging	40	21.2
Unmilled rice only	99	52.4
Total	189	100.0
Ducks		
Commercial feed	7	9.3
Self mixed feed	28	37.3
Kitchen leftovers/free ranging	17	22.7
Unmilled rice only	14	18.7
Grazing in paddy fields	9	12.0
Total	75	100.0
Pet birds		
Commercial feed	6	40.0
Self mixed feed	5	33.3
Kitchen leftovers/free ranging	1	6.7
Unmilled rice only	3	20.0
Total	15	100.0

Table 4.5; Source of water used for poultry

Water source	Frequency (n)	Percent
Pond/Lake	27	11.4
River	13	5.5
Own/private well	14	5.9
Community well	39	16.5
Tap water	124	52.5
Others	9	3.8
Using more than one water source		
Pond/Lake + rain water	5	2.1
Own/private well + rain water	1	0.4
Community well +rain	1	0.4
Rain +Tap water	1	0.4
Rain +Pond/lake	2	0.9
Total	236	100.0

One third (33.1%) of villagers had never sold poultry. Of those selling birds, approximately one third (36.2%) sold birds more than four times a year (Figure 4.1). Most owners sold native chickens/fighting cocks within their own village (42.7%) while most owners of layers, ducks and geese (80%, 69% and 100% respectively) sold them to dealers or middlemen. One third of owners (33.3%) selling poultry sold them to middlemen while 31.6% sold poultry to casual buyers (Table 4.6). When collectors (people who deliver/pick-up poultry) and types of poultry sold were compared (Table 4.6), native/fighting cocks were mostly collected by buyers from the owner's place (45.5% out of 55 households), and the majority of layers (66.7% out of 9) and ducks (53.8% out of 26) sold were delivered to the buyers by the owners. Of 187 households 36.8% reported selling poultry during special ceremonies and festivals such as Chinese festivals and the Thai New Year.

Figure 4.1; Frequency of selling poultry per year

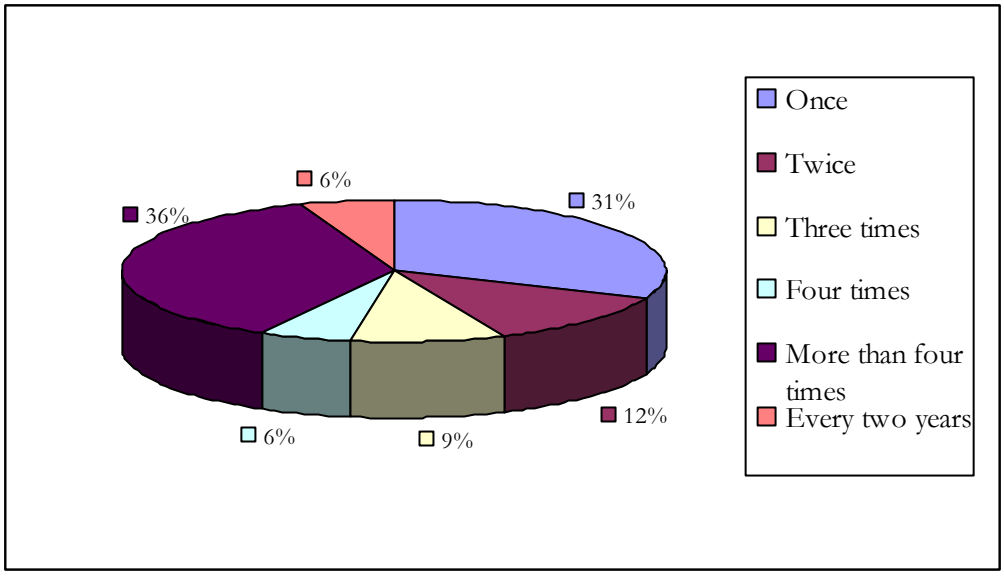


Table 4.6; Comparison of selling procedures and types of poultry sold

Categories	Native / fighting cocks		Layer chickens		Ducks		Geese		Mixed types		N	%
	n	%	n	%	n	%	n	%	n	%		
Poultry sold to												
Markets	1	1.3	-	-	-	-	-	-	-	-	1	0.8
Slaughter	-	-	-	-	1	3.4	-	-	1	7.1	2	1.6
Dealers/ middlemen	10	13.3	8	80.0	20	69.0	1	100.0	4	28.6	43	33.3
In their own village	32	42.7	-	-	1	3.4	-	-	2	14.3	35	27.1
Other villages	18	24.0	-	-	-	-	-	-	3	21.4	21	16.3
Private companies	-	-	2	20.0	4	13.8	-	-	-	-	6	4.7
More than one place	14	18.7	-	-	3	10.3	-	-	4	28.6	21	16.3
Total	**75**	**100**	**10**	**100**	**29**	**100**	**1**	**100**	**14**	**100**	**129**	**100**
Collectors												
Buyers	25	45.5	1	11.1	5	19.2	-	-	5	35.7	36	34.3
Dealers/ middlemen	21	38.2	2	22.2	7	26.9	-	-	4	28.6	34	32.4
Owners	9	16.4	6	66.7	14	53.8	1	100.0	4	28.6	34	32.4
Others	-	-	-	-	-	-	-	-	1	7.1	1	1.0
Total	**55**	**100**	**9**	**100**	**26**	**100**	**1**	**100**	**14**	**100**	**105**	**100**

Poultry were sold at a wide range of ages, depending on the type of poultry and the purpose for keeping them. For example, native chickens or fighting cocks were generally sold between one and 30 months of age, layers between 13 and 20 months of age, and ducks between 15 days to 3 years of age (Table 4.7). Similarly the ages of poultry purchased by households also varied: from one week to one year for native chickens or fighting cocks; one day to one year for layers; and one day to 17 months for ducks. An average of 2 to 3 backyard poultry, such as native chickens or fighting cocks, were sold or purchased per trade. In contrast, commercial poultry, such as layers, ducks and geese, were sold or purchased in larger numbers (Table 4.7). Poultry were purchased from various sources depending on the type of poultry (Table 4.8). Native chickens or fighting cocks were mostly home bred (79.2% out of 154), 7.8% came from other villages, 7.1% were purchased from other households in the same village, and 1.3% were purchased from private companies.

Broilers or layers were mainly supplied by private companies (73.7% of 19) while 10.5% came from households in the same village and from other villages. Only one small flock of eighteen layers were home bred. For ducks, 23.9% of 71 duck owners purchased their ducks from private companies, 19.7% were from other villages or were home bred, and 9.9% from markets or middlemen. Many respondents usually introduced new poultry onto their properties once a year (37.9% out of 103), or up to once every two years (35.0%) (Figure 4.2) Slightly more than half of the 237 respondents (57.4%) ensured that the birds were disease free before purchasing new poultry (Table 4.9). Only 24.8% of 101 households replaced their poultry with an all-in-all-out procedure. Some form of quarantine strategy, including separation of newly received poultry, were applied by 29.4% of the respondents. The period of quarantine ranged from 2 to 120 days, and in some cases poultry were always kept separately (Table 4.10).

Although households kept eggs (n=141) for their own consumption (42.6%) and hatching (46.1%), 34.8% of the households also sold eggs. These eggs were sold to various places including dealers/middlemen (31.7% out of 41 who sold eggs), local and other provincial markets (29.3%), private companies (22.0%), and local shops (17.1%). The frequency of selling eggs varied from daily to weekly and the number sold each time ranged from 4 to 100,000 (Table 4.11).

Approximately half of the respondents (53.5%, n=228) vaccinated their poultry. Newcastle Disease was the most common disease vaccinated against (50% of those households which practiced vaccination). More than half of the vaccinating households (59%, n=100) usually vaccinated their poultry against a variety of other diseases including Fowl cholera, Duck plague, Pox, Infectious coryza, Infectious Bursal Disease and Marek's disease.

The handling and management of poultry manure varied between households. Out of 207 respondents, 42.0% buried or composted their poultry manure and/or litter, 15.9% said that they left it where it was, and 6.3% threw it outside the cages or houses without burying it. Commercial chicken farmers usually burnt the manure from their birds (Table 4.12). Owners of native chickens/fighting cocks, ducks, pet birds or geese discarded the manure in multiple ways as indicated in Table 4.12.

The areas where poultry were kept were cleaned at different intervals. Most poultry keepers cleaned the areas used to house poultry daily (27.0% out of 222 respondents), while 22.1% never cleaned these areas (Table 4.12). Approximately half (46.0%) of 237 respondents used disinfectants when cleaning their poultry keeping areas.

Table 4.7; Ages of poultry traded and number per trade

Poultry trade	Age of poultry (months)			Number of poultry traded at each trade		
	Number of households	Average	Range	Number of households	Average	Range
Sold						
Native/ fighting cocks	89	8.4	1-30	73	3.5	1-25
Layers	11	17.4	13-20	11	8001.8	20-20,000
Ducks	28	15.1	2-36	30	2728.7	2-10,000
Geese	1	4.0	4	1	1000.0	1,000
Purchased						
Native/ fighting cocks	43	7.27	1-12	41	2.2	1-11
Layers	16	3.92	0.03 – 12.1	17	6340.0	30-20,000
Ducks	39	3.38	0.03 – 15.4	47	2149.0	2-10,000
Geese	1	0.03	0.03	1	1000.0	1,000

Figure 4.2; Frequency of purchasing poultry per year

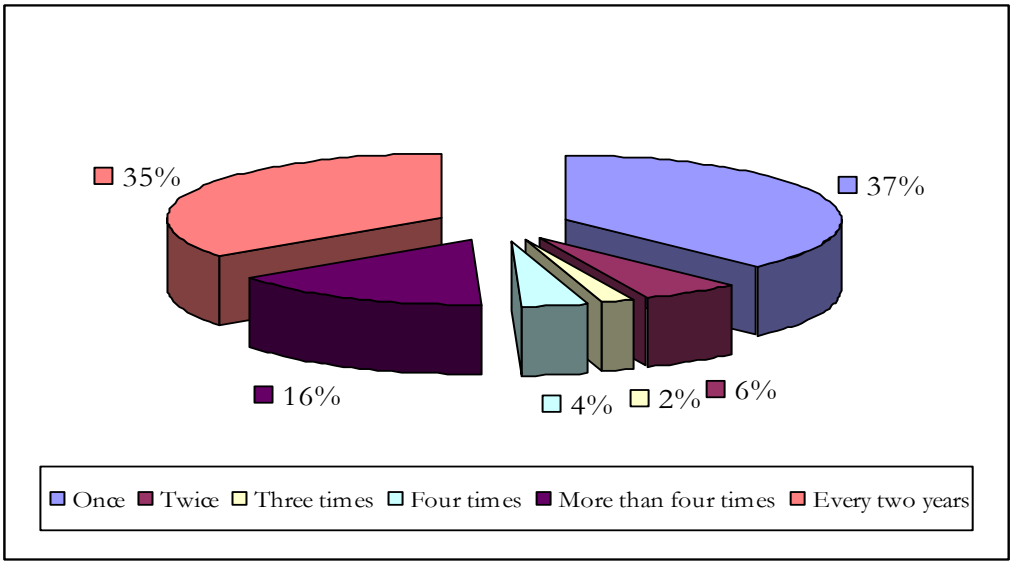


Table 4.8; Sources of new poultry

Poultry source	Native / fighting cocks		Layer/ Broiler chicken		Ducks		Other		Total	
	n	%	n	%	n	%	n	%	n	%
Purchased from one source only										
Bred in house	122	79.2	-	-	14	19.7	-	-	136	52.5
Markets	1	0.6	-	-	7	9.9	4	26.7	12	4.6
Dealers/ middlemen	-	-	-	-	7	9.9	1	6.7	8	3.1
Private companies	2	1.3	14	77.8	15	21.1	-	-	31	12.0
Own village	11	7.1	2	11.1	4	5.6	1	6.7	18	6.9
Other villages	12	7.8	2	11.1	14	19.7	2	13.3	30	11.6
Other	6	3.9	-	-	8	11.3	7	46.7	21	8.1
Purchased from more than one source										
Bred in house +Markets	1	0.6	-	-	-	-	-	-	1	0.4
Bred in house +Private companies	1	0.6	1	5.6	-	-	-	-	2	0.8
Bred in house +Own village	7	4.5	-	-	-	-	-	-	7	2.7
Bred in house +Own village +Other villages	2	1.3	-	-	-	-	-	-	2	0.8
Bred in house +Other villages	4	2.6	-	-	-	-	-	-	4	1.5
Bred in house +Other villages +Others	1	0.6	-	-	-	-	-	-	1	0.4
Dealers/ middlemen +Private companies	-	-	-	-	2	2.8	-	-	2	0.8
Total	**154**	**100.0**	**19**	**100.0**	**71**	**100.0**	**15**	**100.0**	**259**	**100.0**

Table 4.9; Measures implemented to ensure new birds were free from disease

Categories	N	%
How to ensure that new birds are disease free		
Trust seller	30	12.7
Check the birds are healthy	47	19.8
Buy from safe places e.g. standardized company	20	8.4
Trust seller + Check the birds are healthy	22	9.3
Trust seller + Check the birds are healthy + Buy from safe places e.g. standardized company	5	2.1
Trust seller + Check the birds are healthy +concern more on price	1	0.4
Trust seller + Buy from safe places e.g. standardized company	4	1.7
Check the birds are healthy + Buy from safe places e.g. standardized company	2	0.8
Other	5	2.1
Do nothing	101	42.6
Total	237	100.0
Replacement of poultry		
All-in-all-out (whole farm)	25	24.8
Replace birds by house	15	14.9
Replace birds in small batches	15	14.9
Replace birds individually	46	45.5
Total	101	100.0

Table 4.10; Quarantine period

Quarantine period	n	%
≤ 2 wks	10	35.7
> 2 wks – 1 month	4	14.3
> 1 month	2	7.1
Always separated	12	42.9
Total	**28**	**100**

Table 4.11; Frequency and number of eggs traded

Places/persons where eggs sold to	n	Selling frequency (days)	Average number eggs sold per transaction	Range sold per transaction
Dealers/middlemen	13	1 - 7	13,245	30-70,000
Private companies	8	1 - 4	14,381	1250-70,000
Markets	12	1 - 6	12,508	600-100,000
Local shops	5	1 - 3	219	4-1,000

Table 4.12; Management of manure and frequency of cleaning with different poultry types

Categories	Native/ Fighting cocks		Broiler chickens		Layer chickens		Layer ducks		Broiler ducks		Pet birds		Geese		Mixed species	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Manure management																
Throw outside house	12	9.7	-	-	-	-	-	-	-	-	-	-	-	-	1	2.0
Bury or compost	52	41.9	-	-	-	-	8	42.1	1	20.0	2	50.0	1	100.0	23	46.9
Burn on a pile	6	4.8	1	100.0	4	100.0	5	26.3	1	20.0	-	-	-	-	7	14.3
Spread onto fields	3	2.4	-	-	-	-	2	10.5	-	-	-	-	-	-	-	-
Spread around house garden	15	12.1	-	-	-	-	1	5.3	-	-	1	25.0	-	-	2	4.1
Leave where it is	21	16.9	-	-	-	-	2	10.5	3	60.0	1	25.0	-	-	6	12.2
Others	15	12.1	-	-	-	-	1	5.3	-	-	-	-	-	-	10	20.4
Total	**124**	**100.0**	**1**	**100.0**	**4**	**100.0**	**19**	**100.0**	**5**	**100.0**	**4**	**100.0**	**1**	**100.0**	**49**	**100.0**
Frequency of cleaning poultry area																
Every day	46	35.9	-	-	4	44.4	2	8.7	1	16.7	-	-	-	-	7	14.0
Every 2-3 days	17	13.3	-	-	2	22.2	-	-	-	-	-	-	1	100.0	5	10.0
Once a week	15	11.7	-	-	-	-	3	13.0	-	-	1	25.0	-	-	10	20.0
Once a month	10	7.8	1	100.0	1	11.1	1	4.3	-	-	2	50.0	-	-	8	16.0
Never	32	25.0	-	-	-	-	4	17.4	4	66.7	1	25.0	-	-	8	16.0
Every poultry replacement	-	-	-	-	2	22.2	10	43.5	1	16.7	-	-	-	-	8	16.0
When high risk/ had problems	2	1.6	-	-	-	-	-	-	-	-	-	-	-	-	1	2.0
1-4 times a year	6	4.7	-	-	-	-	1	4.3	-	-	-	-	-	-	2	4.0
Not relevant/ grazing	-	-	-	-	-	-	2	8.7	-	-	-	-	-	-	1	2.0
Total	**128**	**100.0**	**1**	**100.0**	**9**	**100.0**	**23**	**100.0**	**6**	**100.0**	**4**	**100.0**	**1**	**100.0**	**50**	**100.0**

The wild birds that were commonly seen (every day) in backyards/ households, farms and paddy fields are listed in Table 4.13 and those seen less frequently (not every day) are recorded in Table 4.14. More than 50% of villagers said that rock pigeons, common mynas and sparrows were commonly seen in backyards, while open bill storks were commonly seen in rice paddy fields. More than 75% of the villagers had seen wild birds feeding together with their poultry. Common wild birds in the areas included pigeons, sparrows, mynas and starlings, doves, bulbuls, pied fantails, koels, magpie-robins, weavers, storks, egrets, water-hens, little grebes, red wattle lapwings and little cormorants.

Nearly all villagers (98.7%) had known about disease outbreaks of avian influenza. Most villagers (39.3%) believed that the disease was introduced by wild birds living close by (Figure 4.3). In Table 4.15 the actions of the villagers if they suspected their birds had avian influenza are outlined. Approximately half (56.5%) would bury the affected birds and 43.9% would report the disease to the authorities.

A range of options were given by villagers on measures necessary to prevent avian influenza affecting their households. More education and awareness towards disease prevention, veterinary advice and a reduction in contact between wild birds and poultry were considered important (Table 4.16). Other preventive measures included vaccination against the disease, having high biosecurity farms, and improvement of hygiene within households/farms including regular disinfection of fomites and poultry keeping areas. Almost 50% of the villagers disinfected their poultry areas regularly; however 30% of villagers did nothing to protect their birds from the disease (Table 4.17).

Washing hands with soap after handling poultry or poultry manure was the most common hygienic procedure that villagers adopted to protect themselves and their family from avian influenza. Approximately 43% of villagers reported that they did not consume sick or dead

birds and they thoroughly cooked poultry products (38.1%; Table 4.18). The most important source of information on the disease for villagers was television (84%) followed by radio, newspaper, and village animal health assistants, respectively (Table 4.19).

Table 4.13; Frequency and percentage of villagers seeing a range of wild birds every day in backyards, farms or paddy fields (n=217)

	Backyard		Farm		Paddy field	
Common name (species/genus)	Frequency	%	Frequency	%	Frequency	%
Asian koel (*Eudynamys scolopaceus*)	69	31.8	4	1.8	22	10.1
Asian open bill stork (*Anastomus oscitans*)	8	3.7	1	0.5	121	55.8
Asian pied starling (*Gracupica contra*)	49	22.6	5	2.3	6	2.8
Black drongo (*Dicrurus macrocercus*)	30	13.8	2	0.9	19	8.8
Bronze winged jacana (*Metopidius indicus*)	1	0.5	1	0.5	24	11.1
Bulbul (*Alophoixus sp.)*	78	35.9	5	2.3	7	3.2
Cattle egret (*Bubulcus ibis*)	4	1.8	1	0.5	35	16.1
Cinnamon bittern (*Ixobrychus cinnamomeus*)	3	1.4	1	0.5	35	16.1
Common moorhen (*Gallinula chloropus*)	5	2.3	2	0.9	30	13.8
Common myna (*Acridotheres tristis*)	116	53.5	13	6	6	2.8
Cotton pygmy goose (*Nettapus coromandelianus*)	3	1.4			11	5.1
Egrets (*Ardea spp.*)	13	6	3	1.4	92	42.4
Greater coucal (*Centropus sinensis*)	76	35	7	3.2	22	10.1
Indian roller (*Coracias benghalensis*)	14	6.5	1	0.5	5	2.3
Lesser whistling duck (*Dendrocygna javanica*)	10	4.6	5	2.3	31	14.3
Little cormorant (*Phalacrocorax niger*)	22	10.1	17	7.8	63	29

Common name (species/genus)	Backyard		Farm		Paddy field	
	Frequency	%	Frequency	%	Frequency	%
Little egret (*Egretta garzetta*)	5	2.3	4	1.8	77	35.5
Little grebe (*Tachybaptus ruficollis*)	25	11.5	6	2.8	52	24
Munia (*Lonchura sp.*)	12	5.5	1	0.5	4	1.8
Night heron (*Nycticorax spp.*)	15	6.9	4	1.8	41	18.9
Oriental magpie-robin (*Copsychus saularis*)	71	32.7	4	1.8	5	2.3
Pied fantail (*Rhipidura javanica*)	40	18.4			2	0.9
Pond heron (*Ardeola spp.*)	19	8.8	10	4.6	78	35.9
Purple swamphen (*Porphyrio porphyrio*)	3	1.4	2	0.9	18	8.3
Red turtle dove (*Streptopelia tranquebarica*)	78	35.9	8	3.7	6	2.8
Red wattled lapwing (*Vanellus indicus*)	7	3.2	3	1.4	48	22.1
Rock pigeon (*Columba livia*)	143	65.9	17	7.8	13	6
Sparrow (*Passer sp.*)	166	76.5	25	11.5	2	0.9
Spotted dove (*Streptopelia chinensis*)	98	45.2	8	3.7	10	4.6
Swallow (*Hirundo spp.*) and swift (*Apus spp.*)	20	9.2	7	3.2	31	14.3
Weaver (*Ploceus sp.*)	41	18.9	3	1.4	22	10.1
White breasted waterhen (*Amaurornis phoenicurus*)	46	21.2	13	6	65	30
White vented myna (*Acridotheres grandis*)	99	45.6	11	5.1	13	6
Zebra doves (*Geopelia striata*)	102	47	9	4.1	5	2.3

Table 4.14; Frequency and percentage of villagers seeing a range of wild birds less frequently than once a day in backyards, farms or paddy fields (n=217)

	Backyard		Farm		Paddy field	
Common name (species/genus)	**Frequency**	**%**	**Frequency**	**%**	**Frequency**	**%**
Asian koel (*Eudynamys scolopaceus*)	3	1.4	1	0.5	2	0.9
Asian open bill stork (*Anastomus oscitans*)	4	1.8	1	0.5	33	15.2
Asian pied starling (*Gracupica contra*)	1	0.5	-	-	1	0.5
Black drongo (*Dicrurus macrocercus*)	3	1.4	-	-	2	0.9
Bulbul (*Alophoixus sp.*)	4	1.8	-	-	-	-
Cattle egret (*Bubulcus ibis*)	1	0.5	-	-	3	1.4
Common moorhen (*Gallinula chloropus*)	1	0.5	-	-	4	1.8
Common myna (*Acridotheres tristis*)	3	1.4	-	-	1	0.5
Cotton pygmy goose (*Nettapus coromandelianus*)	1	0.5	-	-	5	2.3
Egret (*Ardea sp.*)	1	0.5	3	1.4	3	1.4
Greater coucal (*Centropus sinensis*)	3	1.4	1	0.5	-	-
Indian roller (*Coracias benghalensis*)	8	3.7	1	0.5	3	1.4
Lesser whistling duck (*Dendrocygna javanica*)	2	0.9	1	0.5	21	9.7
Little cormorant (*Phalacrocorax niger*)	1	0.5	3	1.4	3	1.4
Little egret (*Egretta garzetta*)	-	-	1	0.5	6	2.8
Little grebe (*Tachybaptus ruficollis*)	1	0.5	1	0.5	3	1.4
Night heron (*Nycticorax spp.*)	-	-	1	0.5	1	0.5

Common name (species/genus)	Backyard		Farm		Paddy field	
	Frequency	%	Frequency	%	Frequency	%
Oriental magpie-robin (*Copsychus saularis*)	2	0.9	1	0.5	-	-
Munia (*Lonchura sp.*)	1	0.5	-	-	-	-
Pied fantail (*Rhipidura javanica*)	2	0.9	216	99.5	1	0.5
Pond heron (*Ardeola sp.*)	1	0.5	2	0.9	4	1.8
Purple swamphen (*Porphyrio porphyrio*)	-	-	-	-	2	0.9
Red turtle dove (*Streptopelia tranquebarica*)	2	0.9	1	0.5	-	-
Red wattled lapwing (*Vanellus indicus*)	1	0.5	1	0.5	11	5.1
Rock pigeon (*Columba livia*)	-	-	-	-	6	2.8
Sparrow (*Passer sp.*)	-	-	-	-	1	0.5
Swallow (*Hirundo sp.*) and swift (*Apus sp.*)	2	0.9	-	-	9	4.1
Weaver (*Ploceus sp.*)	6	2.8	-	-	2	0.9
White breasted waterhen (*Amaurornis phoenicurus*)	4	1.8	-	-	1	0.5
White vented myna (*Acridotheres grandis*)	4	1.8	-	-	1	0.5
Zebra dove (*Geopelia striata*)	2	0.9	1	0.5	-	-

Figure 4.3; Factors considered by villagers to increase the risk of introducing avian influenza to their poultry (n=239)

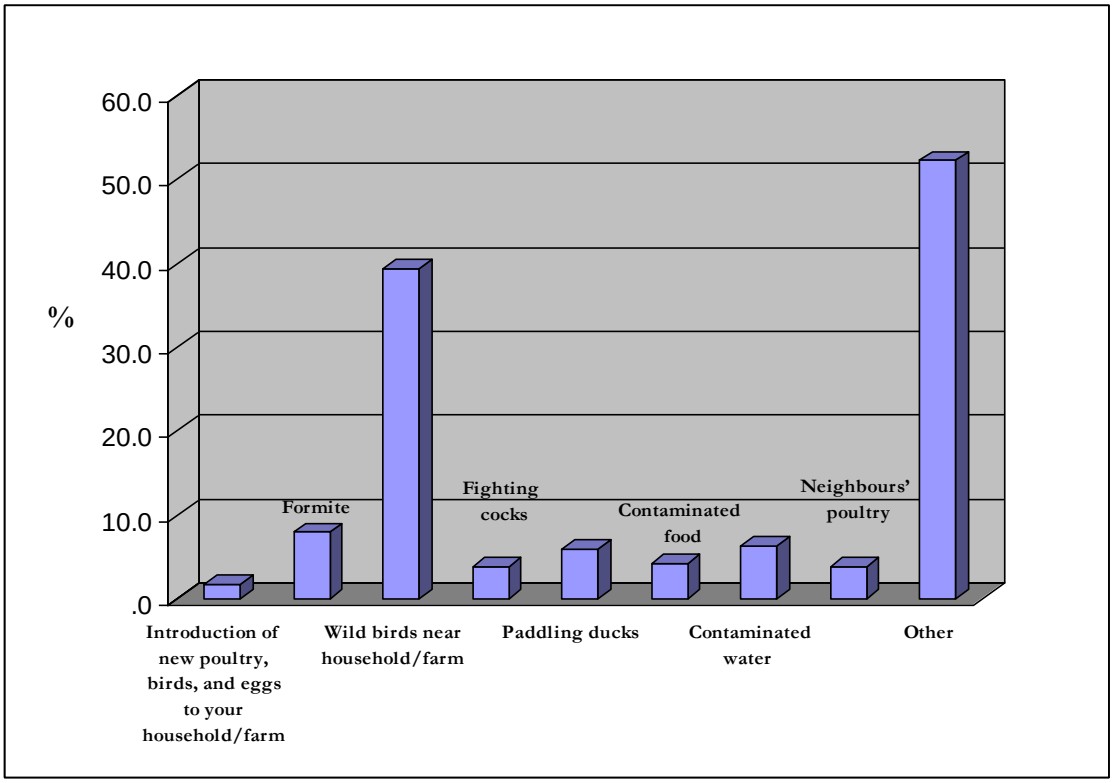


Table 4.15; Villagers' actions if they suspected their birds had avian influenza (n=239)*

Actions taken	Frequency	%
Treat the bird themselves	36	15.1
Throw birds away	6	2.5
Give away or sell birds	2	0.8
Bury birds	135	56.5
Burn birds	22	9.2
Report immediately to authorities	105	43.9
Do nothing	10	4.2
Other	27	11.3

* Some respondents gave more than one answer

Table 4.16; Measures considered by villagers as necessary to prevent/control avian influenza (n=239)

Measures taken	**Frequency**	**%**
Early detection of bird flu in poultry/birds	15	6.3
Higher compensation for culled poultry	4	1.7
Clean feed and water	25	10.5
More education and awareness on disease prevention	38	15.9
Safe source of poultry/birds	14	5.9
Someone to advise them when their birds are sick	36	15.1
Control poultry movement from infected areas	17	7.1
Reduced contact between their poultry and birds from other households	36	15.1
Regular visits from the veterinary department	25	10.5
Others	147	61.5

Table 4.17; Methods villagers were using to protect their birds from avian influenza (n=239)

Methods adopted	**Frequency**	**%**
Regularly disinfect household	112	46.9
Not buying poultry/birds from risky sources	29	12.1
Keeping poultry in protected or fenced areas	40	16.7
Ensuring water and feed was clean	27	11.3
Discouraging casual visitors near poultry	37	15.5

Methods adopted	Frequency	%
Changing clothes and wearing clean shoes after visiting other places	32	13.4
Doing nothing	72	30.1
Other	50	20.9

Table 4.18; Methods adopted by villagers to prevent members of their households from becoming infected with avian influenza (n=239)

Methods adopted	Frequency	%
Not eating poultry that fall sick or die	104	43.5
Eat only well-cooked poultry or eggs	91	38.1
Bury or burn dead poultry	105	43.9
Wash hands with soap after handling poultry or manure	170	71.1
Change clothes after handling poultry or manure	41	17.2
Don't let children play with poultry	86	36.0
Disinfect household regularly	52	21.8
Do nothing	29	12.1
Other	29	12.1

Table 4.19; Sources of information about avian influenza for villagers (N=239)

AI information source	**Frequency***	**%**
Village animal health assistants	72	30.1
Veterinarians or paravets	43	18.0
Village or community leaders	46	19.2
Radio	98	41.0
Television	202	84.5
Newspaper	75	31.4
Pamphlets/brochures/posters	8	3.3
Neighbours, friends or family	44	18.4
Wholesalers or dealers	2	0.8
Other	14	5.9

* Some farmers gave more than one answer

Ninety five (40.1%; 95% CI 33.8, 46.3) of the 237 households surveyed had a history of having an AI outbreak based on animal health history, presence of clinical signs, mortality or morbidity rates, the time sequence of the health problems, and/or a diagnosis by the DLD. In order to classify a household as an outbreak household, evidence of the outbreak described by the respondent needed to be matched with the past history of H5N1 outbreaks in the area for at least two of the three questions in Section 4 of the questionnaire. For example, if respondents said that high morbidity and mortality with clinical signs characteristic of HPAI occurred during the time of a documented HPAI H5N1 outbreak, then the households were classified as AI positive (cases). Households that contained some sick and dead poultry without clinical signs of AI, such as a duck with lameness, were classified as negative (controls or absence of AI). Twenty households could not be classified because they had either moved into the areas after the HPAI outbreaks or had no poultry during the AI outbreak period (Table 4.20).

Layer farms were significantly more likely to be associated with a history of HPAI outbreaks than were other types of poultry (p <0.05) (Tables 4.21 and 4.22). The influence of a range of factors on the presence of outbreaks is outlined in Table 4.23. Factors were classified into three categories: particular farm types, farm practises, and observed wild birds and compared with the presence or absence of disease. The farm type risk factors included poultry farms keeping more than 1,000 birds, layer chicken farms, and commercial farms. The farm practise risk factors included using premixed commercial feed, using water from a community well, selling poultry to dealers or middlemen, sold poultry collected by dealers/middlemen replacing newly received bird into farm individually, selecting healthy birds when purchasing birds, buying poultry from commercial hatcheries, buying poultry from commercialized farms, and spreading poultry manure in a garden and/or selling it as fertilizer. Wild birds likely to be observed in outbreak households included black drongo (seen everyday), greater

coucal (seen everyday), great egret (seen everyday), and little egret (seen less frequently than once a day).

The final multivariable logistic model contained four factors (Table 4.24). The village had no impact on the model when village was added as a random effect to the model. Consequently village was not added to the model. The Hosmer-Lemeshow statistic could not be computed as there were some zero observed values. Seventy percent of cases were correctly identified. Flocks that purchased native chickens/fighting cocks from commercial hatcheries, the replacement of individual birds, and the presence of lesser whistling ducks on the farm were more likely to be associated with a history of an outbreak. In contrast, selecting healthy animals when purchasing animals to ensure they were disease free was protective and consequently less likely to be associated with cases (Table 4.24).

Table 4.20; History of HPAI H5N1outbreak

History of AI outbreak	**Frequency**	**%**	**95%CI**
Controls (no history of outbreak in household)	122	51.5	45.1, 57.8
Cases (history of outbreak)	95	40.1	34.7, 47.2
Unclassified; not included in the model	20	8.4	8.4, 12.0
Total	**237**	**100.0**	**-**

Table 4.21; Types of poultry and HPAI outbreak history

Types of poultry	HPAI outbreak history for flocks			Total
	Negative	Positive	%positive	
Native/ fighting cocks	75	54	41.9	129
Layer chickens	2	12	85.7	14
Broiler chickens	1	0	0.0	1
Layer ducks	14	6	30.0	20
Broiler ducks	3	2	40.0	5
More than one type	22	21	48.8	43
Pet birds	5	0	0.0	5
Total	**122**	**95**	**43.8**	**217**

Table 4.22; The flock size and influence on a history of AI

Poultry type	Mean number in positive flocks	Mean number in negative flocks	*p* value*
Native chicken/fighting cocks	29.23	22.1	0.1
Broiler chickens	0.0	0.1	0.4
Layer chickens	2155.4	141.7	0.1
Breeder chickens	0.1	0.0	0.3
Layer ducks	852.3	446.8	0.3
Broiler ducks	514.2	51.2	0.3

* Based on results from ANOVA's

Table 4.23; Risk factors for the presence of an outbreak in a household/farm (n = 217)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Only one species kept in a household/farm	42.5%		
More than one species kept in a household/farm	48.8%	0.5	0.8 (0.4, 1.5)
More than 1,000 poultry kept	64.9%		
Less than 1,000 poultry kept	39.4%	0.0	2.8 (1.4, 5.9)
Doesn't own native chicken/ fighting cocks	45.8%		
Owns native chicken/ fighting cocks	43.2%	0.8	1.1 (0.6, 2.1)
Doesn't own broiler chickens	44.0%		
Owns broiler chickens	0.0%	1.0	0.6 (0.5, 0.6)
Doesn't own layer chickens	40.4%		
Owns layer chickens	79.0%	0.0	0.2 (0.1, 0.6)
Doesn't own breeder chickens	43.5%		
Owns breeder chickens	100.0%	0.4	0.4 (0.4, 0.5)
Doesn't own layer ducks	44.9%		
Owns layer ducks	40.0%	0.5	1.2 (0.6, 2.3)
Doesn't own broiler ducks	43.5%		
Owns broiler ducks	46.2%	0.8	0.9 (0.4, 2.0)
Doesn't own pet birds	44.6%		
Owns pet birds	40.8%	0.6	1.2 (0.6, 2.2)
Doesn't keep poultry for home consumption	58.4%		
Keeps poultry for home consumption	53.9%	0.5	1.2 (0.7, 2.1)
Doesn't keep poultry to sell as breeders	56.0%		
Keeps poultry to sell as breeders	62.5%	1.0	0.8 (0.2, 3.3)
Doesn't keep poultry as pets	53.7%		
Keeps poultry as pets	61.8%	0.3	0.7 (0.4, 1.3)
Doesn't keep poultry for selling locally	59.1%		
Keeps poultry for selling locally	52.2%	0.3	1.3 (0.8, 2.3)
Doesn't keep poultry for selling commercially	59.1%		
Keeps poultry for selling commercially	33.3%	0.0	2.9 (1.2, 7.1)
Chickens not free ranging	46.5%		
Chickens free ranging	38.7%	0.3	1.4 (0.8, 2.4)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Chickens not kept in a cage/coop	46.5%		
Chickens kept in a cage/coop	38.7%	0.3	1.4 (0.8, 2.4)
Chicken not housed	41.2%		
Chicken housed	53.2%	0.1	0.6 (0.3, 1.2)
Ducks not free ranging	45.0%		
Ducks free ranging	31.6%	0.3	1.8 (0.7, 4.8)
Ducks not kept in a cage/coop	44.1%		
Ducks kept in a cage/coop	33.3%	0.7	1.6 (0.3, 8.8)
Ducks not housed	42.7%		
Ducks housed	50.0%	0.4	0.8 (0.4, 1.6)
Ducks not grazing in rice paddy fields	44.3%		
Ducks grazing in rice paddy fields	20.0%	0.4	3.2 (0.4, 29.0)
Pet birds not free ranging	43.5%		
Pet birds free ranging	100.0%	0.4	0.4 (0.4, 0.5)
Pet birds not kept in a cage/coop	44.8%		
Pet birds kept in a cage/coop	14.3%	0.1	4.9 (0.6, 41.1)
Poultry housing had no roof	40.2%		
Poultry housing had roof	48.4%	0.2	0.7 (0.4, 1.2)
Poultry housing had no solid wall	43.2%		
Poultry housing had solid wall	47.1%	0.7	0.9 (0.4, 1.8)
Poultry housing had no non-solid wall	42.6%		
Poultry housing had non-solid wall	46.4%	0.6	0.9 (0.5, 1.5)
Poultry housing had no solid floor	41.4%		
Poultry housing had solid floor	53.5%	0.2	0.6 (0.3, 1.2)
Poultry housing had no bedding	43.6%		
Poultry housing had bedding	44.8%	0.9	1.0 (0.4, 2.1)
Not sell/offer/give away poultry during festivals	42.0%		
Sell/offer/give away poultry during festivals	46.5%	0.5	0.8 (0.5, 1.4)
No premixed commercial feed fed to chickens	40.5%		
Premixed commercial feed fed to chickens	82.4%	0.0	0.2 (0.0, 0.5)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Self-mixed feed or purchased ingredients not fed to chickens	44.4%		
Self-mixed feed or purchased ingredients fed to chickens	30.0%	0.5	1.9 (0.5, 7.4)
Self-mixed feed and commercial food fed to chickens (not free range)	42.8%		
Self-mixed feed and commercial food not fed to chickens (free range)	52.2%	0.4	0.7 (0.3, 1.6)
No kitchen leftovers/ Chickens provided with feed	45.0%		
Kitchen leftovers/Let chickens find own feed	37.8%	0.4	1.3 (0.7, 2.8)
Chickens not fed only unmilled rice	43.8%		
Chickens only fed unmilled rice	43.8%	1.0	1 (0.6, 1.7)
No premixed commercial feed fed to ducks	43.1%		
Premixed commercial feed fed to ducks	66.7%	0.4	0.4 (0.1, 2.1)
Self-mixed feed or purchased ingredients (self mixed food) fed to ducks	42.8%		
Self-mixed feed or purchased ingredients (self mixed food) not fed to ducks	56.3%	0.3	0.6 (0.2, 1.6)
Self-mixed feed and commercial food fed to ducks (no free range)	44.0%		
Self-mixed feed and commercial food not fed to ducks (free range)	37.5%	1.0	1.3 (0.3, 5.6)
No kitchen leftovers/ducks did not free range	44.8%		
Kitchen leftovers/Let ducks find own feed	28.6%	0.3	2.0 (0.6, 6.7)
Ducks fed unmilled rice with other food	44.6%		
Ducks only fed unmilled rice	30.8%	0.4	1.8 (0.5, 6.1)
Ducks not grazing in rice paddy fields	44.7%		
Ducks grazing in rice paddy fields	27.3%	0.4	2.2 (0.6, 8.3)
No premixed commercial feed fed to pet birds	43.7%		
Premixed commercial feed fed to pet birds	50.0%	1.0	0.8 (0.1, 12.6)
Self-mixed feed or purchased ingredients fed to pet birds	44.4%		
Self-mixed feed or purchased ingredients fed to pet birds	0.0%	0.3	0.6 (0.5, 0.6)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Self-mixed food and commercial food not fed to pet birds (no free range)	44.0%		
Self-mixed feed and commercial food fed to pet birds (free range)	0.0%	1.0	0.6 (0.5, 0.6)
Pond or lake water not used for poultry	44.4%		
Pond or lake water used for poultry	39.3%	0.6	1.2 (0.6, 2.8)
River water not used for poultry	44.4%		
River water used for poultry	30.0%	0.5	1.9 (0.5, 7.4)
Own well water not used for poultry	42.6%		
Own well water used for poultry	60.0%	0.2	0.5 (0.2, 1.4)
Community well water not used for poultry	40.0%		
Community well water used for poultry	62.2%	0.0	0.4 (0.2, 0.8)
Rain water not used for poultry	44.0%		
Rain water used for poultry	0.0%	1.0	0.6 (0.5, 0.6)
Piped or tap water not used for poultry	49.5%		
Piped or tap water used for poultry	39.0%	0.1	1.5 (0.9, 2.6)
Natural water sources e.g. paddy fields were not used	44.3%		
Other natural water sources e.g. paddy fields were used	28.6%	0.5	2.0 (0.4, 10.5)
Poultry not sold to markets	43.9%		
Poultry sold to markets	33.3%	1.0	1.6 (0.1, 17.5)
Poultry not sold for slaughter	44.2%		
Poultry sold for slaughter	0.0%	0.5	0.6 (0.5, 0.6)
Poultry not sold to wholesalers/ dealers	39.3%		
Poultry sold to wholesalers/ dealers	61.4%	0.0	0.4 (0.2, 0.8)
Poultry not sold to people in the same village	45.2%		
Poultry sold to people in the same village	37.5%	0.4	1.4 (0.7, 2.8)
Poultry not sold to people in another village	44.3%		
Poultry sold to people in another village	35.7%	0.5	1.4 (0.5, 4.4)
Poultry not sold to private companies	43.1%		
Poultry sold to private companies	66.7%	0.4	0.4 (0.1, 2.1)
Poultry sold to one place only	44.3%		
Poultry sold to more than one place	41.2%	0.7	1.1 (0.5, 2.4)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Persons who collect sold poultry			
Collectors were not buyers	46.1%		
Collectors were buyers	32.4%	0.1	1.8 (0.8, 3.8)
Collectors were not owners of poultry	44.7%		
Collectors were owners of poultry	39.5%	0.6	1.2 (0.6, 2.5)
Collectors were not dealers/ middlemen	39.4%		
Collectors were dealers/ middlemen	64.9%	0.0	0.4 (0.2, 0.7)
Where do you usually buy or acquire new poultry?			
Native chickens/fighting cocks			
Not bred by the respondent	46.3%		
Bred by the respondent	41.8%	0.5	1.2 (0.7, 2.1)
Not purchased from markets	43.5%		
Purchased from markets	100.0%	0.4	0.4 (0.4, 0.5)
Not buy from commercial hatcheries	40.3%		
Buy from commercial hatcheries	87.5%	0.0	0.1 (0.0, 0.4)
Not buy from the same village houses/farms	44.6%		
Buy from the same village houses/farms	33.3%	0.4	1.6 (0.5, 4.9)
Not buy from other village houses/farms	43.4%		
Buy from other village houses/farms	50.0%	0.7	0.8 (0.2, 2.5)
Layer chickens\broiler chickens			
Not bred by respondent	43.5%		
Bred by respondent	100.0%	0.4	0.4 (0.4, 0.5)
Not bought from commercial hatcheries	42.4%		
Bought from commercial hatcheries	64.3%	0.1	0.4 (0.1, 1.3)
Not bought from the same village house/farm	43.7%		
Bought from the same village house/farm	50.0%	1.0	0.8 (0.1, 12.6)
Not bought from other village houses/farms	43.7%		
Bought from other village houses/farms	50.0%	1.0	0.8 (0.1, 12.6)
Ducks			
Not bred by the respondent	42.7%		
Bred by the respondent	61.5%	0.2	0.5 (0.2, 1.5)
Not bought from markets	44.8%		
Bought from markets	0.0%	0.1	0.6 (0.5, 0.6)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Not purchased from wholesalers/ dealers	43.9%		
Purchased from wholesalers/ dealers	40.0%	1.0	1.2 (0.2, 7.2)
Not purchased from commercial hatcheries	43.5%		
Purchased from commercial hatcheries	47.1%	0.8	0.9 (0.3, 2.3)
Not purchased from the same village house/farm	44.1%		
Purchased from the same village house/farm	25.0%	0.6	2.4 (0.2, 23.2)
Not purchased from houses/farms located in other village	43.4%		
Purchased from houses/farms located in other village	50.0%	0.6	0.9 (0.3, 2.3)
Other birds			
Not purchased from markets	44.1%		
Purchased from markets	25.0%	0.6	2.4 (0.2, 23.2)
Not purchased from the same village houses/farms	44.0%		
Purchased from the same village houses/farms	0.0%	1.0	0.6 (0.5, 0.6)
Not purchased from other village houses/farms	44.2%		
Purchased from other village houses/farms	0.0%	0.5	0.6 (0.5, 0.6)
How do you ensure that birds are healthy when purchased?			
Seller not known	44.6%		
Seller known and trusted	38.7%	0.5	1.3 (0.6, 2.8)
No preference was made in selection	49.3%		
Healthy animals specifically selected	31.3%	0.0	2.1 (1.2, 3.9)
Not only purchase from commercialized farm	40.7%		
Only purchase from commercialized farm	64.3%	0.0	0.4 (0.2, 0.9)
How do you introduce new poultry into your household/ farm?			
All-in-all-out replacement not used	42.3%		
Replace poultry with all-in-all-out system (whole farm)	56.5%	0.2	0.6 (0.2, 1.4)
All of the birds in a house not replaced at one time	43.4%		
All birds in a house replaced at the one time	50.0%	0.6	0.8 (0.3, 2.3)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Birds not replaced in small batches	42.4%		
Birds replaced in small batches	64.3%	0.1	0.4 (0.1, 1.3)
Birds not replaced individually	39.5%		
Individual birds replaced	60.0%	0.0	0.4 (0.2, 0.9)
Newly arrived birds not separated from other birds	44.9%		
Newly arrived birds separated from other birds	36.7%	0.4	1.4 (0.6, 3.1)
Manure management			
Manure not thrown outside	43.8%		
Manure thrown outside	43.8%	1.0	1 (0.4, 2.8)
Manure not buried or composted	46.7%		
Manure buried or composted	40.2%	0.3	1.3 (0.8, 2.2)
Manure not burnt	45.2%		
Manure burnt	35.5%	0.3	1.5 (0.7, 3.3)
Manure not spread on fields	43.4%		
Manure spread on fields	60.0%	0.7	0.5 (0.1, 3.1)
Manure not spread around garden or sold as fertilizer	46.3%		
Manure spread around garden or sold as fertilizer	25.9%	0.1	2.5 (1, 6.1)
Manure not left where it is deposited	44.2%		
Manure left where it is deposited	41.7%	0.8	1.1 (0.5, 2.3)
Eggs not sold	41.0%		
Sell eggs	54.6%	0.1	0.6 (0.3, 1.1)
Poultry keeping area not cleaned every day	43.2%		
Poultry keeping area cleaned every day	45.5%	0.8	0.9 (0.5, 1.7)
Poultry keeping area not cleaned every two - three days	44.1%		
Poultry keeping area cleaned every two-three days	40.9%	0.8	1.1 (0.5, 2.8)
Poultry keeping area not cleaned every week	43.9%		
Poultry keeping area cleaned every week	42.9%	0.9	1.0 (0.5, 2.3)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Poultry keeping area not cleaned every month	45.1%		
Poultry keeping area cleaned every month	31.8%	0.2	1.8 (0.7, 4.5)
Disinfectants not used for cleaning poultry keeping areas	40.4%		
Disinfectants used for cleaning poultry keeping areas	47.6%	0.3	0.8 (0.4, 1.3)
Asian open bill storks were not seen daily in the backyard	42.6%		
Asian open bill storks were seen daily in the backyard	75.0%	0.1	0.3 (0.1, 1.3)
Asian open bill storks were not seen daily in the farm	43.5%		
Asian open bill storks were seen daily in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Asian open bill storks were not seen daily in the paddy fields	46.9%		
Asian open bill storks were seen daily in the paddy fields	41.3%	0.4	1.3 (0.7, 2.2)
Asian pied starlings were not seen daily in the backyard	46.4%		
Asian pied starlings were seen daily in the backyard	34.7%	0.2	1.6 (0.8, 3.2)
Asian pied starlings were not seen daily in the farm	43.9%		
Asian pied starlings were seen daily in the farm	40.0%	1.0	1.2 (0.2, 7.2)
Asian pied starlings were not seen daily in the paddy fields	44.1%		
Asian pied starlings were seen daily in the paddy fields	33.3%	0.7	1.6 (0.3, 8.8)
Common mynas were not seen daily in the backyard	42.6%		
Common mynas were seen daily in the backyard	44.8%	0.7	0.9 (0.53, 1.56)
Common mynas were not seen daily in the farm	43.1%		
Common mynas were seen daily in the farm	53.9%	0.5	0.7 (0.2, 2)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Common mynas were not seen daily in the paddy fields	42.7%		
Common mynas were seen daily in the paddy fields	83.3%	0.1	0.2 (0.0, 1.3)
White vented mynas were not seen daily in the backyard	42.4%		
White vented mynas were seen daily in the backyard	45.5%	0.7	0.9 (0.5, 1.5)
White vented mynas were not seen daily in the farm	42.7%		
White vented mynas were seen daily in the farm	63.6%	0.2	0.4 (0.1, 1.5)
White vented mynas were not seen daily in the paddy fields	42.7%		
White vented mynas were seen daily in the paddy fields	61.5%	0.2	0.5 (0.2, 1.5)
Rock pigeons were not seen daily in the backyard	44.6%		
Rock pigeons were seen daily in the backyard	43.4%	0.9	1.1 (0.6, 1.9)
Rock pigeons were not seen daily in the farm	42.5%		
Rock pigeons were seen daily in the farm	58.8%	0.2	0.5 (0.2, 1.4)
Rock pigeons were not seen daily in the paddy fields	42.2%		
Rock pigeons were seen daily in the paddy fields	69.2%	0.1	0.3 (0.1, 1.1)
Spotted doves were not seen daily in the backyard	47.1%		
Spotted doves were seen daily in the backyard	39.8%	0.3	1.3 (0.8, 2.3)
Spotted doves were not seen daily in the farm	44.5%		
Spotted doves were seen daily in the farm	25.0%	0.5	2.4 (0.5, 12.2)
Spotted doves were not seen daily in the paddy fields	43.0%		
Spotted doves were seen daily in the paddy fields	60.0%	0.3	0.5 (0.1, 1.8)
Zebra doves were not seen daily in the backyard	49.6%		
Zebra doves were seen daily in the backyard	37.3%	0.1	1.7 (1.0, 2.9)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Zebra doves were not seen daily in the farm	43.8%		
Zebra doves were seen daily in the farm	44.4%	1.0	1.0 (0.3, 3.7)
Zebra doves were not seen daily in the paddy fields	43.4%		
Zebra doves were seen daily in the paddy fields	60.0%	0.7	0.5 (0.1, 3.1)
Red collared doves were not seen daily in the backyard	45.3%		
Red collared doves were seen daily in the backyard	41.0%	0.5	1.2 (0.7, 2.1)
Red collared doves were not seen daily in the farm	43.5%		
Red collared doves were seen daily in the farm	50.0%	0.7	0.8 (0.2, 3.2)
Red collared doves were not seen daily in the paddy fields	43.1%		
Red collared doves were seen daily in the paddy fields	66.7%	0.4	0.4 (0.1, 2.1)
Sparrows were not seen daily in the backyard	49.0%		
Sparrows were seen daily in the backyard	42.2%	0.4	1.3 (0.7, 2.5)
Sparrows were not seen daily in f the arm	43.2%		
Sparrows were seen daily in the farm	48.0%	0.7	0.8 (0.4, 1.9)
Sparrows were not seen daily in the paddy fields	43.7%		
Sparrows were seen daily in the paddy fields	50.0%	1.0	0.8 (0.1, 12.6)
Swallows were not seen daily in the backyard	45.7%		
Swallows were seen daily in the backyard	25.0%	0.1	2.5 (0.9, 7.2)
Swallows were not seen daily in the farm	43.3%		
Swallows were seen daily in the farm	57.1%	0.7	0.6 (0.1, 2.6)
Swallows were not seen daily in the paddy fields	43.6%		
Swallows were seen daily in the paddy fields	45.2%	0.9	0.9 (0.4, 2.0)
Munias were not seen daily in the backyard	43.9%		
Munias were seen daily in the backyard	41.7%	0.9	1.1 (0.3, 3.6)
Munias were not seen daily in the farm	44.0%		
Munias were seen daily in the farm	0.0%	0.4	0.6 (0.5, 0.6)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Munias were not seen daily in the paddy fields	43.7%		
Munias were seen daily in the paddy fields	50.0%	1.0	0.8 (0.1, 5.6)
Weavers were not seen daily in the backyard	44.3%		
Weavers were seen daily in the backyard	41.5%	0.7	1.12 (0.6, 2.2)
Weavers were not seen daily in the farm	43.9%		
Weavers were seen daily in the farm	33.3%	1.0	1.6 (0.1, 17.5)
Weavers were not seen daily in the paddy fields	42.6%		
Weavers were seen daily in the paddy fields	54.6%	0.3	0.6 (0.3, 1.5)
Bulbuls were not seen daily in the backyard	43.2%		
Bulbuls were seen daily in the backyard	44.9%	0.8	0.9 (0.5, 1.6)
Bulbuls were not seen daily in the farm	44.8%		
Bulbuls were seen daily in the farm	0.0%	0.1	0.6 (0.5, 0.6)
Bulbuls were not seen daily in the paddy fields	44.3%		
Bulbuls were seen daily in the paddy fields	28.6%	0.5	2.0 (0.4, 10.5)
Lesser whistling ducks were not seen daily in the backyard	44.4%		
Lesser whistling ducks were seen daily in the backyard	30.0%	0.5	1.9 (0.5, 7.4)
Lesser whistling ducks were not seen daily in the farm	42.9%		
Lesser whistling ducks were seen daily in the farm	80.0%	0.2	0.2 (0.0, 1.7)
Lesser whistling ducks were not seen daily in the paddy fields	45.7%		
Lesser whistling ducks were seen daily in the paddy fields	32.3%	0.2	1.8 (0.8, 4.0)
Little grebes were not seen daily in the backyard	44.3%		
Little grebes were seen daily in the backyard	40.0%	0.7	1.2 (0.5, 2.8)
Little grebes were not seen daily in the farm	43.6%		
Little grebes were seen daily in the farm	50.0%	1.0	

Category	% positive farm	*p*	Odds Ratio (95%CI)
Little grebes were not seen daily in the paddy fields	44.2%		
Little grebes were seen daily in the paddy fields	42.3%	0.8	1.1 (0.6, 2.0)
Cotton pygmy geese were not seen daily in the backyard	43.9%		
Cotton pygmy geese were seen daily in the backyard	33.3%	1.0	1.6 (0.1, 17.5)
Cotton pygmy geese were not seen daily in the paddy fields	44.2%		
Cotton pygmy geese were seen daily in the paddy fields	36.4%	0.8	1.4 (0.4, 4.9)
Great egrets were not seen daily in the backyard	45.6%		
Great egrets were seen daily in the backyard	15.4%	0.0	4.6 (1, 2.3)
Great egrets were not seen daily in the farm	43.5%		
Great egrets were seen daily in the farm	66.7%	0.6	0.4 (0.0, 4.3)
Great egrets were not seen daily in the paddy fields	42.4%		
Great egrets were seen daily in the paddy fields	45.7%	0.6	0.9 (0.5, 1.5)
Little egrets were not seen daily in the backyard	44.3%		
Little egrets were seen daily in the backyard	20.0%	0.4	3.2 (0.4, 29.0)
Little egrets were not seen daily in the farm	43.7%		
Little egrets were seen daily in the farm	50.0%	1.0	0.8 (0.1, 5.6)
Little egrets were not seen daily in the paddy fields	40.7%		
Little egrets were seen daily in the paddy fields	49.4%	0.3	0.7 (0.4, 1.2)
Pond herons were not seen daily in the backyard	45.0%		
Pond herons were seen daily in the backyard	31.6%	0.3	1.8 (0.7, 4.8)
Pond herons were not seen daily in the farm	43.5%		
Pond herons were seen daily in the farm	50.0%	0.7	0.8 (0.2, 2.7)
Pond herons were not seen daily in the paddy fields	43.9%		
Pond herons were seen daily in the paddy	43.6%	1.0	1.0 (0.6, 1.8)

Category	% positive farm	*p*	Odds Ratio (95%CI)
fields			
Cattle egrets were not seen daily in the backyard	44.1%		
Cattle egrets were seen daily in the backyard	25.0%	0.6	2.4 (0.2, 23.2)
Cattle egrets were not seen daily in the farm	43.5%		
Cattle egrets were seen daily in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Cattle egrets were not seen daily in the paddy fields	45.1%		
Cattle egrets were seen daily in the paddy fields	37.1%	0.4	1.4 (0.7, 2.9)
Night herons were not seen daily in the backyard	44.6%		
Night herons were seen daily in the backyard	33.3%	0.4	1.6 (0.5, 4.9)
Night herons were not seen daily in the farm	43.2%		
Night herons were seen daily in the farm	75.0%	0.3	0.3 (0.0, 2.5)
Night herons were not seen daily in the paddy fields	44.3%		
Night herons were seen daily in the paddy fields	41.5%	0.7	1.1 (0.6, 2.2)
Cinnamon bitterns were not seen daily in the backyard	43.5%		
Cinnamon bitterns were seen daily in the backyard	66.7%	0.6	0.4 (0.0, 4.3)
Cinnamon bitterns were not seen daily in the farm	43.5%		
Cinnamon bitterns were seen daily in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Cinnamon bitterns were not seen daily in the paddy fields	42.9%		
Cinnamon bitterns were seen daily in the paddy fields	48.6%	0.5	0.8 (0.4, 1.6)
Little cormorants were not seen daily in the backyard	45.1%		
Little cormorants were seen daily in the backyard	31.8%	0.2	1.8 (0.7, 4.5)
Little cormorants were not seen daily in the farm	42.0%		
Little cormorants were seen daily in the farm	64.7%	0.1	0.4 (0.1, 1.1)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Little cormorants were not seen daily in the paddy fields	45.5%		
Little cormorants were seen daily in the paddy fields	39.7%	0.4	1.3 (0.7, 2.3)
Red wattle lapwings were not seen daily in the backyard	43.8%		
Red wattle lapwings were seen daily in the backyard	42.9%	1.0	1.0 (0.2, 4.8)
Red wattle lapwings were not seen daily in the farm	43.5%		
Red wattle lapwings were seen daily in the farm	66.7%	0.6	0.4 (0.0, 4.3)
Red wattle lapwings were not seen daily in the paddy fields	43.8%		
Red wattle lapwings were seen daily in the paddy fields	43.8%	1.0	1 (0.5, 1.9)
Common moorhens were not seen daily in the backyard	43.9%		
Common moorhens were seen daily in the backyard	40.0%	1.0	1.2 (0.2, 7.2)
Common moorhens were not seen daily in the farm	43.3%		
Common moorhens were seen daily in the farm	100.0%	0.2	0.4 (0.4, 0.5)
Common moorhens were not seen daily in the paddy fields	43.9%		
Common moorhens were seen in daily the paddy fields	43.3%	1.0	1.02 (0.5, 2.2)
Bronze winged jacanas were not seen daily in the backyard	43.5%		
Bronze winged jacanas were seen daily in the backyard	100.0%	0.4	0.4 (0.4, 0.5)
Bronze winged jacanas were not seen daily in the farm	43.5%		
Bronze winged jacanas were seen daily in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Bronze winged jacanas were not seen in the paddy fields	44.0%		
Bronze winged jacanas were seen in daily the paddy fields	41.7%	0.8	1.1 (0.5, 2.6)

Category	% positive farm	*p*	Odds Ratio (95%CI)
White breasted waterhens were not seen daily in the backyard	44.4%		
White breasted waterhens were seen daily in the backyard	41.3%	0.7	1.1 (0.6, 2.2)
White breasted waterhens were not seen daily in the farm	43.6%		
White breasted waterhens were seen daily in the farm	46.2%	0.9	0.9 (0.3, 2.8)
White breasted waterhens were not seen daily in the paddy fields	41.5%		
White breasted waterhens were seen daily in the paddy fields	49.2%	0.3	0.7 (0.4, 1.3)
Purple swamp hens were not seen daily in the backyard	43.9%		
Purple swamp hens were seen daily in the backyard	33.3%	1.0	1.6 (0.1, 17.5)
Purple swamp hens were not seen daily in the farm	43.3%		
Purple swamp hens were seen daily in the farm	100.0%	0.2	0.4 (0.4, 0.5)
Purple swamp hens were not seen daily in the paddy fields	44.7%		
Purple swamp hens were seen daily in the paddy fields	33.3%	0.4	1.6 (0.6, 4.5)
Black drongos were not seen daily in the backyard	44.4%		
Black drongos were seen daily in the backyard	40.0%	0.7	1.2 (0.6, 2.6)
Black drongos were not seen daily in the farm	43.7%		
Black drongos were seen daily in the farm	50.0%	1.0	0.8 (0.1, 12.6)
Black drongos were not seen daily in the paddy fields	40.4%		
Black drongos were seen daily in the paddy fields	79.0%	0.0	0.2 (0.1, 0.6)
Asian koels were not seen daily in the backyard	44.6%		
Asian koels were seen daily in the backyard	42.0%	0.7	1.1 (0.6, 2.1)
Asian koels were not daily seen in the farm	44.1%		
Asian koels were seen daily in the farm	25.0%	0.6	2.4 (0.2, 23.2)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Asian koels were not seen daily in the paddy fields	42.6%		
Asian koels were seen daily in the paddy fields	54.6%	0.3	0.6 (0.3, 1.5)
Fantails were not seen daily in the backyard	44.1%		
Fantails were seen daily in the backyard	42.5%	0.9	1.1 (0.5, 2.1)
Fantails were not seen daily in the paddy fields	43.3%		
Fantails were seen daily in the paddy fields	100.0%	0.2	0.4 (0.4, 0.5)
Oriental magpie-robins were not seen daily in the backyard	45.9%		
Oriental magpie-robins were seen daily in the backyard	39.4%	0.4	1.3 (0.7, 2.3)
Oriental magpie-robins were not seen daily in the farm	44.1%		
Oriental magpie-robins were seen daily in the farm	25.0%	0.6	2.4 (0.2, 23.2)
Oriental magpie-robins were not seen daily in the paddy fields	43.4%		
Oriental magpie-robins were seen daily in the paddy fields	60.0%	0.7	0.5 (0.1, 3.1)
Greater coucals were not seen daily in the backyard	46.8%		
Greater coucals were seen daily in the backyard	38.2%	0.2	1.4 (0.8, 2.5)
Greater coucals were not seen daily in the farm	43.8%		
Greater coucals were seen daily in the farm	42.9%	1.0	1.1 (0.2, 4.8)
Greater coucals were not seen daily in the paddy fields	41.0%		
Greater coucals were seen daily in the paddy fields	68.2%	0.0	0.3 (0.1, 0.8)
Indian rollers were not seen daily in the backyard	43.4%		
Indian rollers were seen daily in the backyard	50.0%	0.6	0.8 (0.3, 2.3)
Indian rollers were not seen daily in the farm	44.0%		
Indian rollers were seen daily in the farm	0.0%	1.0	0.6 (0.5, 0.6)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Indian rollers were not seen daily in the paddy fields	43.4%		
Indian rollers were seen daily in the paddy fields	60.0%	0.7	0.5 (0.1, 3.1)
Asian open bill storks were not sometimes seen in the backyard	44.6%		
Asian open bill storks were sometimes seen in the backyard	0.0%	0.1	0.6 (0.5, 0.6)
Asian open bill storks were not sometimes seen in the farm	43.5%		
Asian open bill storks were sometimes seen in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Asian open bill storks were not sometimes seen in the paddy fields	43.5%		
Asian open bill storks were sometimes seen in the paddy fields	45.5%	0.8	0.9 (0.4, 1.9)
Asian pied starlings were not sometimes seen in the backyard	43.5%		
Asian pied starlings were sometimes seen in the backyard	100.0%	0.4	0.4 (0.4, 0.5)
Asian pied starlings were not sometimes seen in the paddy fields	43.5%		
Asian pied starlings were sometimes seen in the paddy fields	100.0%	0.4	0.4 (0.4, 0.5)
Common mynas were not sometimes seen in the backyard	43.9%		
Common mynas were sometimes seen in the backyard	33.3%	1.0	1.6 (0.1, 17.5)
Common mynas were not sometimes seen in the paddy fields	44.0%		
Common mynas were sometimes seen in the paddy fields	0.0%	1.0	0.6 (0.5, 0.6)
White vented mynas were not sometimes seen in the backyard	43.7%		
White vented mynas were sometimes seen in the backyard	50.0%	1.0	0.8 (0.1, 5.6)
White vented mynas were not sometimes seen in the paddy fields	43.5%		
White vented mynas were sometimes seen in the paddy fields	100.0%	0.4	0.4 (0.4, 0.5)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Rock pigeons were not sometimes seen in the paddy fields	44.1%		
Rock pigeons were sometimes seen in the paddy fields	33.3%	0.7	1.6 (0.3, 8.8)
Zebra doves were not sometimes seen in the backyard	43.7%		
Zebra doves were sometimes seen in the backyard	50.0%	1.0	0.8 (0.1, 12.6)
Zebra doves were not sometimes seen in the farm	44.0%		
Zebra doves were sometimes seen in the farm	0.0%	1.0	0.6 (0.5, 0.6)
Red collared doves were not sometimes seen in the backyard	43.7%		
Red collared doves were sometimes seen in the backyard	50.0%	1.0	0.8 (0.1, 12.6)
Red collared doves were not sometimes seen in the farm	44.0%		
Red collared doves were sometimes seen in the farm	0.0%	1.0	0.6 (0.5, 0.6)
Sparrows were not sometimes seen in the paddy fields	44.0%		
Sparrows were sometimes seen in the paddy fields	0.0%	1.0	0.6 (0.5, 0.6)
Swallows were not sometimes seen in the backyard	43.7%		
Swallows were sometimes seen in the backyard	50.0%	1.0	0.8 (0.1, 12.6)
Oriental magpie-robins were not sometimes seen in the backyard	43.7%		
Oriental magpie-robins were sometimes seen in the backyard	50.0%	1.0	0.8 (0.1, 12.6)
Oriental magpie-robins were not sometimes seen in the farm	43.5%		
Oriental magpie-robins were sometimes seen in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Greater coucals were not sometimes seen in the backyard	43.5%		
Greater coucals were sometimes seen in the backyard	66.7%	0.6	0.4 (0.0, 4.3)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Greater coucals were not sometimes seen in the farm	43.5%		
Greater coucals were sometimes seen in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Indian rollers were not sometimes seen in the backyard	45.0%		
Indian rollers were sometimes seen in the backyard	12.5%	0.1	5.7 (0.7, 47.3)
Indian rollers were not sometimes seen in the farm	43.5%		
Indian rollers were sometimes seen in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Indian rollers were not sometimes seen in the paddy fields	43.9%		
Indian rollers were sometimes seen in the paddy fields	33.3%	1.0	1.6 (0.1, 17.5)
Swallows were not sometimes seen in the paddy fields	43.8%		
Swallows were sometimes seen in the paddy fields	44.4%	1.0	1.0 (0.3, 3.7)
Munias were not sometimes seen in the backyard	43.5%		
Munias were sometimes seen in the backyard	100.0%	0.4	0.4 (0.4, 0.5)
Weavers were sometimes not seen in the backyard	44.1%		
Weavers were sometimes seen in the backyard	33.3%	0.7	1.6 (0.3, 8.8)
Weavers were sometimes not seen in the paddy fields	44.2%		
Weavers were sometimes seen in the paddy fields	0.0%	0.5	0.6 (0.5, 0.6)
Bulbuls were not sometimes seen in the backyard	44.1%		
Bulbuls were sometimes seen in the backyard	25.0%	0.6	2.4(0.2, 23.2)
Lesser whistling ducks were not sometimes seen in the backyard	44.2%		
Lesser whistling ducks were sometimes seen in the backyard	0.0%	0.5	0.6 (0.5, 0.6)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Lesser whistling ducks were not sometimes seen in the farm	44.0%		
Lesser whistling ducks were sometimes seen in the farm	0.0%	1.0	0.6 (0.5, 0.6)
Lesser whistling ducks were not sometimes seen in the paddy field	42.9%		
Lesser whistling ducks were sometimes seen in the paddy field	52.4%	0.4	0.7 (0.3, 1.7)
Little grebes were not sometimes seen in the backyard	44.0%		
Little grebes were sometimes seen in the backyard	0.0%	1.0	0.6 (0.5, 0.6)
Little grebes were not sometimes seen in the farm	43.5%		
Little grebes were sometimes seen in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Little grebes were not sometimes seen in the paddy fields	43.5%		
Little grebes were sometimes seen in the paddy fields	66.7%	0.6	0.4 (0.0, 4.3)
Cotton pygmy geese were not sometimes seen in the backyard	44.0%		
Cotton pygmy geese were sometimes seen in the backyard	0.0%	1.0	0.6 (0.5, 0.6)
Cotton pygmy geese were not sometimes seen in the paddy fields	43.4%		
Cotton pygmy geese were sometimes seen in the paddy fields	60.0%	0.7	0.5 (0.1, 3.1)
Great egrets were not sometimes seen in the backyard	43.5%		
Great egrets were sometimes seen in the backyard	100.0%	0.4	0.4 (0.4, 0.5)
Great egrets were not sometimes seen in the farm	43.9%		
Great egrets were sometimes seen in the farm	33.3%	1.0	1.6 (0.1, 17.5)
Great egrets were not sometimes seen in the paddy fields	43.9%		
Great egrets were sometimes seen in the paddy fields	33.3%	1.0	1.6 (0.1, 17.5)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Little egrets were not sometimes seen in the farm	43.5%		
Little egrets were sometimes seen in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Little egrets were not sometimes seen in the paddy fields	45.0%		
Little egrets were sometimes seen in the paddy fields	0.0%	0.0	0.6 (0.5, 0.6)
Pond herons were not sometimes seen in the backyard	43.5%		
Pond herons were sometimes seen in the backyard	100.0%	0.4	0.4 (0.4, 0.5)
Pond herons were not sometimes seen in the farm	43.3%		
Pond herons were sometimes seen in the farm	100.0%	0.2	0.4 (0.4, 0.5)
Pond herons were not sometimes seen in the paddy fields	44.1%		
Pond herons were sometimes seen in the paddy fields	25.0%	0.6	2.4 (0.2, 23.2)
Cattle egrets were not sometimes seen in the backyard	43.5%		
Cattle egrets were sometimes seen in the backyard	100.0%	0.4	0.4 (0.4, 0.5)
Cattle egrets were not sometimes seen in the paddy fields	43.5%		
Cattle egrets were sometimes seen in the paddy fields	66.7%	0.6	0.4 (0.0, 0.5)
Night herons were not sometimes seen in the farm	43.5%		
Night herons were sometimes seen in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Night herons were not sometimes seen in the paddy fields	43.5%		
Night herons were sometimes seen in the paddy fields	100.0%	0.4	0.4 (0.4, 0.5)
Little cormorants were not sometimes seen in the backyard	43.5%		
Little cormorants were sometimes seen in the backyard	100.0%	0.4	0.4 (0.4, 0.5)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Little cormorants were not sometimes seen in the farm	43.5%		
Little cormorants were sometimes seen in the farm	66.7%	0.6	0.4 (0.0, 4.3)
Little cormorants were not sometimes seen in the paddy fields	43.5%		
Little cormorants were sometimes seen in the paddy fields	66.7%	0.6	0.4 (0.0, 4.3)
Red wattle lapwings were not sometimes seen in the backyard	43.5%		
Red wattle lapwings were sometimes seen in the backyard	100.0%	0.4	0.4 (0.4, 0.5)
Red wattle lapwings were not sometimes seen in the farm	43.5%		
Red wattle lapwings were sometimes seen in the farm	100.0%	0.4	0.4 (0.4, 0.5)
Red wattle lapwings were not sometimes seen in the paddy fields	43.7%		
Red wattle lapwings were sometimes seen in the paddy fields	45.5%	0.9	0.9 (0.3, 3.2)
Common moorhens were not sometimes seen in the backyard	43.5%		
Common moorhens were sometimes seen in the backyard	100.0%	0.4	0.4 (0.4, 0.5)
Common moorhens were not sometimes seen in the paddy fields	43.7%		
Common moorhens were sometimes seen in the paddy fields	50.0%	1.0	0.8 (0.1, 5.6)
White breasted waterhen were not sometimes seen in the backyard	43.2%		
White breasted waterhen were sometimes seen in the backyard	75.0%	0.3	0.3 (0.0, 2.5)
White breasted waterhen were not sometimes seen in the paddy fields	43.5%		
White breasted waterhen were sometimes seen in the paddy fields	100.0%	0.4	0.4 (0.4, 0.5)
Purple swamphens were not sometimes seen in the paddy fields	43.7%		
Purple swamphens were sometimes seen in the paddy fields	50.0%	1.0	0.8 (0.1, 12.6)

Category	% positive farm	*p*	Odds Ratio (95%CI)
Black drongos were not sometimes seen in backyard	43.5%		
Black drongos were sometimes seen in backyard	66.7%	0.6	0.4 (0.0, 4.3)
Black drongos were not sometimes seen in the paddy fields	44.2%		
Black drongos were sometimes seen in the paddy fields	0.0%	0.5	0.6 (0.5, 0.6)
Asian koels were sometimes not seen in the backyard	43.9%		
Asian koels were sometimes seen in the backyard	33.3%	1.0	1.6 (0.1, 17.5)
Asian koels were sometimes not seen in the farm	44.0%		
Asian koels were sometimes seen in the farm	0.0%	1.0	0.6 (0.5, 0.6)
Asian koels were sometimes not seen in the paddy fields	43.7%		
Asian koels were sometimes seen in the paddy fields	50.0%	1.0	0.8 (0.1, 12.6)
Fantails were not sometimes seen in the backyard	43.7%		
Fantails were sometimes seen in the backyard	50.0%	1.0	0.8 (0.1, 12.6)
Fantails were not sometimes seen in the paddy fields	44.0%		
Fantails were sometimes seen in the paddy fields	0.0%	1.0	0.6 (0.5, 0.6)

Shading indicated category with $p \leq 0.25$

Table 4.24; Variables included in the final logistic regression model*

Variables	β	Coef/SE	*P* value	Odds ratios	95% Confidence Intervals	
					Lower	Upper
Lesser whistling ducks were commonly seen on the farm	0.860	2.02	0.0431	2.36	1.03	5.44
Select healthy animal to ensure they are disease free	-0.751	-2.28	0.0226	0.47	0.25	0.90
Replace birds individually	0.885	2.37	0.0178	2.42	1.17	5.03
Buy native chickens/fighting cocks from commercial hatcheries	1.935	2.48	0.0133	6.92	1.50	32.01
Constant	-0.449	-2.31	0.0208	-	-	-

*Interaction factors were checked.

4.4 Discussion

Information on farm practices collected in this study should be interpreted with caution as after an outbreak of HPAI H5N1 farm practices may have changed. Most of villagers owned native chickens/fighting cocks with few owning commercial poultry such as layers or ducks. However, the average number of native chickens/fighting cocks owned was small compared with the average number of layers or ducks owned by the commercial units. The system of trading birds varied with the purpose of keeping poultry. Poultry kept for personal consumption and cock fighting were generally traded (purchased and sold) by households/farms in small numbers throughout the year, while poultry kept for commercial purposes were only traded a few times a year but in large numbers. Unlike native chickens/ fighting cocks, commercial poultry are likely to originate from a single source which may supply many farms in an area. Villagers who kept layers and/or ducks for commercial purposes were more likely to be visited regularly by buyers such as dealers/middlemen and private companies. These buyers generally purchase poultry products from a number of farms. The types of poultry were also related to the age of traded poultry. For example, native chickens and fighting cocks were traded at about 7-8 months of age (young adult) while commercial poultry were purchased when they were juveniles and sold as adults. As the trading system varied between households/farms, the risk of infection with H5N1 also would vary depending on the biosecurity practices implemented on those premises.

Some villagers noted that before the HPAI H5N1 outbreaks, equipment, especially plastic egg trays, were re-used without disinfecting between uses. Similar practices were reported to be associated with HPAI cases from the Netherlands (Thomas et al., 2005). Contamination of the environment and/or fomites such as vehicles, equipment, and/or humans travelling between households and farms can increase the risk of infection with avian influenza (Briand and Fukuda, 2009; Tiensin et al., 2009). In this study small commercial farms did not provide

disinfectants for visitors. Schijven, Teunis, and Husman (2005) reported that larger farms (> 10,000 chickens) had a higher risk of having HPAI infection if they had ineffective water treatment. The influenza viruses can persist in the environment, including water sources, for up to 200 days at 17°C (Stallknecht et al., 1990). Poor hygiene could result in contamination of the water from poultry/bird faeces, especially when water was sourced from an open supply such as a pond. Intensive commercial poultry farms with a low biosecurity level had a higher risk of having the disease than did households/farms that had a small number of poultry or those with good biosecurity measures which included good sanitation (Capua and Marangon, 2000) and reduced contacts between poultry and humans or wild birds (Tiensin et al., 2009).

Poor management practices and a lack of knowledge were also noted during the present questionnaire survey. Similar to that reported in a study by Olsen and others (2005), one farmer reported that her family had cooked and consumed suspected H5N1 infected chickens during the 2004 outbreak. Some farmers reported selling their poultry manure as fertiliser which was then transported and sold to farmers in other provinces. However, high temperatures and exposure to UV light can reduce the infectivity of H5N1 viruses present in chicken manure (Chumpolbanchorn et al., 2006). Even though products of plants fertilized by contaminated manure can not spread the H5N1 virus to consumers (Chumpolbanchorn et al., 2006), there is the potential for indirect transmission during transportation and delivery of contaminated manure to the fields.

Some farmers would treat affected poultry themselves, do nothing, or even sell their poultry if their poultry were suspected to be infected with H5N1. Almost 40% of the villagers believed that wild birds were the major risk of introducing the HPAI H5N1 virus to their poultry flocks. However at the time of the interviews, 30% of villagers were doing nothing to prevent their poultry from infection and similarly 12% of villagers were not undertaking any

actions to prevent their families from infection. It is possible that a deficiency of finance resulted in the implementation of poor disease control and prevention practices. The key to a successful control and prevention program for outbreaks of HPAI H5N1 is the implementation of integrated human and veterinary health and response efforts (Witt and Malone, 2005). The study in this chapter showed that there were significant gaps between ideal biosecurity strategies and practises actually adopted by small farm holders.

The logistic regression model revealed that potential risk factors involved in a history of HPAI H5N1 outbreaks were not only farm practices but also the presence of specific wild bird species. Households with outbreaks were seven times more likely to have purchased native chickens and fighting cocks from commercial hatcheries. Generally, hatcheries for commercial poultry, such as layer and broiler ducks and chickens, are intensive with a high standard of biosecurity. However, hatcheries for fighting cocks are relatively small and are more likely to have a low level of biosecurity (Figures 4.5 and 4.6). The hatcheries generally purchase their breeders from cock fighting competitions to improve their breeds (http://www.gaichon.com/porpunkai.html, 2009; R.S.Farm, 2008). The price of fighting cocks also depends upon the achievements in fighting competitions. The purchase of fighting cocks from competitions increases the risk of infection due to the large numbers of birds present in such events from a wide geographical area.

Even though fighting cocks in Thailand have to be registered and tested for HPAI H5N1 by the DLD (Buranathai et al., 2006), it is difficult to control the movements of the birds and their owners who may attend competitions throughout the nation. Based on information from villagers interviewed in this questionnaire, places where the villagers took their cocks for fighting competitions were either within or outside their local districts and could even involve travelling to other provinces. The cock fighting competitions are places where various owners bring their birds together for fighting (Figure 4.7). Contamination with

infectious pathogens can occur if an infected cock has been introduced to the group. Long distant transportations of HPAI H5N1 virus by fighting cocks can be important in the spread of the disease (Gilbert et al., 2006; Sims et al., 2005; Webster et al., 2006). To prevent the spread of the HPAI H5N1 virus from cock fights, further policies, such as vaccination against the virus (Webster and Hulse, 2005), education campaigns, and or efficient law enforcement are required.

Figure 4.5; Housing of breeders and adult cocks in a commercial cock fighting farm

Source: http://photo.lannaphotoclub.com/index.php?topic=5773.0

Figure 4.6; Housing of juvenile birds in a commercial cock fighting farm

Source: http://www.gaichononline.com/smf/index.php?board=11;action=display;threadid=1105

Figure 4.7; A cock fighting competition

Source: http://www.borraped.com/webboard_bn/view.php?category=borraped2&wb_id=1

Another factor that increased the risk of an outbreak was replacing individual birds as compared to using an all-in all-out system. Villagers owning native chickens and/or fighting cocks usually own a small number of birds and were more likely to replace individual birds than all of their birds. These villagers were not likely to apply proper quarantine or biosecurity practises in their households/ farms. Replacing birds individually resulted in twice the risk of having an HPAI H5N1 infection. Understanding the importance of quarantine procedures and the role of farm biosecurity can significantly reduce the risk from this practise. In this study (Table 4.23), selecting healthy birds when purchasing or restocking poultry was identified as a protective factor for HPAI H5N1. Villagers who only purchased disease-free poultry for their households/ farms were two times less likely to have HPAI H5N1 infection than villagers who did not. This farm practise is easy to apply and useful for small poultry owners who purchase poultry individually or in small numbers. This, however, may not be practical for commercial poultry farmers who usually purchase a large number of poultry at a time. Commercialized hatcheries could be an alternative source of replacement birds for commercial poultry farmers in order to ensure new stock are disease free.

Data of wild birds observed in poultry keeping areas in this study was based on observations recalled by the villagers. As only common wild birds were included in the bird photo albums, less common bird species were not included in this study unless the villagers specifically mentioned these birds. A study undertaken by Kung and others (2007) revealed that observation of wild birds in feed troughs was a protective factor for infection. Similar to Kung's study, there was no significant association of observing wild birds feeding together with domestic poultry and a history of outbreaks in this study. Even though the virus has been detected in pigeons and doves (Kou et al., 2005; Mase et al., 2005), none of the terrestrial birds in this study were identified as being a significant risk for outbreaks in the final model.

However, seeing lesser whistling ducks every day in the farms was a significant risk factor. Lesser whistling ducks are common waterfowl living in freshwater wetlands that are widespread throughout Thailand (Lekagul and Round, 1991). In 2005, a report claimed that there were over 40,000 lesser whistling ducks at the Bung Boraphet Non Hunting area, located in the central part of Thailand (BCST, 2005). These ducks can be seen in village and agricultural areas (see Chapter 1). Moreover, an experiment revealed that a lesser whistling duck can be infected by HPAI H5N1 viral inoculation at a dose as low as 10 $TCID_{50}$ and shed the virus through the cloaca and trachea (up to $10^{8.26}$ $TCID_{50}$) with a mortality rate of 73.9% (Wiriyarat, 2009). Infected waterfowls are a potential source of virus for water sources and poultry should be prevented from having contact with these sources. As lesser whistling ducks can be infected with a low dose of the virus and shed a high viral titre in their secretions, it would appear that the presence of these birds on farms would increase the risk of disease. In order to determine level of risk in the study area, the numbers of lesser whistling ducks and other common wild birds and the degree of interaction with poultry in habitats including open system farms, a wild bird observation study was performed and reported in this thesis (Chapter 6).

Similar to the outcomes reported in this chapter, Olsen and others (2005) stated that knowledge on ways to prevent human infection with avian influenza had effectively reached rural people through education campaigns, however, surprisingly, these people had not changed their behaviours. Based on the questionnaire study, reasons for not changing behaviour included not understanding the importance of disease control and prevention due to the low economic value of backyard/free range poultry, as well as the low household income. Education programs focusing on changing attitudes and behaviours to raise understanding and awareness of the importance of adopting effective biosecurity in terms of public health are needed in rural Thailand to reduce the risk of HPAI H5N1 transmission. Recommendations to increase biosecurity for poultry owners include keeping poultry

indoors, disinfecting all equipment regularly, restricting access of people outside the household/enterprise to poultry, and limiting contact between wild birds and domestic poultry (Dierauf et al., 2006).

To understand fully the epidemiology of HPAI H5N1 infection involving domesticated poultry and wild birds, further studies need to be performed on the movements of both commercial (such as grazing ducks) and non-commercial domesticated poultry (such as fighting cocks) and also on the ecology and behaviour of wild birds. A serological and virological investigation of HPAI H5N1 virus in wild bird populations in the study site where the questionnaire study was conducted is described in Chapter 5.

Chapter 5

VIROLOGICAL AND MOLECULAR EPIDEMIOLOGICAL INVESTIGATIONS INTO THE ROLE OF WILD BIRDS IN THE EPIDEMIOLOGY OF INFLUENZA A/H5N1 IN CENTRAL THAILAND

5.1 Introduction

As concerns were raised in Thailand over the spread of H5N1 HPAI virus strains in free flying wild birds, a serological and virological surveillance study of the virus in wild bird populations was undertaken from February 2007 to October 2008. The purpose of the survey was to investigate the disease status in free ranging wild birds in Banglane District, Nakhon Pathom province located in central Thailand. Outbreaks of H5N1 HPAI had affected poultry farms throughout this area during 2004. Consequently a multiple species surveillance scheme, focusing on sampling a variety of common wild birds found in the area, was conducted in order to detect evidence of viral circulation. Gene sequencing and phylogenetic analyses were conducted on viruses isolated from this study to: investigate their relationships to other isolates from Thailand and the general region; determine the origin of the viruses; and gain insights into the epidemiology of these viruses similar to that which has been done in previous studies (Cox and Subbarao, 2000).

5.2 Materials and Methods

5.2.1 Sampling strategy

Collections of samples from wild birds were conducted in the study area in Banglane District, Nakhon Pathom province (Figure 2.1) at two monthly intervals. Samples included tracheal and cloacal swabs, blood, and carcasses (if possible). Details on sample collection procedures are listed in Chapter 2. If the H5N1 virus was detected in any collection trip, repeat survey trips were conducted two weeks and/or four weeks later. A total of 12 field trips were conducted within the study period (February 2007 to October 2008). A minimum of 30 wild birds living in the area covered by the study site were sampled in each sampling trip using techniques that have been described in Chapter 2.

5.2.2 Laboratory procedures

The field sample collection procedures and laboratory procedures for virus isolation and H5 specific NT tests have been described in Chapter 2.

5.2.2.1 Nucleotide sequencing

For sequencing of the HA and ND genes of the isolated H5N1 viruses RT-PCR was conducted using overlapping primers for the HA and NA genes based on consensus sequences of H5 HA and N1 NA genes in Genbank. The PCR products (Chapter 2) were electrophoresed on a 1% agarose gel, the bands visualized and cut under ultraviolet light and the product was purified using the Qaigen Gel Extraction kit (Qaigen®) as described in Puthavathana et al. (2005). The purified c-DNA samples were then submitted to the molecular biology company, Bio Basic Inc. (160 Torbay Road Markham Ontario L3R 1G6 Canada), for gene sequencing. The Cycle Sequencing kit (BigDye Terminator version 3.1; Applied Biosystems) and the ABI PRISM version ABI3730XL DNA sequencer (PE Applied

Biosystems) were used according to standard procedures for nucleotide sequencing and analysis, respectively. The full sequence of the HA and NA genes of these viruses was then determines from the overlapping c-DNA sequence data from these products by colleagues at Faculty of Veterinary Sciences, Mahidol University.

5.2.2.2 Phylogenetic analysis of viruses isolated

Gene sequencing of the RT-PCR products of the H5N1 Haemagglutinin (HA) and Neuraminidase (NA) genes was conducted as above and the cDNA sequences were provided for further phylogenetic analysis. The HA and NA gene sequences provided were compared to sequences from other H5N1 viruses isolated in Thailand that had been submitted to Genbank (www.ncbi.nlm.nih.gov/) between 2004 and 2008 and phylogenetic trees were generated using the programs referred to below. The Software used to generate phylogenetic trees were BioEdit Version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html), MEGA4.1 (http://www.Megasoft ware.net/mega41.html), Expasy translate tool (http://au.expasy.org/tools/dna.html), and CLUSTAL_W (http://www.ebi.ac.uk/Tools/ clustalw2). Nucleotide sequences were translated into amino acid sequences using the Expasy translate tool (Gasteiger et al., 2003). Nucleotide and amino acid sequences were aligned and edited in BioEdit (Hall, 1999). Phylogenetic trees of nucleotide sequences (HA and NA) were generated by MEGA 4.1 (Tamura et al., 2007) applying the neighbour-joining algorithm, bootstrap analysis with 1,000 replicates, and branch swapping rooted by the A/goose/China/Guangdong/1/96 virus (Amonsin et al., 2008). CLUSTAL_W was used for pairwise alignment of amino acid sequences (Weber et al., 2007) of HA and NA genes.

5.3 Results

5.3.1 Results of virus isolation and serological testing of wild birds at the study site

A total of 421 apparently healthy birds (44 species; Table 5.1) were sampled from February 2007 to October 2008. From the H5 NT testing the overall seroprevalence was 2.1% (8 out of 385 samples; with 95%CI 0.7, 3.5). The species that tested H5 antibody positive by NT are listed in Table 5.2. From the virus isolation procedures on the 421 swabs from wild birds, H5N1 viruses were isolated from two samples at a detection rate of 0.5% (2 out of 421 samples; 95%CI 0.0, 1.1). The positive samples were from an Asian pied starling (*Gracupica contra*) and a white vented myna (*Acidotheres grandis*) collected on June 7th 2007 (Table 5.3; Figure 5.1). The serum samples from these two birds were negative for H5 antibody by NT and all the birds that were H5 antibody positive by NT were negative on viral isolation.

The serum samples that were H5 antibody positive by NT had been collected between March and December 2007 (Figure 5.1). The first serological positive sample was detected from an oriental magpie robin (*Copsychus saularis*) in the sample collection trip conducted on March 7th 2007 On May 23rd a serum sample from a rock pigeon (*Columba livia*) was tested H5 antibody positive. H5 antibody positive samples from a rock pigeon and spotted dove (*Streptopelia chinensis*) were also identified on June 7th. In the following sample collection trip on July 16th, samples from an Asian pied starling (*Gracupica contra*) and a starling or myna* (*Acridotheres sp*) were H5 antibody positive. On August 14th, a sample from a blue-tailed bee-eater (*Merops philippinus)* was H5 antibody positive. No serum samples were H5 antibody positive from the sample collection in October. However, a sample from a pond heron was H5 antibody positive on December 26th. Subsequently no H5 antibody positive serum samples were detected and no further viruses were isolated from swab samples collected in this study.

Eighty-seven percent (366 out of 421) of the sampled birds were adults while three percent were juveniles and in ten percent of birds the age was not identified. Most sampled birds

were apparently healthy (99.5%, 419 birds) while two of the birds had clinical signs of disease. However, all positive samples (on both serology and virology) were from apparently healthy birds. Both of the virological positive samples were collected from adults. Six out of eight of the seropositive samples were also from adults. One seropositive sample was from a juvenile pond heron but the age of a rock pigeon which was seropositive was unidentified

Table 5.1; Common name and species of wild birds sampled in the survey

Common name	**Species, Genus, or Family**	**Number of samples**
Ashy wood swallow	*Artamus fuscus*	2
Asian golden weaver	*Ploceus hypoxanthus*	2
Asian koel	*Eudynamys scolopaceus*	4
Asian open bill stork	*Anastomus oscitans*	1
Asian pied starling	*Gracupica contra*	21
Barn swallow	*Hirundo rustica*	3
Black drongo	*Dicrurus macrocercus*	1
Black-crowned night heron	*Nycticorax nycticorax*	3
Blue-tailed bee eater	*Merops philippinus*	2
Bronze-winged jacana	*Metopidius indicus*	4
Bulbul	*Alophoixus sp.*	2
Cattle egret	*Bubulcus ibis*	1
Chinese pond heron	*Ardeola bacchus*	16
Common flameback	*Dinopium javanense*	1
Common myna	*Acridotheres tristis*	11
Dove	*Columbinae sp.*	1
Egret	*Ardeidae sp.*	1
Great egret	*Mesophoyx intermedia*	1
Greater coucal	*Centropus sinensis*	2

Common name	Species, Genus, or Family	Number of samples
Grey-capped woodpecker	*Dendrocopos canicapillus*	1
Herons	*Ardeidae sp.*	1
House sparrow	*Passer domesticus*	9
House swift	*Apus affinis*	1
Intermediate egret	*Casmerodius albus*	1
Javan pond heron	*Ardeola speciosa*	3
Leaf warblers	*Phylloscopus sp.*	1
Lesser whistling duck	*Dendrocygna javanica*	25
Little cormorant	*Phalacrocorax niger*	7
Little egret	*Egretta garzetta*	2
Little grebe	*Tachybaptus ruficollis*	4
Munia	*Lonchura sp.*	4
Myna	*Acridotheres sp.*	3
Oriental magpie-robin	*Copsychus saularis*	3
Oriental pratincole	*Glareola maldivarum*	1
Pheasant-tailed jacana	*Hydrophasianus chirurgus*	1
Pied fantail	*Rhipidura javanica*	6
Plain-backed Sparrow	*Passer flaveolus*	1
Pond heron	*Ardeola sp.*	20
Prinia	*Prinia sp.*	1
Red turtle dove	*Streptopelia tranquebarica*	59
Rock pigeon	*Columba livia*	94
Sandpiper	*Scolopacidae sp.*	2
Spotted dove	*Streptopelia chinensis*	21
Streak-eared bulbul	*Pycnonotus blanfordi*	18

Common name	Species, Genus, or Family	Number of samples
Tree sparrow	*Passer montanus*	7
Wagtail	*Motacilla sp.*	1
White-breasted waterhen	*Amaurornis phoenicurus*	1
White-vented myna	*Acridotheres grandis*	30
Zebra dove	*Geopelia striata*	14

Table 5.2; Seroprevalence to H5N1 virus for wild birds tested in the survey

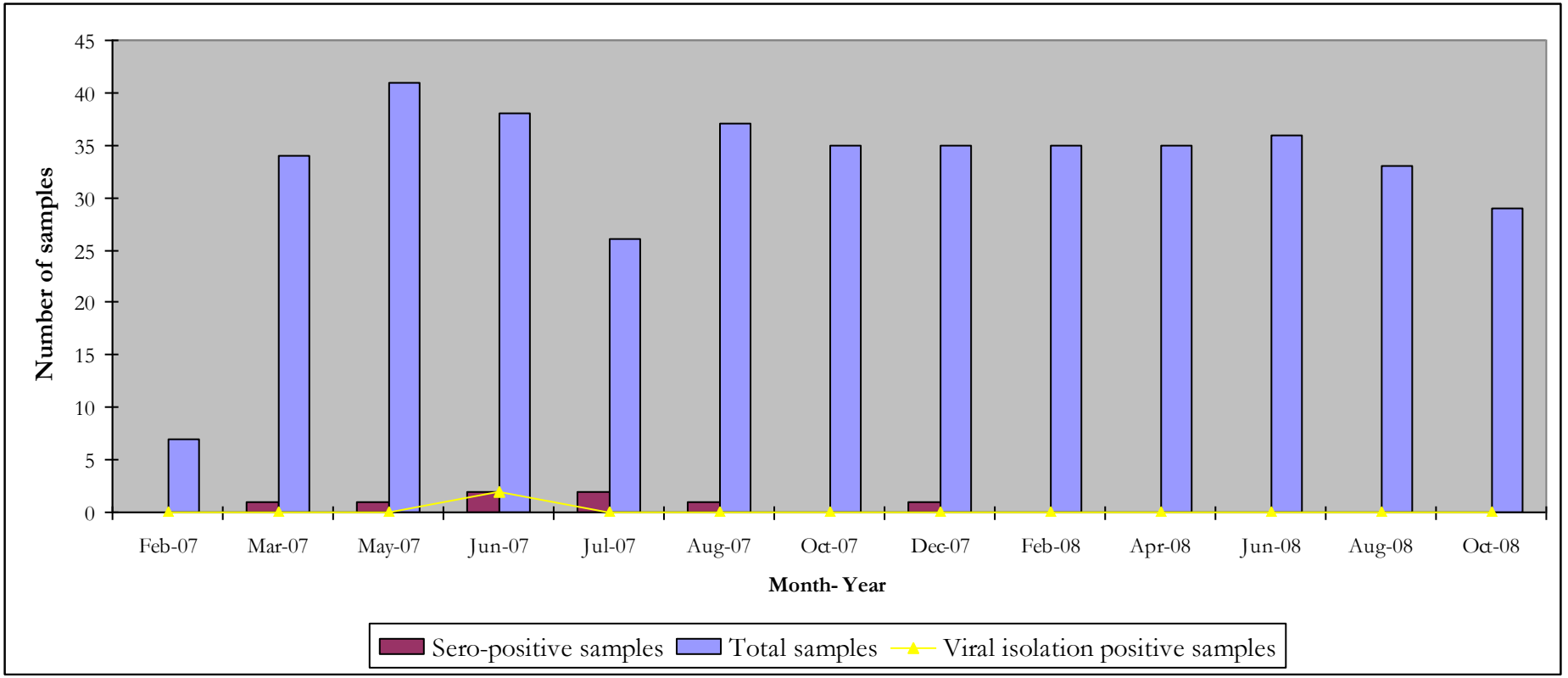
Common name/ Species	Positive samples	Total samples	Sero-prevalence	95% CI
Rock pigeon (*Columba livia*)	2	93	2.2 %	0.0, 5.1
Asian pied starling (*Gracupica contra*)	1	18	5.6%	0.0, 16.1
Spotted dove (*Streptopelia chinensis*)	1	20	5.0%	0.0, 14.6
Oriental magpie robin (*Copsychus saularis*)	1	3	33.3%	0.0, 86.7
Blue-tailed bee-eater (*Merops philippinus)*	1	2	50.0%	0.0, 100.0
Starling and/or myna* (*Acridotheres spp*)	2	37	5.4%	0.0, 12.7
Pond heron (*Ardeola sp*)	1	19	5.3%	0.0, 15.3

* According to the database, only the Thai common name was noted. The Thai common name can mean either myna or starling.

Table 5.3; Prevalence of viral isolation in the survey

Common name/ Species	Positive samples	Total samples	Prevalence (%)	95% CI
White vented Myna (*Acridotheres grandis*)	1	30	3.3	0.0, 9.8
Asian pied starling (*Gracupica contra*)	1	21	4.8	0.0, 13.9

Figure 5.1; Number of samples collected in the study during February 2007 to October 2008 and the timing of serological and virological positive samples



5.3.2 Results of genetic characterization and phylogenetic analysis

The complete nucleotide sequences of the HA and NA genes of the H5N1 viruses from the White vented myna (WMV/07) and Asian pied starling (APS/07) viruses that were isolated from the surveillance study in Banglane District are shown in Figure 5.2. These HA and NA gene sequences were submitted to Genbank (Accession numbers 1278033, 1278041, 1277938, and 1278028). Both viruses have gene sequences that translate to give multiple basic amino acids at the HA cleavage site at amino acid positions 341 to 346 (Figure 5.3) indicating that they are H5N1 HPAI viruses (Hoffmann et al., 2007; Steinhauer, 1999). Phylogenetic analysis showed that the nucleotide sequences of the HA genes of both viruses found in this study (WVM/07 and APS/07) were most closely related to the H5N1 virus (A/chicken/Thailand/PC-168/2006) isolated on July, 23rd 2006 from a chicken in Pichit province, in the Northern part of Thailand, reported by Chutinimitkul and others (2007) (Figure 5.4). The nucleotide sequences of the NA genes of WVM/07 and APS/07 were clustered in a group of Thai H5N1 viruses isolated between 2004 and 2005 (Figure 5.5). The alignment scores expressed as the percentage similarity of amino acid sequences of HA and NA genes of these viruses compared to other H5N1 viruses are shown in Tables 5.4 and 5.5. The similarity rates of amino acid sequences of HA genes (both WVM/07 and APS/07) show between 98% - 99% when compared to other Thai isolates. Amino acid sequences of the NA gene (both WVM/07 and APS/07) were 100% similar to the gene of the viruses including A/chicken/Thailand/PC-168/2006, A/chicken/Phichit /NIAH 6069 88/2006, and A/ quail/Thailand/CU-333/06. Moreover, WVM/07 and APS/07 were clustered with Thai isolates belonged to genotypes Z, clade 1.

Comparisons of amino acid sequences of HA genes of WVM/07, APS/07, and A/chicken/ Thailand/PC-168/2006 are shown in Figure 5.3 (99.5% and 99.3% homogeneous, respectively). Position 3 on HA amino acid sequences of WVM/07 and

APS/07 viruses had a Lysine residue which is different from the Arginine residue of the A/chicken/Thailand/PC-168/2006 sequence. A Tyrosine residue was present at position 210 of WVM/07 while an Asparagine residue was present in the others. Positions 455 and 474 of the APS/07 sequence had Glutamic acid and Glycine residues respectively, while an Aspartic acid residue was found at these positions in the others. An Arginine residue was present at position 473 of WVM/07 and A/chicken/Thailand/PC-168/2006 while APS/07 had a Threonine at that position. The NA genes of WVM/07 and APS/07 contained a 60 nucleotide deletion from position 145 to 204 which corresponds to a 20 amino acid deletion at position 49-68 of the neuraminidase protein. The NA of viruses WVM/07 and APS/07 did not have the histidine to tyrosine mutation at amino acid position 274 (H247Y) which is associated with resistance to the antiviral drug oseltamivir.

Figure 5.2; Complete nucleotide sequences of HA genes (A) and NA genes (B) of WVM/07 and APS/07 viruses

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A)                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             10         20         30         40         50
WVM/07                ATGGAGAAAA TAGTGCTTCT TTTTGCAATA GTCAGTCTTG TTAAAAGTGA
APS/07                ATGGAGAAAA TAGTGCTTCT TTTTGCAATA GTCAGTCTTG TTAAAAGTGA
Clustal Consensus     ********** ********** ********** ********** **********


                      ....|....| ....|....| ....|....| ....|....| ....|....|
                             60         70         80         90        100
WVM/07                TCAGATTTGC ATTGGTTACC ATGCAAACAA CTCGACAGAG CAGGTTGACA
APS/07                TCAGATTTGC ATTGGTTACC ATGCAAACAA CTCGACAGAG CAGGTTGACA
Clustal Consensus     ********** ********** ********** ********** **********


                      ....|....| ....|....| ....|....| ....|....| ....|....|
                            110        120        130        140        150
WVM/07                CAATAATGGA AAGGAACGTT ACTGTTACAC ATGCCCAAGA CATACTGGAA
APS/07                CAATAATGGA AAGGAACGTT ACTGTTACAC ATGCCCAAGA CATACTGGAA
Clustal Consensus     ********** ********** ********** ********** **********


                      ....|....| ....|....| ....|....| ....|....| ....|....|
                            160        170        180        190        200
WVM/07                AAGACACACA ACGGGAAGCT CTGCGATCTA GATGGAGTGA AGCCTCTAAT
APS/07                AAGACACACA ACGGGAAGCT CTGCGATCTA GATGGAGTGA AGCCTCTAAT
Clustal Consensus     ********** ********** ********** ********** **********


                      ....|....| ....|....| ....|....| ....|....| ....|....|
                            210        220        230        240        250
WVM/07                TTTGAGAGAC TGTAGTGTAG CTGGATGGCT CCTCGGAAAC CCAATGTGTG
APS/07                TTTGAGAGAC TGTAGTGTAG CTGGATGGCT CCTCGGAAAC CCAATGTGTG
Clustal Consensus     ********** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            260        270        280        290        300
WVM/07              ACGAATTCAT TAATGTGCCG GAATGGTCTT ACATAGTGGA GAAGGCCAAT
APS/07              ACGAATTCAT TAATGTGCCG GAATGGTCTT ACATAGTGGA GAAGGCCAAT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            310        320        330        340        350
WVM/07              CCAGTCAATG ACCTCTGTTA CCCAGGGGAT TTCAATGACT ATGAAGAATT
APS/07              CCAGTCAATG ACCTCTGTTA CCCAGGGGAT TTCAATGACT ATGAAGAATT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            360        370        380        390        400
WVM/07              GAAACACCTA TTGAGCAGAA TAAACCATTT TGAGAAAATT CAGATCATCC
APS/07              GAAACACCTA TTGAGCAGAA TAAACCATTT TGAGAAAATT CAGATCATCC
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            410        420        430        440        450
WVM/07              CTAAAAGTTC TTGGTCCAGT CATGAAGCCT CATTAGGGGT GAGCTCAGCA
APS/07              CTAAAAGTTC TTGGTCCAGT CATGAAGCCT CATTAGGGGT GAGCTCAGCA
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            460        470        480        490        500
WVM/07              TGTCCATACC TGGGAAAGTC CTCCTTTTTC AGAAATGTGG TATGGCTCAT
APS/07              TGTCCATACC TGGGAAAGTC CTCCTTTTTC AGAAATGTGG TATGGCTCAT
Clustal Consensus   ********** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            510        520        530        540        550
WVM/07              CAAAAAGAAC AGTACATACC CAACAATAAA GAGGAGCTAC AATAATACCA
APS/07              CAAAAAGAAC AGTACATACC CAACAATAAA GAGGAGCTAC AATAATACCA
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            560        570        580        590        600
WVM/07              ACCAAGAAGA TCTTTTGGTA CTGTGGGGGA TTCACCATCC TAATGATGCG
APS/07              ACCAAGAAGA TCTTTTGGTA CTGTGGGGGA TTCACCATCC TAATGATGCG
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            610        620        630        640        650
WVM/07              GCAGAGCAGA CAAAGCTCTA TCAATACCCA ACCACCTATA TTTCTGTTGG
APS/07              GCAGAGCAGA CAAAGCTCTA TCAAAACCCA ACCACCTATA TTTCTGTTGG
Clustal Consensus   ********** ********** **** ***** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            660        670        680        690        700
WVM/07              GACATCAACA CTAAACCAGA GATTGGTACC AAGAATAGCT ACTAGATCCA
APS/07              GACATCAACA CTAAACCAGA GATTGGTACC AAGAATAGCT ACTAGATCCA
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            710        720        730        740        750
WVM/07              AAGTAAACGG GCAAAGTGGA AGGATGGAGT TCTTCTGGAC AATTTTAAAA
APS/07              AAGTAAACGG GCAAAGTGGA AGGATGGAGT TCTTCTGGAC AATTTTAAAA
Clustal Consensus   ********** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            760        770        780        790        800
WVM/07              CCGAATGATG CAATCAACTT CGAGAGTAAT GGAAATTTCA TTGCTCCAGA
APS/07              CCGAATGATG CAATCAACTT CGAGAGTAAT GGAAATTTCA TTGCTCCAGA
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            810        820        830        840        850
WVM/07              ATATGCATAC AAAATTGTTA AGAAAGGGGA CTCAACAATT ATGAAAAGTG
APS/07              ATATGCATAC AAAATTGTTA AGAAAGGGGA CTCAACAATT ATGAAAAGTG
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            860        870        880        890        900
WVM/07              AATTGGAATA TGGTAACTGC AACACCAAGT GTCAAACTCC AATGGGGGCG
APS/07              AATTGGAATA TGGTAACTGC AACACCAAGT GTCAAACTCC AATGGGGGCG
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            910        920        930        940        950
WVM/07              ATAAACTCTA GTATGCCATT CCACAATATA CACCCTCTCA CTATCGGGGA
APS/07              ATAAACTCTA GTATGCCATT CCACAATATA CACCCTCTCA CTATCGGGGA
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            960        970        980        990       1000
WVM/07              ATGCCCCAAA TATGTGAAAT CAAACAGATT AGTCCTTGCG ACTGGGCTCA
APS/07              ATGCCCCAAA TATGTGAAAT CAAACAGATT AGTCCTTGCG ACTGGGCTCA
Clustal Consensus   ********** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           1010       1020       1030       1040       1050
WVM/07              GAAATAGCCC TCAAAGAGAG AGA------- --AGAAGAAA AAAGAGAGGA
APS/07              GAAATAGCCC TCAAAGAGAG AGA------- --AGAAGAAA AAAGAGAGGA
Clustal Consensus   ********** ********** ***          ******** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           1060       1070       1080       1090       1100
WVM/07              TTATTTGGAG CTATAGCTGG TTTTATAGAG GGGGGATGGC AGGGAATGGT
APS/07              TTATTTGGAG CTATAGCTGG TTTTATAGAG GGGGGATGGC AGGGAATGGT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           1110       1120       1130       1140       1150
WVM/07              AGATGGTTGG TATGGGTACC ACCATAGCAA TGAGCAGGGG AGTGGGTACG
APS/07              AGATGGTTGG TATGGGTACC ACCATAGCAA TGAGCAGGGG AGTGGGTACG
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           1160       1170       1180       1190       1200
WVM/07              CTGCAGACAA AGAATCCACT CAAAAGGCAA TAGATGGAGT CACCAATAAG
APS/07              CTGCAGACAA AGAATCCACT CAAAAGGCAA TAGATGGAGT CACCAATAAG
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           1210       1220       1230       1240       1250
WVM/07              GTCAACTCGA TAATTGACAA AATGAACACT CAGTTTGAGG CCGTTGGAAG
APS/07              GTCAACTCGA TAATTGACAA AATGAACACT CAGTTTGAGG CCGTTGGAAG
Clustal Consensus   ********** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1260       1270       1280       1290       1300
WVM/07              GGAATTTAAC AACTTAGAAA GGAGAATAGA GAATTTAAAC AAGAAGATGG
APS/07              GGAATTTAAC AACTTAGAAA GGAGAATAGA GAATTTAAAC AAGAAGATGG
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1310       1320       1330       1340       1350
WVM/07              AAGACGGGTT CCTAGATGTC TGGACTTATA ATGCTGAACT TCTGGTTCTC
APS/07              AAGACGGGTT CCTAGATGTC TGGACTTATA ATGCTGAACT TCTGGTTCTC
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1360       1370       1380       1390       1400
WVM/07              ATGGAAAATG AGAGAACCCT AGACTTTCAT GACTCAAATG TCAAGAACCT
APS/07              ATGGAAAATG AGAGAACCCT AGAATTTCAT GACTCAAATG TCAAGAACCT
Clustal Consensus   ********** ********** *** ****** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1410       1420       1430       1440       1450
WVM/07              TTACGACAAG GTCCGACTAC AGCTTAGGGA TAATGCAAAG GAGCTGGGTA
APS/07              TTACGACAAG GTCCGACTAC AGCTTACGGG TAATGCAAAG GAGCTGGGTA
Clustal Consensus   ********** ********** ****** **  ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1460       1470       1480       1490       1500
WVM/07              ACGGTTGTTT CGAGTTCTAT CATAAGTGTG ATAATGAATG TATGGAAAGT
APS/07              ACGGTTGTTT CGAGTTCTAT CATAAGTGTG ATAATGAATG TATGGAAAGT
Clustal Consensus   ********** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1510       1520       1530       1540       1550
WVM/07              GTGAGAAACG GAACGTATGA CTACCCGCAG TATTCAGAAG AAGCAAAACT
APS/07              GTGAGAAACG GAACGTATGA CTACCCGCAG TATTCAGAAG AAGCAAAACT
Clustal Consensus   ********** ********** ********** ********** **********

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1560       1570       1580       1590       1600
WVM/07              AAAAAGAGAG GAAATAAGTG GAGTAAAATT GGAATCAATA GGAATTTACC
APS/07              AAAAAGAGAG GAAATAAGTG GAGTAAAATT GGAATCAATA GGAATTTACC
Clustal Consensus   ********** ********** ********** ********** **********

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1610       1620       1630       1640       1650
WVM/07              AAATACTGTC AATTTATTCT ACAGTGGCGA GTTCCCTAGC ACTGGCAATC
APS/07              AAATACTGTC AATTTATTCT ACAGTGGCGA GTTCCCTAGC ACTGGCAATC
Clustal Consensus   ********** ********** ********** ********** **********

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1660       1670       1680       1690       1700
WVM/07              ATGGTAGCTG GTCTATCCTT ATGGATGTGC TCCAATGGGT CGTTACAATG
APS/07              ATGGTAGCTG GTCTATCCTT ATGGATGTGC TCCAATGGGT CGTTACAATG
Clustal Consensus   ********** ********** ********** ********** **********

                    ....|....| ....|.
                            1710
WVM/07              CAGAATTTGC ATTTAA
APS/07              CAGAATTTGC ATTTAA
Clustal Consensus   ********** ******
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B)                    ....|....| ....|....| ....|....| ....|....| ....|....|
                               10         20         30         40         50
WVM/07                ATGAATCCAA ATAAGAAGAT AATAACCATC GGATCAATCT GTATGGTAAC
APS/07                ATGAATCCAA ATAAGAAGAT AATAACCATC GGATCAATCT GTATGGTAAC
Clustal Consensus     ********** ********** ********** ********** **********

                      ....|....| ....|....| ....|....| ....|....| ....|....|
                               60         70         80         90        100
WVM/07                TGGAATGGTT AGCTTAATGT TACAAATTGG GAACTTGATC TCAATATGGG
APS/07                TGGAATGGTT AGCTTAATGT TACAAATTGG GAACTTGATC TCAATATGGG
Clustal Consensus     ********** ********** ********** ********** **********

                      ....|....| ....|....| ....|....| ....|....| ....|....|
                              110        120        130        140        150
WVM/07                TCAGTCATTC AATTCACACA GGGAATCAAC ACAAAGCTGA ACCA------
APS/07                TCAGTCATTC AATTCACACA GGGAATCAAC ACAAAGCTGA ACCA------
Clustal Consensus     ********** ********** ********** ********** ****

                      ....|....| ....|....| ....|....| ....|....| ....|....|
                              160        170        180        190        200
WVM/07                ---------- ---------- ---------- ---------- ----------
APS/07                ---------- ---------- ---------- ---------- ----------
Clustal Consensus

                      ....|....| ....|....| ....|....| ....|....| ....|....|
                              210        220        230        240        250
WVM/07                ----ATCAGC AATACTAATT TTCTTACTGA GAAAGCTGTG GCTTCAGTAA
APS/07                ----ATCAGC AATACTAATT TTCTTACTGA GAAAGCTGTG GCTTCAGTAA
Clustal Consensus         ****** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            260        270        280        290        300
WVM/07              AATTAGCGGG CAATTCATCT CTTTGCCCCA TTAATGGCTG GGCTGTATAC
APS/07              AATTAGCGGG CAATTCATCT CTTTGCCCCA TTAATGGCTG GGCTGTATAC
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            310        320        330        340        350
WVM/07              AGTAAGGACA ACAGTATAAG GATCGGTTCC AAGGGGGATG TGTTTGTTAT
APS/07              AGTAAGGACA ACAGTATAAG GATCGGTTCC AAGGGGGATG TGTTTGTTAT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            360        370        380        390        400
WVM/07              AAGAGAGCCA TTCATCTCAT GCTCCCACTT GGAATGCAGA ACTTTCTTTT
APS/07              AAGAGAGCCA TTCATCTCAT GCTCCCACTT GGAATGCAGA ACTTTCTTTT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            410        420        430        440        450
WVM/07              TGACTCAGGG AGCCTTGCTG AATGACAAGC ACTCCAATGG GAGTGTCAAA
APS/07              TGACTCAGGG AGCCTTGCTG AATGACAAGC ACTCCAATGG GAGTGTCAAA
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            460        470        480        490        500
WVM/07              GACAGGAGCC CTCACAGAAC ATTAATGAGT TGTCCTGTGG GTGAGGCTCC
APS/07              GACAGGAGCC CTCACAGAAC ATTAATGAGT TGTCCTGTGG GTGAGGCTCC
Clustal Consensus   ********** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            510        520        530        540        550
WVM/07              CTCCCCATAT AACTCAAGGT TTGAGTCTGT TGCTTGGTCA GCAAGTGCTT
APS/07              CTCCCCATAT AACTCAAGGT TTGAGTCTGT TGCTTGGTCA GCAAGTGCTT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            560        570        580        590        600
WVM/07              GCCATGATGG CACCAGTTGG TTGACAATTG GAATTTCTGG CCCAGACAAT
APS/07              GCCATGATGG CACCAGTTGG TTGACAATTG GAATTTCTGG CCCAGACAAT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            610        620        630        640        650
WVM/07              GGGGCTGTGG CTGTATTGAA ATACAATGGC ATAATAACAG ACACTATCAA
APS/07              GGGGCTGTGG CTGTATTGAA ATACAATGGC ATAATAACAG ACACTATCAA
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            660        670        680        690        700
WVM/07              GAGTTGGAGG AATAACATAC TGAGAACTCA AGAGTCTGAA TGTGCATGTG
APS/07              GAGTTGGAGG AATAACATAC TGAGAACTCA AGAGTCTGAA TGTGCATGTG
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            710        720        730        740        750
WVM/07              TAAATGGCTC TTGCTTTACT GTAATGACTG ACGGACCAAG TAATGGTCAG
APS/07              TAAATGGCTC TTGCTTTACT GTAATGACTG ACGGACCAAG TAATGGTCAG
Clustal Consensus   ********** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            760        770        780        790        800
WVM/07              GCATCACATA AGATCTTCAA AATGGAAAAA GGGAAAGTGG TTAAATCAGT
APS/07              GCATCACATA AGATCTTCAA AATGGAAAAA GGGAAAGTGG TTAAATCAGT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            810        820        830        840        850
WVM/07              CGAGTTGGAT GCTCCTAATT ATCACTATGA GGAATGCTCC TGTTATCCTG
APS/07              CGAGTTGGAT GCTCCTAATT ATCACTATGA GGAATGCTCC TGTTATCCTG
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            860        870        880        890        900
WVM/07              ATGCTGGCGA AATCACATGT GTGTGCAGGG ATAATTGGCA TGGCTCAAAT
APS/07              ATGCTGGCGA AATCACATGT GTGTGCAGGG ATAATTGGCA TGGCTCAAAT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            910        920        930        940        950
WVM/07              CGGCCATGGG TATCTTTCAA TCAAAATTTG GAGTATCAAA TAGGATATAT
APS/07              CGGCCATGGG TATCTTTCAA TCAAAATTTG GAGTATCAAA TAGGATATAT
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            960        970        980        990        1000
WVM/07              ATGCAGTGGA GTTTTCGGAG ACAATCCACG CCCCAATGAT GGAACAGGTA
APS/07              ATGCAGTGGA GTTTTCGGAG ACAATCCACG CCCCAATGAT GGAACAGGTA
Clustal Consensus   ********** ********** ********** ********** **********
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1010       1020       1030       1040       1050
WVM/07              GTTGTGGTCC GGTGTCCTCT AACGGAGCAT ATGGGGTAAA AGGGTTTTCA
APS/07              GTTGTGGTCC GGTGTCCTCT AACGGAGCAT ATGGGGTAAA AGGGTTTTCA
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1060       1070       1080       1090       1100
WVM/07              TTTAAATACG GCAATGGTGT CTGGATCGGG AGAACAAAAA GCACTAATTC
APS/07              TTTAAATACG GCAATGGTGT CTGGATCGGG AGAACAAAAA GCACTAATTC
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1110       1120       1130       1140       1150
WVM/07              CAGGAGCGGC TTTGAAATGA TTTGGGATCC AAATGGGTGG ACTGAAACGG
APS/07              CAGGAGCGGC TTTGAAATGA TTTGGGATCC AAATGGGTGG ACTGAAACGG
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1160       1170       1180       1190       1200
WVM/07              ACAGTAGCTT TTCAGTGAAA CAAGATATCG TAGCAATAAC TGATTGGTCA
APS/07              ACAGTAGCTT TTCAGTGAAA CAAGATATCG TAGCAATAAC TGATTGGTCA
Clustal Consensus   ********** ********** ********** ********** **********


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1210       1220       1230       1240       1250
WVM/07              GGATATAGCG GGAGTTTTGT CCAGCATCCA GAATTGACAG GACTAGATTG
APS/07              GGATATAGCG GGAGTTTTGT CCAGCATCCA GAATTGACAG GACTAGATTG
Clustal Consensus   ********** ********** ********** ********** **********
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```
                        ....|....| ....|....| ....|....| ....|....| ....|....|
                                1260       1270       1280       1290       1300
WVM/07                  CATAAGACCT TGTTTCTGGG TTGAGTTGAT CAGAGGGCAG CCCAAAGAGA
APS/07                  CATAAGACCT TGTTTCTGGG TTGAGTTGAT CAGAGGGCAG CCCAAAGAGA
Clustal Consensus       ********** ********** ********** ********** **********


                        ....|....| ....|....| ....|....| ....|....| ....|....|
                                1310       1320       1330       1340       1350
WVM/07                  -GCACAATTT GGACTA--GT GGGAGCAG-C ATATCTTTTT GTGGTGTAGA
APS/07                  -GCACAATTT GGACTA--GT GGGAGCAG-C ATATCTTTTT GTGGTGTAGA
Clustal Consensus        ********* ******  ** ******** * ********** **********


                        ....|....| ....|....| ....|....| ....|....| ....|....|
                                1360       1370       1380       1390       1400
WVM/07                  -TAGTGAC-A CTGTGGGTTG GTCCTGGCCA GACGGTGCTG AGTTGCCATT
APS/07                  -TAGTGAC-A CTGTGGGTTG GTCCTGGCCA GACGGTGCTG AGTTGCCATT
Clustal Consensus        ******* * ********** ********** ********** **********


                        ....|....| ....|....| ....|....| ....|....| ....
                                1410       1420       1430       1440
WVM/07                  CATCATTGAC AAGTAG---- ---------- ---------- ----
APS/07                  CATCATTGAC AAGTAG---- ---------- ---------- ----
Clustal Consensus       ********** ******
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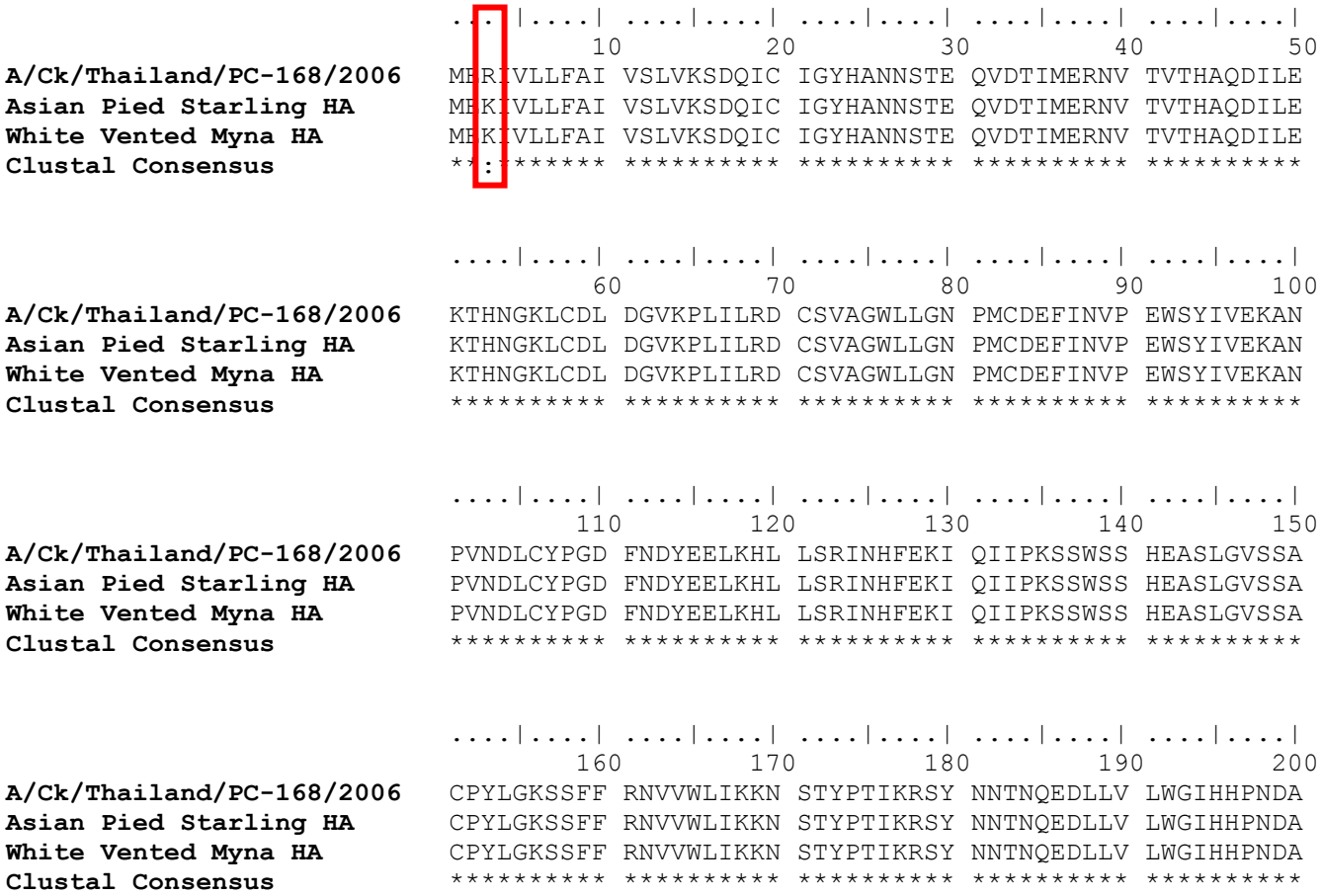
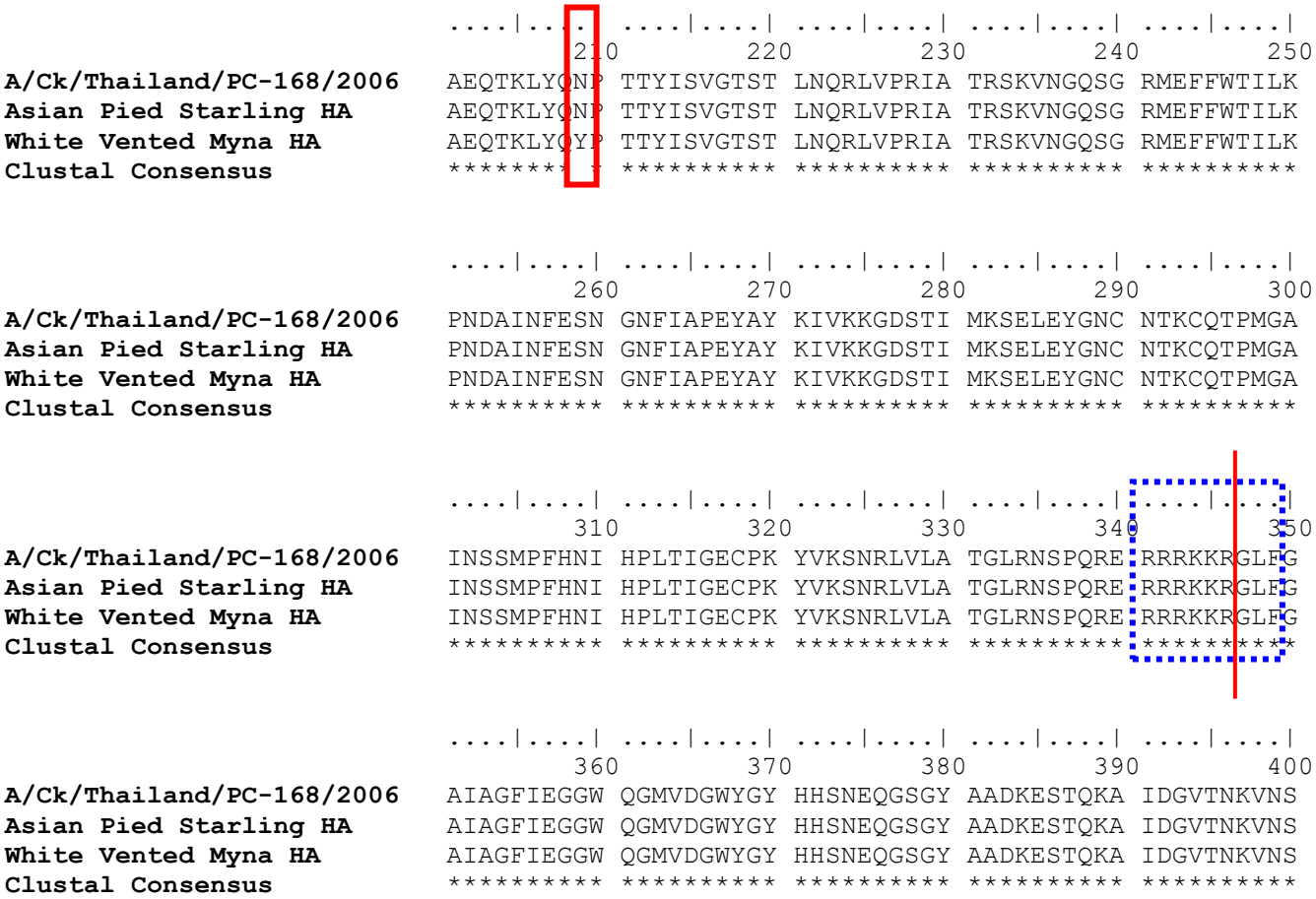
Figure 5.3; Alignment of amino acid sequences of Haemagglutinin (HA) gene




....|....| ....|....| ....|....| ....|....| ....|....|
210 220 230 240 250
A/Ck/Thailand/PC-168/2006 AEQTKLYQNP TTYISVGTST LNQRLVPRIA TRSKVNGQSG RMEFFWTILK
Asian Pied Starling HA AEQTKLYQNP TTYISVGTST LNQRLVPRIA TRSKVNGQSG RMEFFWTILK
White Vented Myna HA AEQTKLYQYP TTYISVGTST LNQRLVPRIA TRSKVNGQSG RMEFFWTILK
Clustal Consensus ********.* ********** ********** ********** **********
....|....| ....|....| ....|....| ....|....| ....|....|
260 270 280 290 300
A/Ck/Thailand/PC-168/2006 PNDAINFESN GNFIAPEYAY KIVKKGDSTI MKSELEYGNC NTKCQTPMGA
Asian Pied Starling HA PNDAINFESN GNFIAPEYAY KIVKKGDSTI MKSELEYGNC NTKCQTPMGA
White Vented Myna HA PNDAINFESN GNFIAPEYAY KIVKKGDSTI MKSELEYGNC NTKCQTPMGA
Clustal Consensus ********** ********** ********** ********** **********
....|....| ....|....| ....|....| ....|....| ....|....|
310 320 330 340 350
A/Ck/Thailand/PC-168/2006 INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQRE RRRKKRGLFG
Asian Pied Starling HA INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQRE RRRKKRGLFG
White Vented Myna HA INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQRE RRRKKRGLFG
Clustal Consensus ********** ********** ********** ********** **********
....|....| ....|....| ....|....| ....|....| ....|....|
360 370 380 390 400
A/Ck/Thailand/PC-168/2006 AIAGFIEGGW QGMVDGWYGY HHSNEQGSGY AADKESTQKA IDGVTNKVNS
Asian Pied Starling HA AIAGFIEGGW QGMVDGWYGY HHSNEQGSGY AADKESTQKA IDGVTNKVNS
White Vented Myna HA AIAGFIEGGW QGMVDGWYGY HHSNEQGSGY AADKESTQKA IDGVTNKVNS
Clustal Consensus ********** ********** ********** ********** **********

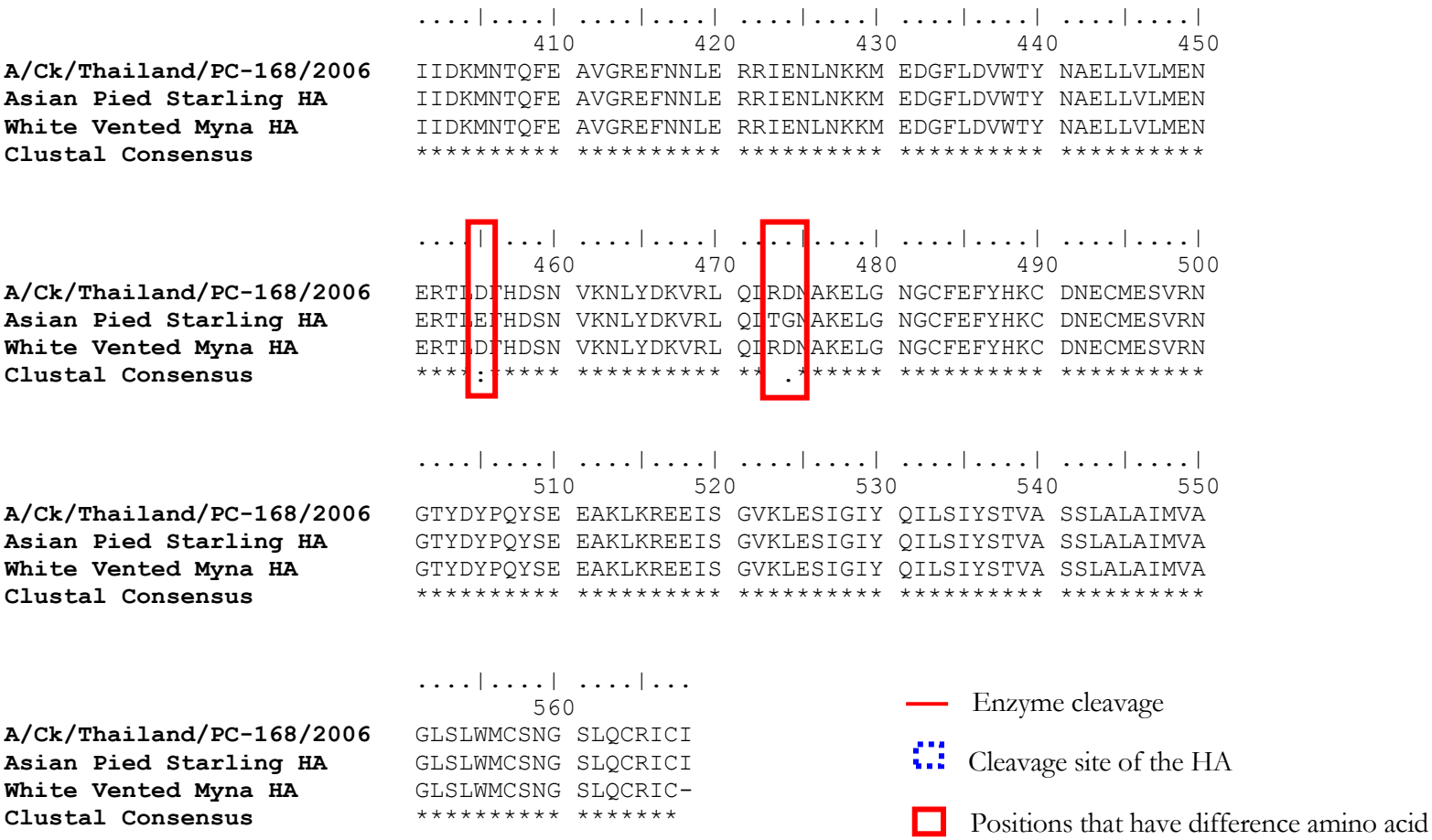
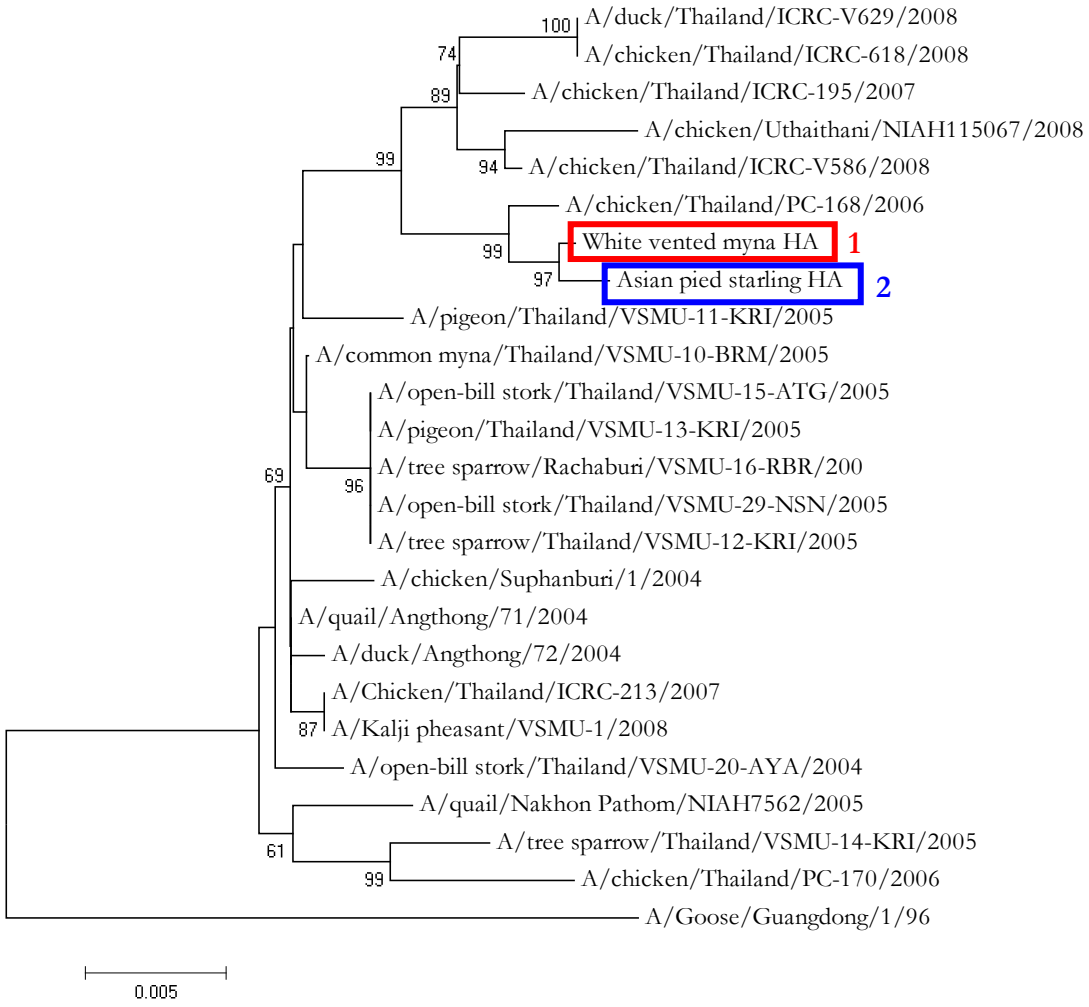

....|....| ....|....| ....|....| ....|....| ....|....|
410 420 430 440 450
A/Ck/Thailand/PC-168/2006 IIDKMNTQFE AVGREFNNLE RRIENLNKKM EDGFLDVWTY NAELLVLMEN
Asian Pied Starling HA IIDKMNTQFE AVGREFNNLE RRIENLNKKM EDGFLDVWTY NAELLVLMEN
White Vented Myna HA IIDKMNTQFE AVGREFNNLE RRIENLNKKM EDGFLDVWTY NAELLVLMEN
Clustal Consensus ********** ********** ********** ********** **********
....|....| ....|....| ....|....| ....|....| ....|....|
460 470 480 490 500
A/Ck/Thailand/PC-168/2006 ERTLDFHDSN VKNLYDKVRL QLRDNAKELG NGCFEFYHKC DNECMESVRN
Asian Pied Starling HA ERTLEFHDSN VKNLYDKVRL QLTGNAKELG NGCFEFYHKC DNECMESVRN
White Vented Myna HA ERTLDFHDSN VKNLYDKVRL QLRDNAKELG NGCFEFYHKC DNECMESVRN
Clustal Consensus ***:****** ********** ** .****** ********** **********
....|....| ....|....| ....|....| ....|....| ....|....|
510 520 530 540 550
A/Ck/Thailand/PC-168/2006 GTYDYPQYSE EAKLKREEIS GVKLESIGIY QILSIYSTVA SSLALAIMVA
Asian Pied Starling HA GTYDYPQYSE EAKLKREEIS GVKLESIGIY QILSIYSTVA SSLALAIMVA
White Vented Myna HA GTYDYPQYSE EAKLKREEIS GVKLESIGIY QILSIYSTVA SSLALAIMVA
Clustal Consensus ********** ********** ********** ********** **********
....|....| ....|...
560
A/Ck/Thailand/PC-168/2006 GLSLWMCSNG SLQCRICI
Asian Pied Starling HA GLSLWMCSNG SLQCRICI
White Vented Myna HA GLSLWMCSNG SLQCRIC-
Clustal Consensus ********** *******
Enzyme cleavage
Cleavage site of the HA
Positions that have difference amino acid

Figure 5.4; Phylogenetic tree of nucleotide sequences of the HA gene of Thai isolates and isolates from the wild bird surveillance



White vented myna (1) and Asian pied starling (2) isolated from this study

Figure 5.5; Phylogenetic tree of nucleotide sequences of NA gene of Thai isolates and isolates from the wild bird surveillance

White vented myna (1) and Asian pied starling (2) isolated from this study

Table 5.4; Comparison of amino acid sequences of Haemagglutinin (HA) genes

Sequence	Number of amino acids	Aligned score %	
		White vented myna HA	Asian pied starling HA
A/Goose/Guangdong/1/96	568	95.6	95.8
A/cat/Thailand/KU-02/04	568	99.1	98.9
A/chicken/Bangkok/Thailand/CU-3/04	568	99.1	98.9
A/chicken/Kalasin/NIAH317/2004	568	99.1	98.9
A/chicken/Nakhonsawan/NIAH6006587/2008	568	98.9	98.8
A/chicken/Phichit/NIAH1/2006	568	99.6	99.5
A/chicken/Phichit/NIAH606988/2006	568	99.6	99.5
A/chicken/Sukhothai/NIAH6-3-0005/2005	568	98.9	98.8
A/chicken/Suphanburi/1/2004	568	98.8	98.6
A/chicken/Suphanburi/137/2005	568	99.1	98.9
A/chicken/Thailand/CU-321/06	568	97.4	97.0
A/chicken/Thailand/ICRC-195/2007	568	98.8	98.6
A/chicken/Thailand/ICRC-213/2007	568	99.1	98.9
A/chicken/Thailand/ICRC-618/2008	568	98.4	98.2
A/chicken/Thailand/ICRC-V586/2008	568	98.9	98.6
A/chicken/Thailand/Kamphaengphet/NIAH6-3-0009/2005	568	98.9	98.8
A/chicken/Thailand/NS-339/2008	572	98.9	98.8
A/chicken/Thailand/PC-168/2006	568	99.5	99.3
A/chicken/Thailand/PC-170/2006	568	98.1	97.9
A/chicken/Thailand/PC-340/2008	568	99.1	98.9
A/chicken/Thailand/Phitsanulok/NIAH6-3-0012/2005	568	98.9	98.8
A/chicken/Uthaithani/NIAH115067/2008	568	98.4	98.2
A/common myna/Thailand/VSMU-10-BRM/2005	568	99.1	98.9
A/duck/Angthong/72/2004	568	99.1	98.9
A/duck/Thailand/ICRC-V629/2008	568	98.4	98.2
A/Kalij pheasant/Thailand/vsmu-1/2008	568	99.1	98.9
A/moorhen/Thailand/CU-317/06	568	97.0	96.7
A/open bill stork/Thailand/VSMU-15-ATG/2005	568	98.8	98.6
A/open bill stork/Thailand/VSMU-20-AYA/2004	568	98.9	98.8
A/open bill stork/Thailand/VSMU-29-NSN/2005	568	98.8	98.6
A/pigeon/Thailand/VSMU-11-KRI/2005	568	98.8	98.6
A/pigeon/Thailand/VSMU-13-KRI/2005	568	98.8	98.6
A/quail/Angthong/71/2004	568	99.1	98.9
A/quail/Nakhon Pathom/NIAH7562/2005	568	98.8	98.6
A/quail/Thailand/CU-330/06	568	99.1	98.9
A/quail/Thailand/CU-331/06	568	99.1	98.9
A/quail/Thailand/CU-332/06	568	99.1	98.9
A/Thailand/SP83/2004	568	99.1	98.9

Sequence	Number of amino acids	Aligned score %	
		White vented myna HA	Asian pied starling HA
A/Thailand/5(KK-494)/2004	568	99.1	98.9
A/tiger/Suphanburi/Thailand/Ti-1/04	568	99.1	98.9
A/tiger/Thailand/SPB-1	568	99.1	98.9
A/tree sparrow/Rachaburi/VSMU-16-RBR/2005	568	98.8	98.6
A/tree sparrow/Thailand/VSMU-12-KRI/2005	568	98.8	98.6
A/tree sparrow/Thailand/VSMU-14-KRI/2005	568	98.6	98.4
A/watercock/Thailand/CU-319/06	568	97.4	97.0
Asian Pied Starling HA	568	99.1	-
White Vented Myna HA	568	-	99.1

Table 5.5; Comparison of amino acid sequences of Neuraminidase (NA) genes

Sequence	Number of amino acids	Aligned score %	
		White vented myna NA	Asian pied starling NA
A/G oose/Guangdong/1/96	469	94.9	94.9
A/bird/Thailand/3.1/2004	449	99.1	99.1
A/brown-head gull/Thailand/vsmu-4/2008	450	95.5	95.5
A/chicken/Bangkok/Thailand/CU-6/04	449	99.1	99.1
A/chicken/Chachoengsao/Thailand/CU-11/04	441	99.3	99.3
A/chicken/Kohn Kaen/NIAH330/2004	449	99.1	99.1
A/chicken/Nakhon Sawan/Thailand/CU-12/04	439	99.3	99.3
A/chicken/Nakhon Sawan/Thailand/CU-13/04	439	99.3	99.3
A/chicken/Phichit/NIAH606988/2006	449	100	100
A/Ck/Sukhothai/NIAH114843/2008	449	96	96
A/chicken/Suphanburi/1/2004	449	99.1	99.1
A/chicken/Suphanburi/Thailand/CU-1/04	449	99.1	99.1
A/chicken/Thailand/CH-2/2004	449	99.1	99.1
A/chicken/Thailand/ICRC-195/2007	449	97.1	97.1
A/chicken/Thailand/ICRC-618/2008	449	96.4	96.4
A/chicken/Thailand/ICRC-V143/2007	449	99.1	99.1
A/chicken/Thailand/ICRC-V586/2008	451	93.1	93.1
A/chicken/Thailand/PC-168/2006	391	100	100
A/chicken/Thailand/PC-170/2006	449	97.6	97.6
A/chicken/Uthaithani/NIAH115067/2008	449	96.9	96.9
A/Ck/Thailand/9.1/2004	449	99.1	99.1
A/common myna/Thailand/VSMU-10-BRM/2005	449	97.8	97.8
A/duck/Angthong/72/2004	449	99.1	99.1

Sequence	Number of amino acids	Aligned score %	
		White vented myna NA	Asian pied starling NA
A/duck/Chonburi/Thailand/CU-5/04	446	99.1	99.1
A/duck/Thailand/ICRC-V629/2008	449	96.4	96.4
A/Gs/Thailand/79/2004	449	99.1	99.1
A/Kalij pheasant/Thailand/vsmu-1/2008	449	98.7	98.7
A/moorhen/Thailand/CU-317/06	449	98.7	98.7
A/open bill stork/Thailand/VSMU-20-AYA/2004	449	98.7	98.7
A/open bill stork/Thailand/VSMU-29-NSN/2005	449	98.4	98.4
A/open bill stork/Thailand/VSMU-9-BKK/2004	449	98.7	98.7
A/pigeon/Thailand/VSMU-11-KRI/2005	449	98.7	98.7
A/pigeon/Thailand/VSMU-13-KRI/2005	449	98.4	98.4
A/quail/Angthong/71/2004	449	98.7	98.7
A/quail/Nakhon Pathom/NIAH7562/2005	449	98.2	98.2
A/quail/Phathumthani/NIAH2711/2004	449	99.1	99.1
A/quail/Thailand/CU-320/06	449	98.7	98.7
A/quail/Thailand/CU-330/06	446	99.6	99.6
A/quail/Thailand/CU-332/06	440	99.8	99.8
A/quail/Thailand/CU-333/06	441	100	100
A/Tiger/Thailand/VSMU-1-SPB/2004	449	99.1	99.1
A/tiger/Thailand/VSMU-11-SPB/2004	449	99.1	99.1
A/tree sparrow/Rachaburi/VSMU-16-RBR/2005	449	98.4	98.4
A/tree sparrow/Thailand/VSMU-12-KRI/2005	449	98.4	98.4
A/tree sparrow/Thailand/VSMU-14-KRI/2005	449	98.4	98.4
A/watercock/Thailand/CU-319/06	444	98.6	98.6
Asian pied starling NA	449	100	-
White vented myna NA	449	-	100

5.4 Discussion

The virus surveillance conducted in Banglane District demonstrated that H5N1 HPAI viruses could be isolated from wild birds in this area. As well as showing the multiple basic amino acids at the HA cleavage site that is characteristic of H5N1 HPAI viruses, the viruses also had the deletion of 20 amino acids at positions 49-68 in the NA stalk that is similar to previous Z genotype clade 1 H5N1 HPAI viruses isolated in Thailand (Amonsin et al., 2006; Viseshakul et al., 2004). This is considered to be correlated with an adaptation of aquatic bird avian influenza viruses to chickens (Matrosovich et al., 1999). The mutation H247Y in the NA, that is associated with resistance of influenza A viruses to Oseltamivir (Collins et al., 2008; Deyde et al., 2009; Mihajlovic and Mitrasinovic, 2008), was not present, as has been the case with other Thai H5N1 viruses (Chutinimitkul et al., 2007).

The wild birds in this survey that tested positive for antibody to H5 virus or were infected with H5N1 viruses were common residential species in the district, except for the pond heron which can be either a resident or a winter visitor (Robson 2004). These residential species (except for the blue-tailed bee-eater) were commonly observed wandering in villages and households where backyard poultry and low bio-security farms were present (Details in Chapter 6). Previous literature has reported that some terrestrial bird species are less susceptible to infection with H5N1 HPAI viruses compared to others (Boon et al., 2007; Perkins and Swayne, 2003a). Clinically healthy resident wild bird species showed evidence of previous or current infection with H5N1 HPAI viruses, albeit at a low prevalence. This wild bird surveillance also showed that some terrestrial birds that were exposed to H5N1 viruses became infected, but were clinically healthy, had developed H5

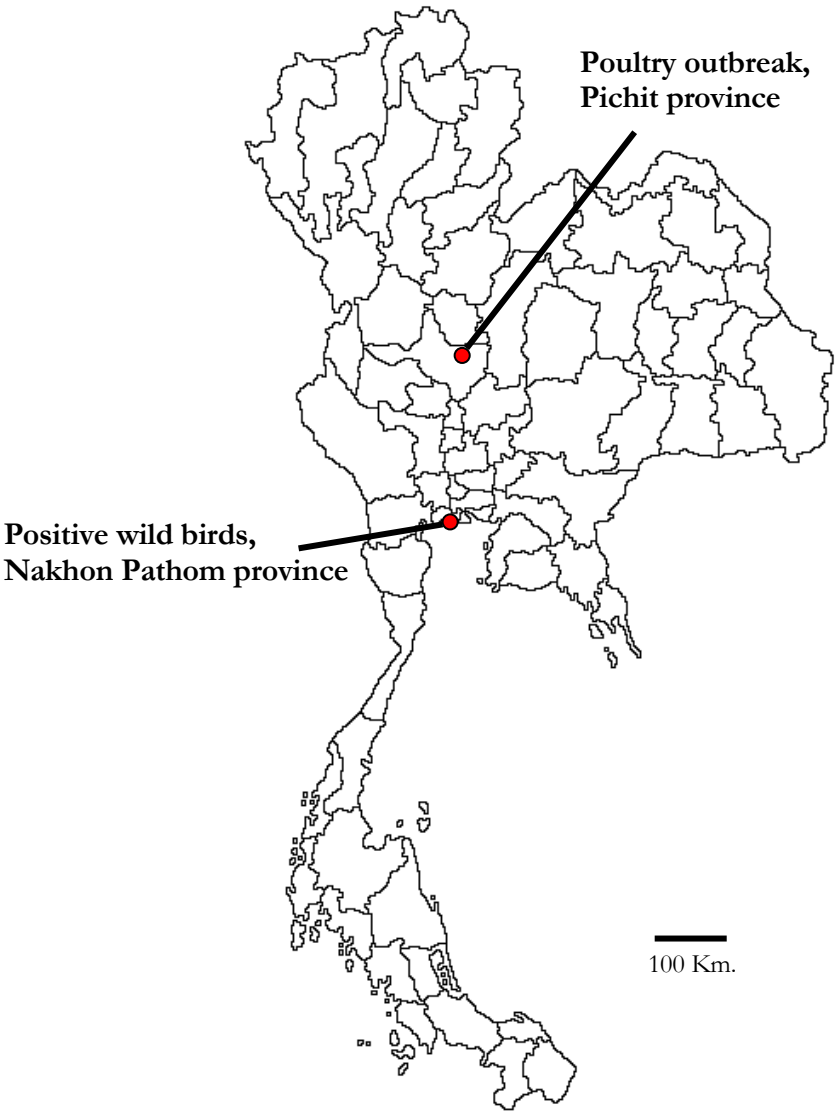
neutralising antibody and at the time of swabbing were not shedding virus. Such birds appear to be healthy survivors that would not act as prolonged virus carriers.

There were no reports of HPAI H5N1 outbreaks in poultry during the study period. However, outbreaks of HPAI H5N1 virus in domesticated poultry are generally detected by the presence of clinical signs and a high mortality rate (DLD, 2008). A surveillance study in live bird and food markets, where live poultry and products originate from backyards and/or non-commercial farms, that was conducted in the ten central provinces of Thailand during July 2006 to August 2007 detected H5N1 viruses in live chickens, moorhen (visceral organs), water cock (visceral organs), and quail (visceral organs) (Amonsin et al., 2008). Thus, undetected H5N1 infections in poultry may exist in some areas without the presence of obvious outbreaks of disease. The wild bird surveillance program reported in this chapter detected H5N1 virus infection in June 2007 and serological evidence of H5 infection in wild bird samples collected between March and December 2007 but not in 2008 indicating that there did not appear to be prolonged virus circulation at a substantial level. However, the sample size collected at the bi-monthly sampling times (35 birds on average) was small and may not detect a seroprevalence of 2.1% for H5 or a prevalence of 0.5% for H5N1 virus isolation found in these studies. Assuming a prevalence of 2.1% and a sample size of 35 birds the probability of detecting 1 or more positive birds is 0.53, and consequently the power of this study is low (Calculations using Survey Toolbox version 1.0; Cameron (1999)).

The phylogenetic relationship showed that both H5N1 viral samples that were isolated from wild birds clustered with other samples isolated in Thailand between 2004 and 2006. The study revealed that nucleotide sequences of the HA genes were closely related to

those of a H5N1 virus (A/chicken/Thailand/PC-168/2006) from Pichit province, central Thailand in 2006, while the NA genes were closely related to a virus (A/duck/Angthong/72/2004) isolated from a duck in Angthong province located in the central part of Thailand in 2004. The deduced amino acid sequences of the HA genes showed a close relationship (99% homology) between the viruses and three poultry viruses isolated from Pichit province in 2006 (A/chicken/Thailand/PC-168/2006, A/Ck/Phichit/NIAH1/2006, and A/Ck/ Phichit/NIAH606988/06). The NA genes also showed similarity (100% homogeny) at the amino acid level to poultry viruses (two chicken viruses isolated from Pichit province and one quail virus from a market survey in Central Thailand). In order to transmit to a new host species, a high number of mutations are often required (Kuiken et al., 2006). The question that arises is how viruses causing disease in poultry in Pichit province in 2006 spread to infect residential wild birds in Nakhon Pathom province in 2007 with little change in their gene sequences. There were no concurrent H5N1 outbreaks reported in poultry in Nakhon Pathom province during the study period. Figure 5.6 illustrates the location of the two provinces, which are approximately 230 kilometres apart (GoogleEarth, 2007). Possible explanations for this would include spill-over of H5N1 viruses from contaminated sources resulting from the movements of domestic poultry and/or fomites from outbreak areas to distant locations and thence to wild bird populations or infection of wild birds within the outbreak locations and then translocation by wild bird movement to distant locations.

Figure 5.6; Map of Thailand shows locations where the poultry outbreak in Pichit province and the positive wild birds were detected



There were limitations in the collection of field samples in this wild bird surveillance program due to difficulties in trapping wild birds, the inability to apply true random sampling to a wild population, and problems with ensuring an adequate sample size. Since the surveillance program involved a multiple species sample collection scheme, the number of samples per species also varied considerably. The proportion of samples collected per species was dependant upon the species' population sizes present in the area. For example, more samples were collected from the terrestrial birds commonly seen in the area including rock pigeons, white vented mynas, red turtle doves, spotted doves, and Asian pied starlings. Thus, the outcomes and results of this study should be interpreted with caution. This study does not elucidate the direction of movement or source of virus transmission to the wild birds (domestic poultry to resident wild birds or vice versa; origin from migratory birds, domestic poultry or indirectly via human movements).

To gain a wider picture of the disease ecology and its epidemiology, serological and virological surveillance and molecular studies in both poultry and wild birds need to be conducted in parallel. In future studies, a reduction in the biases caused by field sample collection from wild birds needs to be taken into account and further studies and knowledge of wild bird ecology and behaviour should be applied to address relevant epidemiological questions. The potential for viral transmission from wild birds to poultry is affected by the interactions between the species and a study to investigate these interactions is reported in the following chapter.

Chapter 6

OBSERVATIONAL STUDY TO INVESTIGATE INTERACTIONS OF WILD BIRDS WITH POULTRY SPECIES

6.1 Introduction

The most likely route of avian influenza transmission is contact between poultry and wild birds (Koch and Elbers, 2006). An observational study was undertaken to observe the ecology and behaviours of wild birds living in a study site in order to gain understanding of the interactions between wild birds and domesticated species and to determine the possibility of spill back and/or spill over of influenza viruses between these species. Bridge species are species that act as a bridge for the viral transmission from water birds to domestic poultry and/or from domestic poultry to water birds (Pfeiffer et al., 2006). Three main categories used to classify bridge species are considering them as: feral species, which are no longer wild; as species that share habitats and/or live reasonably close to domestic poultry; or as species living in areas where domestic poultry range widely (Pfeiffer, 2006). Bridge species were identified by analysis of data from this observational study. This study was designed to collect data on interactions between wild birds and domestic poultry in areas where bridge species are likely to be present. The observed sites were located within Banglane and Bangsripa districts at the locations where the questionnaire study (details in Chapter 4) and/or the surveillance program (details in Chapter 5) were conducted. The study involved collecting field data every two weeks

throughout the year at different time periods in the day. Results and outcomes of this study were used to provide quantitative or qualitative data for the risk assessments for the transmission of H5N1 virus from wild birds to domestic poultry described in Chapter 8.

6.2 Materials and Methods

6.2.1 Study design

As indicated in Chapter 2, this was an observational study to investigate the frequency and level of interaction between wild birds and poultry in a number of habitats in Central Thailand where previous outbreaks of H5N1 HPAI had occurred. The observation times were divided into eight periods; 6.00 a.m. – 7.30 a.m. (T1), 7.30 - 9.00 a.m. (T2), 9.00 a.m. -10.30 a.m. (T3), 10.30 a.m. - 12.00 p.m. (T4), 12.00 p.m. – 1.30 p.m. (T5), 1.30 p.m. - 3.00 p.m. (T6), 3.00 p.m. - 4.30 p.m. (T7), and 4.30 p.m. - 6.00 p.m. (T8). Each site was observed for 30 minutes per visit and all wild birds and domesticated species found in the site, the interaction between the species, and activities observed, including flying-in-flying-out, feeding, perching (off the ground), standing (on the ground), and direct contact, were recorded on a field data sheet (Appendix IV). The time that the birds spent (more or less than 30 seconds) in the area also were recorded. Date, start and finish time, temperature, humidity, and description of the site were recorded. Observational data were collected twice a month throughout the year. At the completion of the study, each site had been observed 24 times to cover all the time periods during daylight hours (6.00am to 6.00pm). The eight sites were pre-surveyed and coded as A1, A2, B1, B2, C1, C2, D1, and D2. The schedule for a four month period is shown in Table 6.1.

Table 6.1; Observed sites and time periods in one season (four months)

Months	**T1 (6.00 - 7.30)**	**T2 (7.30 - 9.00)**	**T3 (9.00 - 10.30)**	**T4 (10.30 -12.00)**	**T5 (12.00 -13.30)**	**T6 (13.30 -15.00)**	**T7 (15.00 -16.30)**	**T8 (16.30 -18.00)**
A season/four months	A1A2	A1A2	A1A2	A1A2	A1A2	A1A2	A1A2	A1A2
	B1B2	B1B2	B1B2	B1B2	B1B2	B1B2	B1B2	B1B2
	C1C2	C1C2	C1C2	C1C2	C1C2	C1C2	C1C2	C1C2
	D1D2	D1D2	D1D2	D1D2	D1D2	D1D2	D1D2	D1D2

6.2.2 Observation site selection

The sites were defined based on the wild bird's habitat types including wild bird roosting areas, rice paddies and/or ponds, low bio-security poultry farms, and backyard/household areas. Each of the four habitat types was replicated (a total of eight observation sites in the study). The description of each study sites is as follows;

Site A1: Pigeon roosting site (Figure 6.1)

A block of abandoned buildings was surrounded by rice paddy fields with a main road at the front. A number of pigeons roosted and nested on the buildings with an estimated population size of 100. GPS location: X47P0623050, Y1549677

Site A2: Rice paddy field with a natural pond (Figure 6.2)

Rice paddy fields beside a natural pond represented a natural feeding ground for wild birds. The site was located in the middle of several households next to a rice processor

with a small road across the area. Various species of wild birds were seen at the site with a variety of population sizes depending on the time, season, and bird species. GPS location: X47P0631774, Y1550139

Site B1: Backyard behind a group of factory workers' homes (Figure 6.3)

A backyard behind a village where chickens were kept by residents who lived nearby was surrounded by bush with a small access road through resident households. Approximately 20 native chickens were raised at this site. Some chickens were kept in coops and small cages while some were allowed to roam freely. Wild birds were commonly seen in the area. GPS location: X47P0631119, Y1549981

Site B2: An open system duck farm at a duck slaughter house (Figure 6.4)

The farm belonged to the duck slaughter house where both ducks for slaughter and growing broiler ducks and geese were accommodated. The slaughter area was separated from housing areas. There were two houses with a pond in the middle. In general, one of the houses was used to keep ducks waiting for slaughter. Ducks were housed here for up to 7 days depending on market demands. Another poultry house was used for raising broiler ducks and/or geese. The farm's system for bringing poultry in and out and housing varied through out the year. GPS location: X47P0632594, Y1550161

Figure 6.1; Abandoned building where pigeons were nesting (site A1)

Figure 6.2; Rice paddy fields and a natural pond (site A2)

Figure 6.3; Backyard areas behind accommodation for factory workers (site B1)

Figure 6.4; An open system duck farm in a slaughter house (site B2)

Site C1: Asian open bill stork roosting site (Figure 6.5)

This observed site was based on a location where the storks roosted and raised their chicks. The storks first roosted at an abandoned agricultural area and then moved to the bush on the Mae-Klong river bank. The observed site was moved following the movement of the storks. The observation spot was located on the opposite side of the river bank where the storks nested (under a road bridge). The estimate stork population size was 600. There were also pigeons and white vented mynas nesting under the bridge with an estimated population size of 100. GPS location: X47P0621218, Y1560894

Site C2: Rice paddy fields and an abandoned lotus farm (Figure 6.6)

This site was located in the middle of villages. Rice was grown in the paddy fields three to four times a year. The abandoned lotus ponds had become a habitat for water birds and some terrestrial birds. Various birds fed and nested in the ponds. GPS location: X47P0632647, Y1550113

Site D1: Backyard in a local village (Figure 6.7)

The backyard was located in the cluster of four residential houses. Native chickens/ fighting cocks (approximately 20 adults and 30 juveniles) ranged freely in the household and backyard area. Some fighting cocks were kept in coops and these birds were fed with unmilled rice and supplements. The coops had a small bucket of water hanging on the side. There was a small pig enclosure for piglets and a separate pen for a sow in the backyard area. Pig food was left in food containers inside the enclosure. GPS location: X47P0627406, Y1548816

Site D2: An open system layer duck farm (Figure 6.8)

The farm had two houses with a duck pond in the middle. The pond was used by the ducks during the day-time. The housing was 50x10 metres in size and had a ceramic tile roof and could house 2000 ducks. Feed was provided to ducks in containers which were permanently located in the middle of the housing. Permanent water containers were kept around the edges of the building. Wild birds were commonly seen feeding on the duck food. Rice paddy fields were next to the farm (on the opposite road side). GPS location: X47P0627311, Y1551572

Figure 6.5; Asian open bill stork roosting site (site C1)

Figure 6.6; Abandoned lotus pond next to rice paddy fields (site C2)

Figure 6.7; A household with backyard poultry and pigs (site D1)

Figure 6.8; An open system layer duck farm (site D2)

6.2.3 Observation field data collection

Equipment used in each observation trip included cameras (compact and SLR) and tripod, binoculars (10x42), bird guide books (Lekagul and Round, 1991), observation data collection forms, pens/ markers, portable GPS, thermometer and humidity recorders (Kestrel® 4000 Pocket wind metre), and a timer. During data collection, each site was observed for approximately 30 minutes. Approximately 15 minutes was required to travel between sites. Sections A and B of the data collection form were filled in during the first five minutes after arrival at the observation site. Photos of the sites and birds were also taken. Wild birds were then observed and data on species, activities, and interactions were noted in section C of the

data sheet during the next 25 minutes. All wild birds that were visible in the areas through a 360 degree radius were recorded.

6.2.4 Statistical analysis

Observational data were entered into a Microsoft Excel spreadsheet and analysed with SPSS. Temperature and humidity of each time period recorded was averaged. Frequencies and percentages of wild birds and their behaviours were calculated and described in the Tables. Pearson correlation coefficients (r) between the number of birds counted in each habitat type (1 = A1 and C1, 2 = A2 and C2, 3 = B1 and D1, and 4 = B2 and D2) and observation time period of the days were calculated. All significant ($p \leq 0.05$) variables were reported.

6.3 Results

The average temperature was 32.3 °C and humidity was 52.0% at the study sites (Table 6.2). The maximum temperatures were recorded between 1.30 p.m. - 3.00 p.m. Sites A1, A2, and D2 were observed a total of 24 times while B1, B2, C1, C2, and D1 were observed 23 times. More than eighty species of wild birds were observed in the study. The majority of the birds observed were terrestrial birds with rock pigeons (20%) and sparrows (19.3%) being the most common (Table 6.3). Some waterfowls such as lesser whistling ducks, cotton pygmy goose, and garganey and waders such as Asian open bill storks, black-winged stilt, bronze-winged jacana, and greater painted-snipe were also seen at the sites. However, the frequencies of observing water birds were small when compared to the numbers of terrestrial birds observed.

Table 6.2; Average temperature and humidity at the study sites in each time period (T1 – T8)

Category	Minimum	Maximum	Mean	Std. Deviation
T1; 6.00-7.30 temperature (°C)	23	31	27.2	2.0
T1; 6.00-7.30 humidity (%)	50.3	90.3	74.6	11.4
T2; 7.30 - 9.00 temperature (°C)	23	40	29.0	3.7
T2; 7.30 - 9.00 humidity (%)	26.0	89.2	64.1	17.7
T3; 9.00 -10.30 temperature (°C)	27	42	31.0	3.1
T3; 9.00 -10.30 humidity (%)	17.0	77.8	55.9	15.6
T4; 10.30 -12.00 temperature (°C)	28	39	33.5	2.4
T4; 10.30 -12.00 humidity (%)	37.8	60.0	49.5	7.4
T5; 12.00 -13.30 temperature (°C)	27	47	34.8	4.5
T5; 12.00 -13.30 humidity (%)	23.1	66.7	44.0	13.2
T6; 13.30 -15.00 temperature (°C)	28	47	35.5	5.2
T6; 13.30 -15.00 humidity (%)	24.4	59.2	41.56	10.3
T7; 15.00 -16.30 temperature (°C)	28	41	34.7	3.4
T7; 15.00 -16.30 humidity (%)	24.9	52.0	38.3	9.4
T8; 16.30 -18.00 temperature (°C)	28	39	32.6	3.1
T8; 16.30 -18.00 humidity (%)	31.3	60.6	47.6	9.1
Overall temperature (°C)	23	47	32.3	4.5
Overall humidity (%)	17.0	90.3	52.0	16.4

Table 6.3; Frequency of wild birds observed and percentage of them being observed

Common name (species, genus, or family)	Observed frequency	Percent of all observations
Asian brown flycatcher (*Muscicapa dauurica*)	7	0.07
Asian koel (*Eudynamys scolopaceus*)	26	0.27
Asian openbill stork (*Anastomus oscitans*)	233	2.39
Asian palm-swift (*Cypsiurus balasiensis*)	4	0.04
Asian pied starling (*Gracupica contra*)	208	2.13
Babbler (Family: *Timaliidae*)	1	0.01
Baillons crake (*Porzana pusilla*)	1	0.01
Bee-eater (*Nyctyornis sp. or Merops sp.*)	2	0.02
Bittern (*Ixobrychus sp.*)	4	0.04
Black bittern (*Ixobrychus flavicollis*)	3	0.03
Black capped kingfisher (*Halcyon pileata*)	4	0.04
Black drongo (*Dicrurus macrocercus*)	173	1.77
Black-shouldered Kite (*Elanus axillaris*)	7	0.07
Black-winged Stilt (*Himantopus himantopus*)	6	0.06
Bronze-winged jacana (*Metopidius indicus*)	165	1.69
Brown shrike (*Lanius cristatus*)	48	0.49
Streak-eared bulbul (*Pycnonotus blanfordi*)	302	3.10
Bushchat (*Saxicola sp.*)	5	0.05
Cattle egret (*Bubulcus ibis*)	28	0.29
Chestnut - headed bee – eater (*Merops leschenaulti*)	1	0.01
Cinnamon bittern (*Ixobrychus cinnamomeus*)	1	0.01
Common moorhen (*Gallinula chloropus*)	6	0.06
Common myna (*Acridotheres tristis*)	294	3.01
Common stonechat (*Saxicola torquata*)	9	0.09
Common tailorbird (*Orthotomus sutorius*)	26	0.27
Cotton pygmy goose (*Nettapus coromandelianus*)	17	0.17
Cuckoo (*Cuculus sp. or Clamator sp.*)	1	0.01
Dark - necked tailorbird (*Orthotomus atrogularis*)	2	0.02
Dove (*Columbinae sp.*)	3	0.03
Egret (*Ardeidae sp.*)	179	1.84
Flycatcher (Family: *Muscicapidae*)	4	0.04
Garganey (*Anas querquedula*)	1	0.01
Asian golden weaver (*Ploceus hypoxanthus*)	108	1.11
Greater cormorant (*Phalacrocorax carbo*)	1	0.01
Greater coucal (*Centropus sinensis*)	33	0.34
Greater painted-snipe (*Rostratula benghalensis*)	4	0.04
Grey heron (*Ardea cinerea*)	2	0.02
Grey-headed lapwing (*Vanellus cinereus*)	4	0.04
Gull (*Larus sp.*)	4	0.04
Indian roller (*Coracias benghalensis*)	6	0.06
Intermediate egret (*Casmerodius albus*)	13	0.13
Iora (*Aegithina sp.*)	3	0.03
Kingfisher (Suborder: *Alcedines*)	3	0.03

Common name (species, genus, or family)	Observed frequency	Percent of all observations
Lesser cormorant (*Phalacrocorax niger*)	229	2.35
Lesser whistling duck (*Dendrocygna javanica*)	133	1.36
Little egret (*Egretta garzetta*)	85	0.87
Little grebe (*Tachybaptus ruficollis*)	23	0.24
Malkoha (*Phaenicophaeus sp.*)	8	0.08
Myna (*Acridotheres sp.*)	3	0.03
Night heron (*Nycticorax sp.*)	40	0.41
Olive-backed sunbird (*Nectarinia jugularis*)	5	0.05
Oriental magpie-robin (*Copsychus saularis*)	91	0.93
Oriental pratincole (*Glareola maldivarum*)	3	0.03
Pheasant-tailed jacana (*Hydrophasianus chirurgus*)	103	1.06
Pied fantail (*Rhipidura javanica*)	135	1.38
Rock pigeon (*Columba livia*)	2009	20.60
Plain prinia (*Prinia inornata*)	1	0.01
Plain-backed sparrow (*Passer flaveolus*)	22	0.23
Plaintive cuckoo (*Cacomantis merulinus*)	5	0.05
Pond heron (*Ardeola sp.*)	563	5.77
Prinia (*Prinia sp.*)	94	0.96
Purple heron (*Ardea purpurea*)	1	0.01
Purple swamphens (*Porphyrio porphyrio)*)	6	0.06
Racket-tailed drongo (*Dicrurus paradiseus*)	1	0.01
Red - rumped swallow (*Cecropis daurica*)	1	0.01
Red turtle dove (*Streptopelia tranquebarica*)	568	5.82
Red wattle lapwings (*Vanellus indicus*)	37	0.38
Scaly breasted munia (*Lonchura punctulata*)	24	0.25
Scarlet-backed flowerpecker (*Dicaeum cruentatum*)	1	0.01
Sparrow (*Passer sp.*)	1879	19.26
Spotted dove (*Streptopelia chinensis*	162	1.66
Sunbird (Family: *Nectariniidae*)	2	0.02
Swallow and swift (Families: *Hirundinidae and Apodidae*)	373	3.82
Tailorbird (*Orthotomus sp.*)	42	0.43
Tern (*Sterna sp. or Chlidonias sp.*)	1	0.01
Warbler (*Phylloscopus sp.*)	9	0.09
Watercock (*Gallicrex cinerea*)	1	0.01
Weaver (Family: *Ploceidae*)	34	0.35
White-breasted waterhen (*Amaurornis phoenicurus*)	41	0.42
White-vented myna (*Acridotheres grandis*)	853	8.75
White-throated Kingfisher (*Halcyon smyrnensis*)	11	0.11
Yellow - vented Bulbul (*Pycnonotus goiavier*)	23	0.24
Yellow Bittern (*Ixobrychus sinensis*)	3	0.03
Zebra dove (*Geopelia striata*)	177	1.81
Total number of observations	**9754**	**100**

The majority of the birds seen at site A1 (pigeon roosting site) were terrestrial species although some waders and water birds were also observed (Table 6.4). The species that were commonly observed at this site included pigeons (31%), sparrows (22%), white vented mynas (15%), and red turtle doves (9%). The percentages of a species observed were similar to the percentage of observation frequency of the same species. At the paddy field and water source study sites (Site A2 and C2), pond herons were the most frequently observed species (14%; Table 6.4). Even though pigeons (9%) were observed regularly at sites A2 and C2, water birds, such as bronze-winged jacanas and lesser cormorants, were also frequently seen. As well, common terrestrial birds, such as white vented mynas and sparrows, were also seen at these sites. However, the total number of pigeons was much higher than the total number of pond herons in these two sites. The most commonly seen species in backyard areas (sites B1 and D1) were sparrows (29%) followed by pigeons, red turtle doves, white vented mynas, and bulbuls (Table 6.4). Water birds, such as pond herons, egrets, Asian open bill storks, lesser cormorants, and lesser whistling ducks, were occasionally observed in the backyard areas.

Wild birds seen in the open system duck farms (sites B2 and D2) were mainly common terrestrial birds including pigeons (31%), sparrows (29%), red turtle doves (6%), white vented mynas (5%), and common mynas (4%; Table 6.5). Groups of water birds, such as lesser cormorant, lesser whistling ducks, egrets, lapwings, and waterhens, were also seen at these sites; however, the percentage of frequencies of water birds being observed were very low (less than 1%; Table 6.5). At site C1 (Asian open bill stork roosting site) 57% of the birds in the site were Asian open bill storks and 18% of the total birds observed were pigeons.

However pigeons (23%) and white vented mynas (20%) were the most frequently observed species at the site (Table 6.5).

Table 6.4; Summary of total observed numbers (TN) and frequency observed (FO) of wild birds at the study sites

Common name	Site A1				Sites A2 and C2				Sites B1and D1			
	TN	%TN	FO	%FO	TN	%TN	FO	%FO	TN	%TN	FO	%FO
Asian brown flycatcher	1	0.03	1	0.08	-	-	-	-	6	0.13	6	0.25
Asian koel	6	0.19	6	0.47	3	0.05	3	0.12	12	0.25	12	0.51
Asian open bill stork	54	1.69	18	1.42	460	8.17	82	3.39	164	3.46	30	1.26
Asian palm-swift	1	0.03	1	0.08	1	0.02	1	0.04	3	0.06	1	0.04
Asian pied starling	15	0.47	11	0.87	186	3.30	94	3.89	43	0.91	27	1.14
Babbler	-	-	-	-	1	0.02	1	0.04	-	-	-	-
Baillon's crake	-	-	-	-	2	0.04	1	0.04	-	-	-	-
Bittern	-	-	-	-	3	0.05	3	0.12	-	-	-	-
Bee eater	1	0.03	1	0.08	-	-	-	-	4	0.08	1	0.04
Black bittern	-	-	-	-	1	0.02	1	0.04	1	0.02	1	0.04
Black capped kingfisher	-	-	-	-	1	0.02	1	0.04	-	-	-	-
Black drongo	1	0.03	1	0.08	54	0.96	50	2.07	81	1.71	65	2.74
Black-shouldered kite	-	-	-	-	1	0.02	1	0.04	5	0.11	5	0.21
Black-winged stilt	-	-	-	-	17	0.30	6	0.25	-	-	-	-
Bronze-winged jacana	-	-	-	-	261	4.63	160	6.61	-	-	-	-
Brown shrike	1	0.03	1	0.08	20	0.36	20	0.83	20	0.42	20	0.84
Bulbul	52	1.63	34	2.68	40	0.71	31	1.28	214	4.52	157	6.61
Bushchat	-	-	-	-	3	0.05	3	0.12	-	-	-	-
Cattle egret	2	0.06	2	0.16	33	0.59	19	0.79	1	0.02	1	0.04
Chestnut - headed bee – eater		-	-	-	-	-	-	-	1	0.02	1	0.04
Common moorhen	-	-	-	-	14	0.25	6	0.25	-	-	-	-
Common myna	61	1.91	43	3.39	92	1.63	52	2.15	92	1.94	69	2.91
Common Stonechat	-	-	-	-	9	0.16	9	0.37	-	-	-	-

Common name	Site A1				Sites A2 and C2				Sites B1and D1			
	TN	%TN	FO	%FO	TN	%TN	FO	%FO	TN	%TN	FO	%FO
Common tailorbird	7	0.22	6	0.47	3	0.05	3	0.12	13	0.27	13	0.55
Cotton pygmy goose	-	-	-	-	75	1.33	17	0.70	-	-	-	-
Dark - necked tailorbird	-	-	-	-	-	-	-	-	2	0.04	2	0.08
Dove	-	-	-	-	-	-	-	-	2	0.04	1	0.04
Egret	12	0.38	10	0.79	131	2.33	82	3.39	88	1.86	36	1.52
Flycatcher	6	0.19	3	0.24	-	-	-	-	1	0.02	1	0.04
Golden weaver	-	-	-	-	126	2.24	78	3.22	12	0.25	9	0.38
Greater cormorant	-	-	-	-	-	-	-	-	-	-	-	-
Greater coucal	1	0.03	1	0.08	6	0.11	6	0.25	9	0.19	9	0.38
Greater painted-snipe	-	-	-	-	5	0.09	3	0.12	-	-	-	-
Grey-headed lapwing	-	-	-	-	4	0.07	4	0.17	-	-	-	-
Grey heron	-	-	-	-	2	0.04	2	0.08	-	-	-	-
Gull	-	-	-	-	5	0.09	4	0.17	-	-	-	-
Indian rollers	-	-	-	-	3	0.05	3	0.12	2	0.04	2	0.08
Intermediate egret	2	0.06	2	0.16	11	0.20	8	0.33	6	0.13	3	0.13
Iora	-	-	-	-	-	-	-	-	3	0.06	2	0.08
Lesser cormorant	13	0.41	10	0.79	403	7.15	139	5.75	42	0.89	37	1.56
Lesser whistling duck	6	0.19	2	0.16	574	10.19	96	3.97	121	2.55	10	0.42
Little egret	5	0.16	5	0.39	68	1.21	53	2.19	11	0.23	8	0.34
Little grebe	-	-	-	-	32	0.57	17	0.70	-	-	-	-
Malkoha	1	0.03	1	0.08	2	0.04	2	0.08	4	0.08	3	0.13
Myna	2	0.06	1	0.08	-	-	-	-	4	0.08	2	0.08
Night heron	1	0.03	1	0.08	46	0.82	32	1.32	9	0.19	5	0.21
Olive-backed sunbird	1	0.03	1	0.08	-	-	-	-	3	0.06	3	0.13
Oriental magpie-robin	8	0.25	8	0.63	2	0.04	2	0.08	63	1.33	58	2.44
Oriental pratincole	-	-	-	-	4	0.07	3	0.12	-	-	-	-
Pheasant-tailed jacana	-	-	-	-	185	3.28	97	4.01	-	-	-	-

Common name	Site A1				Sites A2 and C2				Sites B1and D1			
	TN	%TN	FO	%FO	TN	%TN	FO	%FO	TN	%TN	FO	%FO
Pied fantail	20	0.63	15	1.18	3	0.05	3	0.12	77	1.63	71	2.99
Pigeon	1567	48.97	389	30.63	1358	24.11	209	8.64	739	15.60	362	15.24
Plain-backed sparrow	1	0.03	1	0.08	27	0.48	17	0.70	-	-	-	-
Plaintive cuckoo	-	-	-	-	-	-	-	-	-	-	-	-
Plain prinia	-	-	-	-	2	0.04	1	0.04	-	-	-	-
Pond heron	7	0.22	6	0.47	527	9.36	351	14.51	53	1.12	48	2.02
Prinia	-	-	-	-	55	0.98	47	1.94	12	0.25	11	0.46
Purple heron	-	-	-	-	1	0.02	1	0.04	-	-	-	-
Purple swamphen	-	-	-	-	11	0.20	5	0.21	-	-	-	-
Racket-tailed drongo	-	-	-	-	-	-	-	-	1	0.02	1	0.04
Red - rumped swallow	-	-	-	-	1	0.02	1	0.04	-	-	-	-
Red turtle dove	202	6.31	110	8.66	120	2.13	79	3.27	300	6.33	173	7.28
Red wattle lapwings	-	-	-	-	54	0.96	32	1.32	2	0.04	2	0.08
Scaly breast munia	4	0.13	2	0.16	4	0.07	2	0.08	6	0.13	3	0.13
Scarlet-backed flowerpecker	-	-	-	-	-	-	-	-	1	0.02	1	0.04
Sparrow	636	19.88	281	22.13	282	5.01	104	4.30	1851	39.08	697	29.35
Spotted dove	13	0.41	12	0.94	64	1.14	50	2.07	61	1.29	52	2.19
Sunbird	-	-	-	-	-	-	-	-	1	0.02	1	0.04
Swallow	72	2.25	51	4.02	250	4.44	99	4.09	176	3.72	88	3.71
Tailorbird	10	0.31	10	0.79	22	0.39	12	0.50	14	0.30	13	0.55
Tern	-	-	-	-	5	0.09	1	0.04	-	-	-	-
Warbler	7	0.22	5	0.39	-	-	-	-	3	0.06	3	0.13
Watercock	-	-	-	-	1	0.02	1	0.04	-	-	-	-
Weaver	5	0.16	2	0.16	26	0.46	17	0.70	18	0.38	12	0.51
White breast waterhen	-	-	-	-	45	0.80	31	1.28	7	0.15	6	0.25
White-throated kingfisher	-	-	-	-	4	0.07	4	0.17	-	-	-	-

Common name	Site A1				Sites A2 and C2				Sites B1and D1			
	TN	%TN	FO	%FO	TN	%TN	FO	%FO	TN	%TN	FO	%FO
White vented myna	364	11.38	192	15.12	238	4.23	125	5.17	277	5.85	161	6.78
Yellow bittern	-	-	-	-	-	-	-	-	1	0.02	1	0.04
Yellow - vented bulbul	-	-	-	-	-	-	-	-	30	0.63	23	0.97
Zebra dove	32	1.00	25	1.97	44	0.78	32	1.32	65	1.37	46	1.94
Total	3200	100.00	1270	100.00	5633	100.00	2419	100.00	4737	100.00	2375	100.00

Table 6.5; Summary of total observed numbers (TN) and frequency observed (FO) of wild birds at the study sites B2, D2 and C1

Common name	Sites B2 and D2				Site C1			
	TN	%TN	FO	%FO	TN	%TN	FO	%FO
Asian koel	2	0.03	2	0.08	3	0.04	3	0.25
Asian open bill stork	141	2.39	19	0.77	4555	57.75	84	7.00
Asian palm-swift	-	-	-	-	3	0.04	1	0.08
Asian pied starling	73	1.24	57	2.30	60	0.76	19	1.58
Bittern	-	-	-	-	2	0.03	1	0.08
Black bittern	1	0.02	1	0.02	-	-	-	-
Black capped kingfisher	-	-	-	-	3	0.04	3	0.25
Black drongo	26	0.44	23	0.93	41	0.52	34	2.83
Black-shouldered kite	-	-	-	-	1	0.01	1	0.08
Bronze-winged jacana	-	-	-	-	6	0.08	5	0.42
Brown shrike	-	-	-	-	7	0.09	7	0.58
Bulbul	57	0.97	43	1.73	49	0.62	37	3.08
Bushchat	-	-	-	-	2	0.03	2	0.17

Common name	Sites B2 and D2				Site C1			
	TN	%TN	FO	%FO	TN	%TN	FO	%FO
Cattle egret	3	0.05	3	0.12	4	0.05	3	0.25
Cinnamon bittern	-	-	-	-	2	0.03	1	0.08
Common myna	197	3.34	116	4.68	27	0.34	14	1.17
Common stonechat	-	-	-	-	-	-	-	-
Common tailorbird	1	0.02	1	0.04	3	0.04	3	0.25
Cuckoo	-	-	-	-	2	0.03	1	0.08
Dove	-	-	-	-	2	0.03	2	0.17
Egret	40	0.68	31	1.25	243	3.08	20	1.67
Garganey	-	-	-	-	1	0.01	1	0.08
Golden weaver	23	0.39	19	0.77	4	0.05	2	0.17
Greater cormorant	-	-	-	-	1	0.01	1	0.08
Greater coucal	6	0.10	5	0.20	14	0.18	12	1.00
Greater painted-snipe	-	-	-	-	6	0.08	1	0.08
Indian rollers	-	-	-	-	1	0.01	1	0.08
Iora	-	-	-	-	1	0.01	1	0.08
Kingfisher	-	-	-	-	3	0.04	3	0.25
Lesser cormorant	87	1.47	17	0.69	66	0.84	24	2.00
Lesser whistling duck	93	1.58	12	0.48	129	1.64	13	1.08
Little egret	10	0.17	8	0.32	11	0.14	11	0.92
Little grebe	4	0.07	2	0.08	5	0.06	3	0.25
Malkoha	-	-	-	-	2	0.03	2	0.17
Night heron	4	0.07	2	0.08	-	-	-	-
Olive-backed sunbird	1	0.02	1	0.04	-	-	-	-
Oriental magpie-robin	17	0.29	16	0.65	9	0.11	7	0.58
Pheasant-tailed jacana	-	-	-	-	11	0.14	5	0.42
Pied fantail	40	0.68	33	1.33	17	0.22	13	1.08
Pigeon	2021	34.24	769	31.02	1441	18.27	277	23.08

Common name	Sites B2 and D2				Site C1			
	TN	%TN	FO	%FO	TN	%TN	FO	%FO
Plain-backed sparrow	3	0.05	3	0.12	1	0.01	1	0.08
Plaintive cuckoo	-	-	-	-	11	0.14	5	0.42
Pond heron	74	1.25	65	2.62	159	2.02	93	7.75
Prinia	13	0.22	12	0.48	31	0.39	24	2.00
Purple swamphen					1	0.01	1	0.08
Red turtle dove	246	4.17	163	6.58	59	0.75	42	3.50
Red wattle lapwings	5	0.08	3	0.12	-	-	-	-
Scaly breast munia	7	0.12	3	0.12	38	0.48	14	1.17
Sparrow	2136	36.19	737	29.73	108	1.37	59	4.92
Spotted dove	45	0.76	37	1.49	14	0.18	11	0.92
Sunbird	-	-	-	-	1	0.01	1	0.08
Swallow	176	2.98	73	2.94	197	2.50	62	5.17
Tailorbird	5	0.08	4	0.16	3	0.04	3	0.25
Warbler	-	-	-	-	1	0.01	1	0.08
Weaver	2	0.03	1	0.04	3	0.04	2	0.17
White breast waterhen	2	0.03	2	0.08	2	0.03	2	0.17
White-throated kingfisher	-	-	-	-	7	0.09	7	0.58
White vented myna	255	4.32	133	5.37	499	6.33	241	20.08
Yellow bittern	-	-	-	-	2	0.03	2	0.17
Zebra dove	86	1.46	63	2.54	13	0.16	11	0.92
Total	5902	100.00	2479	100.00	7887	100.00	1200	100.00

A variety of birds, both terrestrial and water birds, were observed in the wild bird roosting/nesting sites (A1 and C1). Asian open bill storks and pigeons had the highest total numbers (n) while white vented mynas, sparrows, swallows and red turtle doves were observed commonly but in smaller numbers (Table 6.6). More than 80% of the storks were observed perching in trees where they nested. The storks were rarely observed landing/standing on the ground, feeding, or having direct contact with other birds. Most of the storks (97%) spent longer then 30 seconds at the site while some (15%) flew in and out. Similarly most pigeons (67%) at the sites were observed perching, while 44% were observed flying in and out and less than 1% were seen to have direct contact with other birds. Ten percent of pigeons were observed to be close (<1 metre) to other pigeons, while 6% were observed standing on the ground with 2% feeding. More than half of the observed pigeons spent more than 30 seconds at the site while 44% flew in and out (Table 6.6).

Some birds were observed flying in and out without stopping over or landing at the sites. The birds that made direct contact with other birds (either of the same or different species) were sparrows, pigeons, white vented mynas, red turtle doves, pied fantails, common mynas, oriental magpie-robins, scaly breast munias, and a pond heron. Small numbers of white vented mynas (10), sparrows (5), pigeons (2), and pond heron (1) were observed close to (<1 metre) and/or feeding together with backyard chickens (Table 6.6). The wild birds observed close to (<1 metre) and/or feeding together with other birds of the same species included sparrows, scaly breast munias, and prinias. While the wild birds observed close to (<1 metre) and/or feeding together with birds belonging to other species included Asian pied starlings, pigeons, sparrows, white vented mynas, red turtle

doves, lesser whistling ducks, egrets, common mynas, scaly breast munias, greater painted-snipes, prinias, pond herons, pied fantails, zebra doves, golden weavers, weavers, oriental magpie-robins, tailorbirds, spotted doves, and lesser cormorants. Of these species the white vented mynas and pigeons were observed most frequently close to (<1 metre) and/or feeding with other bird species.

In total, 77% of wild birds were observed perching and 83% spent more than 30 seconds at the sites (Table 6.6). One-third of the birds were flying in and out. Small numbers of the birds were feeding and/or standing (or landing on the ground) at these sites. Direct contact between birds was observed (2%) while few birds were in close proximity (<1 metre) and/or feeding together with other birds either of the same or different species.

Table 6.6; Numbers of wild birds seen and their behaviours at the wild bird roosting /nesting areas (sites A1 and C1)

Common name	n	Behaviour*												Close contact/ close proximity (< 1 metre)*					
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CC	%CC	CSB	%CSB	CDB	%CDB
Asian brown flycatcher	1	1	100	-	-	1	100	-	-	-	-	1	100	-	-	-	-	-	-
Asian koel	9	5	56	-	-	5	56	-	-	-	-	5	56	-	-	-	-	-	-
Asian open bill stork	4609	702	15	1	0	4051	88	3	0	-	-	4471	97	-	-	211	5	3	0
Asian palm-Swift	4	4	100	-	-	-	-	-	-	-	-	3	75	-	-	-	-	-	-
Asian pied starling	75	44	59	12	16	40	53	9	12	-	-	50	67	-	-	11	15	26	35
Bee eater	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bittern	2	2	100	-	-	-	-	2	100	-	-	2	100	-	-	-	-	-	-
Black capped kingfisher	3	2	67	-	-	1	33	-	-	-	-	1	33	-	-	-	-	-	-
Black drongo	42	20	48	2	5	32	76	1	2	-	-	33	79	-	-	1	2	3	7
Black-shouldered kite	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bronze-winged jacana	6	5	83	3	50	-	-	3	50	-	-	4	67	-	-	-	-	-	-
Brown shrike	8	2	25	-	-	8	100	-	-	-	-	8	100	-	-	-	-	-	-
Bulbul	101	51	50	8	8	70	69	1	1	2	2	68	67	-	-	5	5	5	5
Bushchat	2	2	100	-	-	2	100	-	-	-	-	2	100	-	-	-	-	-	-
Cattle egret	6	6	100	-	-	-	-	-	-	-	-	0	0	-	-	-	-	-	-
Cinnamon bittern	2	-	-	-	-	2	100	-	-	-	-	2	100	-	-	-	-	-	-

Common name	n	Behaviour*												Close contact/ close proximity (< 1 metre)*					
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CC	%CC	CSB	%CSB	CDB	%CDB
Common myna	88	60	68	8	9	46	52	6	7	3	3	50	57	-	-	7	8	11	13
Common tailorbird	10	2	20	4	40	8	80	-	-	-	-	7	70	-	-	-	-	-	-
Cuckoo	2	2	100	2	100	2	100	-	-	-	-	2	100	-	-	-	-	-	-
Dove	2	-	-	-	-	2	100	-	-	-	-	2	100	-	-	-	-	2	100
Egret	255	231	91	225	88	-	-	204	80	-	-	224	88	-	-	20	8	2	1
Flycatcher	6	6	100	-	-	6	100	-	-	-	-	6	100	-	-	-	-	-	-
Garganey	1	-	-	1	100	-	-	1	100	-	-	1	100	-	-	-	-	1	100
Golden weaver	4	4	100	-	-	2	50	-	-	-	-	2	50	-	-	2	50	-	-
Greater cormorant	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Greater coucal	15	10	67	6	40	11	73	6	40	-	-	12	80	-	-	-	-	1	7
Greater painted-snipe	6	-	-	6	100	-	-	6	100	-	-	6	100	-	-	6	100	6	100
Indian rollers	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Intermediate egret	2	2	100	-	-	-	-	-	-	-	-	0	0	-	-	-	-	-	-
Iora	1	-	-	-	-	1	100	-	-	-	-	1	100	-	-	-	-	-	-
Kingfisher	3	2	67	-	-	1	33	-	-	-	-	1	33	-	-	-	-	1	33
Lesser cormorant	79	74	94	-	-	6	8	0	0	-	-	7	9	-	-	2	3	2	3
Lesser whistling duck	135	54	40	-	-	-	-	81	60	-	-	81	60	-	-	21	16	10	7
Little egret	16	14	88	-	-	4	25	-	-	-	-	3	19	-	-	-	-	1	6
Little grebe	5	-	-	4	80	-	-	5	100	-	-	5	100	-	-	-	-	-	-

Common name	n	Behaviour*												Close contact/ close proximity (< 1 metre)*					
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CC	%CC	CSB	%CSB	CDB	%CDB
Malkoha	3	2	67	-	-	1	33	-	-	-	-	1	33	-	-	-	-	-	-
Myna	2	2	100	-	-	-	-	-	-	-	-	0	0	-	-	-	-	-	-
Night heron	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Olive-backed sunbird	1	1	100	-	-	1	100	-	-	-	-	1	100	-	-	-	-	-	-
Oriental magpie-robin	17	11	65	4	24	10	59	2	12	2	12	12	71	-	-	2	12	1	6
Pheasant-tailed jacana	11	6	55	5	45	-	-	5	45	-	-	5	45	-	-	0	0	2	18
Pied fantail	37	15	41	11	30	25	68	5	14	6	16	37	100	-	-	4	11	1	3
Pigeon	3008	1335	44	80	3	2037	68	206	7	58	2	2158	72	2	0	317	11	119	4
Plain-backed sparrow	2	1	50	-	-	1	100	-	-	-	-	1	50	-	-	-	-	-	-
Plaintive cuckoo	11	7	64	-	-	11	100	-	-	-	-	11	100	-	-	-	-	-	-
Pond heron	166	120	72	69	42	641	84	81	49	1	1	121	73	1	1	5	3	11	7
Prinia	31	15	48	11	35	18	64	1	3	-	-	26	84	-	-	5	16	-	-
Purple swamphen	1	-	-	1	100	-	-	1	100	-	-	1	100	-	-	-	-	-	-
Red turtle dove	261	67	26	11	4	219	84	12	5	17	7	222	85	-	-	32	12	18	7
Scaly breast munia	42	23	55	10	24	38	90	11	26	2	5	41	98	-	-	8	19	10	24
Sparrow	744	496	67	167	22	489	66	177	24	103	14	593	80	5	1	178	24	66	9
Spotted dove	27	13	48	2	7	19	70	2	7	-	-	21	78	-	-	2	7	1	4
Sunbird	1	-	-	-	-	1	100	-	-	-	-	1	100	-	-	-	-	-	-
Swallow	269	237	88	13	5	37	14	-	-	-	-	161	60	-	-	-	-	7	3
Tailorbird	13	9	69	-	-	7	54	2	15	-	-	7	54	-	-	2	15	1	8

Common name	n	Behaviour*												Close contact/ close proximity (< 1 metre)*					
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CC	%CC	CSB	%CSB	CDB	%CDB
Warbler	8	5	63	4	50	5	63	-	-	-	-	8	100	-	-	-	-	-	-
Weaver	8	3	38	-	-	5	63	-	-	-	-	7	88	-	-	2	25	-	-
White breast waterhen	2	-	-	2	100	-	-	2	100	-	-	2	100	-	-	-	-	-	-
White vented myna	863	506	59	57	7	591	68	88	10	33	4	633	73	10	1	110	13	158	18
White-thoated kingfisher	7	2	29	-	-	6	86	-	-	-	-	6	86	-	-	-	-	-	-
Yellow bittern	2	2	100	-	-	-	-	-	-	-	-	0	0	-	-	-	-	-	-
Zebra dove	45	12	27	6	13	29	64	5	11	-	-	32	71	-	-	4	9	1	2
Total	11087	4202	38	735	7	8492	77	928	8	227	2	9168	83	18	0	957	9	470	4

*a bird can perform more than one behaviour and/or close contact

FiFo = Fly in Fly out, F = Feeding, P = Perching, S = standing/landed on the ground or water bodies, DC = Direct contact with other wild and/or domestic birds, >30 = spending more than 30 seconds at the site, CC = close (<1 metre) and/or feeding together with domestic chicken, CSB = close (<1 metre) and/or feeding together with the same species of wild birds, and CDB = close (<1 metre) and/or feeding together with other different species wild birds

There was no domesticated poultry observed at the pond and rice paddy field sites (A2 and C2). Even though the majority of wild birds observed at the sites were pigeons, water birds, such as lesser whistling ducks, pond herons, Asian open bill storks, lesser cormorants, bronze-winged jacanas, Asian pied starlings, pheasant-tailed jacanas, and egret,s were also observed in relatively high numbers (Table 6.7). Water birds such as white breast waterhens, cattle egrets, bronze-winged jacanas, and pheasant-tailed jacanas mostly feed and spent a longer period of time (>30 seconds) at these sites (Table 6.7). Terrestrial birds like sparrows, white vented mynas, Asian pied starlings, red turtle doves, common mynas, and spotted doves were also commonly observed in the areas. Wild birds observed standing (landing) on the ground and/or in water bodies were red wattle lapwings, bronze winged jacanas, cotton pygmy geese, pheasant-tailed jacanas, white breast waterhens, and pond herons. Birds living close and/or feeding together with other bird species at these sites were common mynas, pheasant-tailed jacanas, bronze-winged jacanas, Asian pied starlings, lesser cormorants, white vented myna, sparrows, pond herons, red turtle doves, and lesser whistling ducks (Table 6.7).

Sixty percent of the observed pigeons were flying in and out and/or perching on trees and residential buildings while 12% were standing on the ground and 8% were feeding. Direct contact with other pigeons was not seen. Feeding together with other pigeons and/or other bird species was relatively infrequent (< 5%). Thirty-two percent of lesser whistling ducks were observed landing at these sites and 19% were observed feeding. Some of the ducks were observed in close proximity to (<1 metre) and/or feeding together with other ducks (12%) or with other bird species (4%; Table 6.7).

Apart from pigeons, wild birds that had direct contact with either the same or different species were Asian open bill storks, Asian pied starlings, Baillon's crakes, bronze-winged jacanas, common mynas, golden weavers, lesser cormorants, lesser whistling ducks, a pheasant-tailed jacana, plain-backed sparrows, pond herons, red turtle doves, sparrows, spotted doves, swallows, tailorbirds, weavers, white vented mynas, and zebra doves. Moreover, the birds in close proximity to (<1 metre) and/or feeding together with other bird species included Asian open bill storks, Asian pied starling, black drongos, bronze-winged jacanas, bulbuls, cattle egrets, common moorhens, common mynas, cotton pygmy geese, a golden weaver, greater painted-snipes, a grey-headed lapwing, intermediate egrets, lesser cormorants, lesser whistling ducks, night herons, pheasant-tailed jacana, pond herons, purple swamphens, red turtle doves, red wattle lapwings, sparrows, spotted doves, swallows, white breast waterhens, white vented mynas and zebra doves.

More than half of the total birds were flying in and out and/or spending more than thirty seconds at the sites. A quarter of the birds were feeding while thirty percent were perching and/or standing (landing on the ground). Less than ten percent of the birds were close to (<1 metre) and/or feeding together with other birds of either the same or different species and/or having direct contacts with other birds.

Table 6.7; Numbers of observed wild birds and their behaviours at the pond and rice paddy field sites (A2 and C2)

		Behaviour*												Close contact/ living close approximately less than 1 metre*			
Common name	n	FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CSB	%CSB	CDB	%CDB
Asian koel	3	2	67	-	-	1	33	-	-	-	-	1	33	-	-	-	-
Asian open bill stork	460	227	49	154	33	40	9	203	44	2	0	381	83	62	13	8	2
Asian palm-swift	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asian pied starling	186	110	59	85	46	76	41	58	31	6	3	154	83	17	9	24	13
Babbler	1	-	-	-	-	1	100	-	-	-	-	-	-	-	-	-	-
Baillon's crake	2	-	-	2	100	-	-	-	-	2	100	2	100	2	100	-	-
Bittern	3	1	33	1	33	2	67	-	-	-	-	-	-	-	-	-	-
Black bittern	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Black capped kingfisher	1	-	-	1	100	-	-	-	-	-	-	-	-	-	-	-	-
Black drongo	54	24	44	4	7	41	76	3	6	-	-	46	85	-	-	2	4
Black-shouldered kite	1	1	100	-	-	-	-	-	-	-	-	1	100	-	-	-	-
Black-winged stilt	17	6	35	11	65	-	-	11	65	-	-	11	65	-	-	-	-
Bronze-winged jacana	261	81	31	178	68	1	0	211	81	8	3	249	95	12	5	44	17
Brown shrike	20	8	40	-	-	19	95	1	5	-	-	18	90	-	-	-	-
Bulbul	40	30	75	1	3	27	68	3	8	-	-	30	75	2	5	3	8
Bushchat	3	2	67	-	-	3	100	-	-	-	-	2	67	-	-	-	-
Cattle egret	33	9	27	24	73	-	-	7	21	-	-	26	79	-	-	2	6

		Behaviour*												Close contact/ living close approximately less than 1 metre*			
Common name	n	FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CSB	%CSB	CDB	%CDB
Common moorhen	14	1	7	12	86	-	-	-	-	-	-	14	100	-	-	2	14
Common myna	92	55	60	46	50	40	43	42	46	2	2	68	74	11	12	24	26
Common stonechat	9	2	22	1	11	8	89	1	11	-	-	8	89	-	-	-	-
Common tailorbird	3	2	67	1	33	1	33	-	-	-	-	2	67	-	-	-	-
Cotton pygmy goose	75	29	39	15	20	2	3	56	75	-	-	68	91	10	13	8	11
Egret	131	71	54	40	31	16	12	44	34	-	-	70	53	6	5	2	2
Golden weaver	126	87	69	1	1	94	75	8	6	2	2	84	67	4	3	1	1
Greater coucal	6	4	67	1	17	-	-	3	50	-	-	5	83	-	-	-	-
Greater painted-snipe	5	-	-	3	60	-	-	3	60	-	-	5	100	-	-	2	40
Grey heron	2	2	100	-	-	-	-	1	50	-	-	6	300	-	-	-	-
Grey-headed lapwing	4	-	-	4	100	-	-	4	100	-	-	-	-	-	-	1	25
Gull	5	4	80	-	-	1	20	-	-	-	-	3	60	-	-	-	-
Indian rollers	3	3	100	-	-	2	67	-	-	-	-	-	-	-	-	-	-
Intermediate egret	11	4	36	7	64	-	-	4	36	-	-	7	64	1	9	4	36
Lesser cormorant	403	148	37	4	1	244	61	29	7	2	0	272	67	65	16	45	11
Lesser whistling duck	574	414	72	113	20	9	2	187	33	2	0	276	48	74	13	26	5
Little egret	68	25	37	31	46	8	12	36	53	-	-	46	68	-	-	6	9
Little grebe	32	1	3	13	41	-	-	25	78	-	-	32	100	-	-	-	-
Malkoha	2	1	50	-	-	1	50	-	-	-	-	1	50	-	-	-	-

		Behaviour*												Close contact/ living close approximately less than 1 metre*			
Common name	n	FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CSB	%CSB	CDB	%CDB
Night heron	46	39	85	-	-	8	17	1	2	-	-	9	20	-	-	5	11
Oriental magpie-robin	2	-	-	-	-	2	100	-	-	-	-	2	100	-	-	-	-
Oriental pratincole	4	2	50	-	-	-	-	4	100	-	-	4	100	-	-	-	-
Pheasant-tailed jacana	185	57	31	101	55	-	-	131	71	1	1	169	91	10	5	39	21
Pied fantail	3	2	67	2	67	1	33	2	67	-	-	3	100	-	-	-	-
Pigeon	1358	825	61	117	9	828	61	164	12	-	-	1014	75	55	4	14	1
Plain prinia	2	2	100	2	100	-	-	-	-	-	-	-	-	-	-	-	-
Plain-backed sparrow	27	16	59	6	22	20	74	6	22	2	7	21	78	2	7	-	-
Pond heron	527	207	39	234	44	68	13	328	62	2	0	392	74	13	2	33	6
Prinia	55	19	35	7	13	41	75	1	2	-	-	46	84	-	-	1	2
Purple heron	1	1	100	-	-	-	-	1	100	-	-	1	100	-	-	-	-
purple swamphen	11	-	-	7	64	-	-	11	100	-	-	11	100	3	27	4	36
Red - rumped swallow	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Red turtle dove	120	57	48	28	23	57	48	36	30	6	5	86	72	12	10	6	5
Red wattle lapwings	54	9	17	15	28	-	-	49	91	-	-	53	98	-	-	11	20
Scaly breast munia	4	4	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sparrow	282	149	53	105	37	125	44	92	33	22	8	230	82	32	11	18	6
Spotted dove	64	37	58	11	17	29	45	17	27	2	3	51	80	7	11	2	3
Swallow	250	234	94	14	6	28	11	-	-	2	1	143	57	11	4	3	1
Tailorbird	22	16	73	-	-	9	41	3	14	4	18	9	41	-	-	-	-

		Behaviour*												Close contact/ living close approximately less than 1 metre*			
Common name	n	FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CSB	%CSB	CDB	%CDB
Tern	5	5	100	-	-	-	-	-	-	-	-	5	100	-	-	-	-
Watercock	1	-	-	1	100	-	-	-	-	-	-	1	100	-	-	-	-
Weaver	26	17	65	-	-	21	81	1	4	2	8	23	88	2	8	-	-
White breast waterhen	45	5	11	38	84	2	4	30	67	-	-	45	100	9	20	2	4
White vented myna	238	150	63	62	26	56	24	62	26	11	5	131	55	35	15	24	10
White-throated kingfisher	4	-	-	-	-	4	100	-	-	-	-	4	100	-	-	-	-
Zebra dove	44	30	68	18	41	11	25	16	36	3	7	30	68	3	7	2	5
Total	2765	1072	18	1001	17	666	11	1276	21	45	1	2229	37	231	4	238	4

*a bird can perform more than one behaviour and/or close contact

FiFo = Fly in Fly out, F = Feeding, P = Perching, S = standing/landed on the grounds or water bodies, DC = direct contacting or close (<1 metre) to other wild and/or domestic birds, >30 = spending more than 30 seconds at the site, CSB = close and/or feeding together (<1 metre) with other same species wild birds, and CDB = close (<1 metre) and/or feeding together with other different species wild birds

Both terrestrial and water birds were seen in the backyard areas (B1 and D1). Most water birds including Asian open bill storks, a black bittern, egrets, intermediate egrets, lesser whistling ducks, little egrets, night herons, red wattle lapwings, and a yellow bittern that presented at the sites were observed flying in and out without stopping (Table 6.8). However, some pond herons were observed feeding (6%), standing (8%), and or perching (21%) in the backyards. Unlike waterbirds, terrestrial birds were likely to stop over at the sites. The most observed birds in the backyards were sparrows, pigeons, red turtle doves, white vented mynas, and bulbuls, respectively (Table 6.8). Forty-two percent of sparrows were observed feeding. Some sparrows were also seen close to and/or feeding together with the other sparrows (19%) and wild birds (3%; Table 6.8). However, most pigeons (75%) were observed flying in and out, while about 30% were perching and/or spending longer than 30 seconds at the sites. However, only 3% of all pigeons (n=739) were feeding while 3% and 2% were seen close to (<1 metre) and/or feeding together with other pigeons and other wild birds respectively.

Close contact between wild birds, domestic poultry, and pigs were observed. Wild birds close to (<1 metre) and/or feeding together with backyard chickens were sparrows (31%), common mynas, pied fantails, red turtle doves, pigeons, zebra doves, a white breast waterhen, a tailorbird, and an oriental magpie-robin while the birds observed close to and/or feeding together with pigs were sparrows (8%), pied fantails, common mynas, and white vented mynas (Table 6.8). However, only a couple of pigeons were observed having direct contact with poultry and pigs at these sites. Wild birds in close proximity to (<1 metre) and/or feeding together with other bird species included an Asian koel, an Asian pied starling, black drongos, brown shrikes, bulbuls, common mynas, a common

tailorbird, oriental magpies-robins, pied fantails, pigeons, a prinia, red turtle doves, sparrows, spotted doves, white vented mynas, and zebra doves.

Seventy-three percent of all wild birds seen in the backyards were flying in and out while 59% were spending more than 30 seconds and 50% were perching (Table 6.8). Twenty and 17 percent of all birds were observed feeding and perching respectively. Overall the percentage of wild birds observed close to (< 1 metre) and/or feeding together with backyard chickens, pigs, ducks, other birds (same species), and other birds (different species) were 13, 3, <1, 10, and 3, respectively.

Table 6.8; Numbers of observed wild birds and their behaviours in the backyard areas (site B1 and D1)

Common name	n	Behaviour*												Close contact/living close approximately less than 1 metre*									
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CD	%CD	CC	%CC	CP	%CP	CSB	%CSB	CDB	%CDB
Asian brown flycatcher	6	2	33	-	-	6	100	-	-	-	-	4	67	-	-	-	-	-	-	-	-	-	-
Asian koel	12	8	67	-	-	8	67	-	-	1	8	7	58	-	-	-	-	-	-	-	-	1	8
Asian open bill stork	164	164	100	-	-	-	-	-	-	13	8	23	14	-	-	-	-	-	-	-	-	-	-
Asian palm-swift	3	3	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asian pied starling	43	33	77	-	-	25	58	-	-	-	-	24	56	-	-	-	-	-	-	2	5	1	2
Bee eater	4	4	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Black bittern	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Black drongo	81	57	70	-	-	43	53	3	4	4	5	49	60	-	-	-	-	-	-	6	7	3	4
Black-shouldered kite	5	4	80	-	-	1	20	-	-	-	-	2	40	-	-	-	-	-	-	-	-	-	-
Brown shrike	20	4	20	-	-	20	100	-	-	-	-	19	95	-	-	-	-	-	-	-	-	2	10
Bulbul	214	154	72	30	14	112	52	6	3	4	2	120	56	-	-	-	-	-	-	22	10	5	2
Cattle egret	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chestnut - headed bee – eater	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Common myna	92	63	68	19	21	59	64	17	18	-	-	61	66	-	-	7	8	2	2	13	14	9	10

Common name	n	Behaviour*												Close contact/living close approximately less than 1 metre*									
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CD	%CD	CC	%CC	CP	%CP	CSB	%CSB	CDB	%CDB
Common tailorbird	13	9	69	5	38	11	85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	8
Dark - necked tailorbird	2	1	50	-	-	2	100	-	-	-	-	2	100	-	-	-	-	-	-	-	-	-	-
Dove	2	2	100	-	-	2	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Egret	88	87	99	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flycatcher	1	-	-	-	-	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Golden weaver	12	8	67	2	17	4	33	1	8	0	0	6	50	-	-	-	-	-	-	-	-	-	-
Greater coucal	9	5	56	2	22	5	56	1	11	0	0	6	67	-	-	-	-	-	-	-	-	-	-
Indian rollers	2	-	-	-	-	2	100	-	-	-	-	2	100	-	-	-	-	-	-	-	-	-	-
Intermediate egret	6	6	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Iora	3	3	100	-	-	3	100	-	-	-	-	3	100	-	-	-	-	-	-	-	-	-	-
Lesser cormorant	42	42	100	-	-	1	2	-	-	-	-	2	5	-	-	-	-	-	-	-	-	-	-
Lesser whistling duck	121	121	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Little egret	11	11	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Malkoha	4	2	50	-	-	2	50	-	-	-	-	2	50	-	-	-	-	-	-	-	-	-	-
Myna	4	3	75	-	-	4	100	4	100	-	-	4	100	-	-	-	-	-	-	-	-	-	-
Night heron	9	9	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	22	-	-
Olive-backed sunbird	3	-	-	-	-	3	100	-	-	-	-	3	100	-	-	-	-	-	-	-	-	-	-

Common name	n	Behaviour*												Close contact/living close approximately less than 1 metre*									
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CD	%CD	CC	%CC	CP	%CP	CSB	%CSB	CDB	%CDB
Oriental magpie-robin	63	34	54	7	11	32	51	-	-	2	3	36	57	-	-	1	2	-	-	2	3	3	5
Pied fantail	77	40	52	32	42	50	65	19	25	8	10	64	83	-	-	6	8	3	4	-	-	7	9
Pigeon	739	555	75	20	3	250	34	27	4	19	3	280	38	2	0	6	1	0	0	27	4	15	2
Pond heron	53	47	89	3	6	11	21	4	8	0	0	16	30	-	-	-	-	-	-	-	-	-	-
Prinia	12	6	50	-	-	9	75	-	-	-	-	12	100	-	-	-	-	-	-	-	-	1	8
Racket-tailed drongo	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Red turtle dove	300	127	42	7	2	224	75	10	3	2	1	218	73	-	-	6	2	-	-	33	11	18	6
Red wattle lapwings	2	2	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scaly breast munia	4	4	100	-	-	4	100	-	-	-	-	4	100	-	-	-	-	-	-	-	-	-	-
Scarlet-backed flowerpecker	1	1	100	-	-	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sparrow	1851	1367	74	772	42	1234	67	663	36	165	9	1498	81	-	-	573	31	148	8	355	19	47	3
Spotted dove	61	19	31	2	3	49	80	4	7	4	7	53	87	-	-	-	-	-	-	6	10	6	10
Sunbird	1	-	-	-	-	1	100	-	-	-	-	1	100	-	-	-	-	-	-	-	-	-	-
Swallow	176	176	100	-	-	-	-	-	-	-	-	63	36	-	-	-	-	-	-	-	-	-	-
Tailorbird	14	4	29	1	7	11	79	-	-	-	-	12	86	-	-	1	7	-	-	-	-	-	-
Warbler	3	-	-	1	33	2	67	-	-	-	-	2	67	-	-	-	-	-	-	-	-	-	-
Weaver	18	9	50	1	6	10	56	-	-	-	-	11	61	-	-	-	-	-	-	3	17	-	-
White breast waterhen	7	1	14	5	71	1	14	6	86	2	29	7	100	-	-	1	14	-	-	2	29	-	-

Common name	n	Behaviour*												Close contact/living close approximately less than 1 metre*									
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CD	%CD	CC	%CC	CP	%CP	CSB	%CSB	CDB	%CDB
White vented myna	277	232	84	22	8	97	35	8	3	9	3	103	37	-	-	1	0	2	1	12	4	6	2
Yellow - vented bulbul	30	16	53	5	17	21	70	1	3	4	13	24	80	-	-	-	-	-	-	7	23	-	-
Yellow bittern	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Zebra dove	65	18	28	25	38	27	42	28	43	2	3	48	74	-	-	3	5	-	-	2	3	3	5
Total	4735	3468	73	968	20	2348	50	802	17	239	5	2791	59	2	0	605	13	155	3	494	10	128	3

*a bird can perform more than one behaviour and/or close contact

FiFo = Fly in Fly out, F = Feeding, P = Perching, S = standing/landed on the ground or water bodies, DC = direct contact or close to (<1 metre) other wild and/or domestic birds, >30 = spending more than 30 seconds at the site, CD = close to and/or feeding together (<1 metre) with duck, CC = close to and/or feeding together (<1 metre) with chickens, CP = close to and/or feeding together (<1 metre) with pigs, CSB = close to and/or feeding together (<1 metre) with the same species of wild birds, and CDB = close to (<1 metre) and/or feeding together with different species of wild birds

Wild birds and their behaviours observed at the open system duck farms (site B2 and D2) are summarised in Table 6.9. The majority of birds found at the sites were terrestrial birds which were dominated by sparrows and pigeons. Water birds present at the sites included Asian open bill storks, black bitterns, cattle egrets, egrets, lesser cormorants, lesser whistling ducks, little egrets, little grebes, night herons, pond herons, red wattle lapwings, and white breast waterhens. However, only pond herons, egrets and herons and little grebes were seen inside the farms while other water birds tended to fly past and/or land in the agricultural land outside the farm areas. For example, all black bitterns, lesser whistling ducks, and night herons flew in and out without stopping or landing in the sites (Table 6.9). Water birds in close proximity to (<1 metre) and/or feeding together with ducks included an egret, a little egret, and pond herons. For water birds, there was only one bird performing direct contact recorded at the sites. As well, egrets and pond herons were observed close to (<1 metre) and/or feeding together with other birds (same or different species) .

Common terrestrial birds including Asian pied starlings, bulbuls, common mynas, a common tailorbird, an oriental magpie-robins, pied fantails, pigeons, red turtle doves, a swallow, sparrows, white vented mynas, and zebra doves observed close to (<1 metre) and/or feeding together with farmed ducks were also likely to be feeding, standing or landing (Table 6.9). Some terrestrial birds that were noted feeding in the farms without close contact to the ducks were black drongos, greater coucals, a prinia, scaly breast munias, and spotted doves. Most terrestrial birds spent longer than 30 seconds at the sites with a high proportion being involved in feeding activities. Direct contact between wild

birds and other birds was observed in Asian pied starlings, bulbuls, a common myna, golden weavers, oriental magpie-robins, pied fantails, pigeons, red turtle doves, sparrows, spotted doves, and white vented mynas. Wild birds observed close to (<1 metre) and/or feeding together with other bird species (not included the ducks) at the farms were Asian pied starlings, black drongos, common mynas, egrets, a lesser cormorant, oriental magpie-robins, pied fantails, pigeons, pond herons, red turtle doves, sparrows, spotted doves, a tailorbird, white vented mynas, and zebra doves.

The majority of all wild birds (81%) were spent more than 30 seconds at the open system duck farms (Table 6.9). Sixty-five percent, 59%, and 50% of the birds were observed flying in and out, feeding, and standing respectively. More than 30% of the birds observed at these sites were close to (<1 metre) and/or feeding together with farm ducks while 13% were close to and/ or feeding with geese.

When the observed wild bird behaviours and activities in all study sites were compared (Figure 6.9), feeding and standing on the ground were mostly observed at open system duck farms. Close contact between wild birds and domestic animals, including backyard chickens, ducks, geese, and pigs, were mainly observed in backyard/ household sites and open system duck farms. Close contacts between wild birds and other wild birds (both same and different species) were observed in all study sites infrequently.

Table 6.9; Numbers of observed wild birds and their behaviours at the open system duck farms (site B2 and D2)

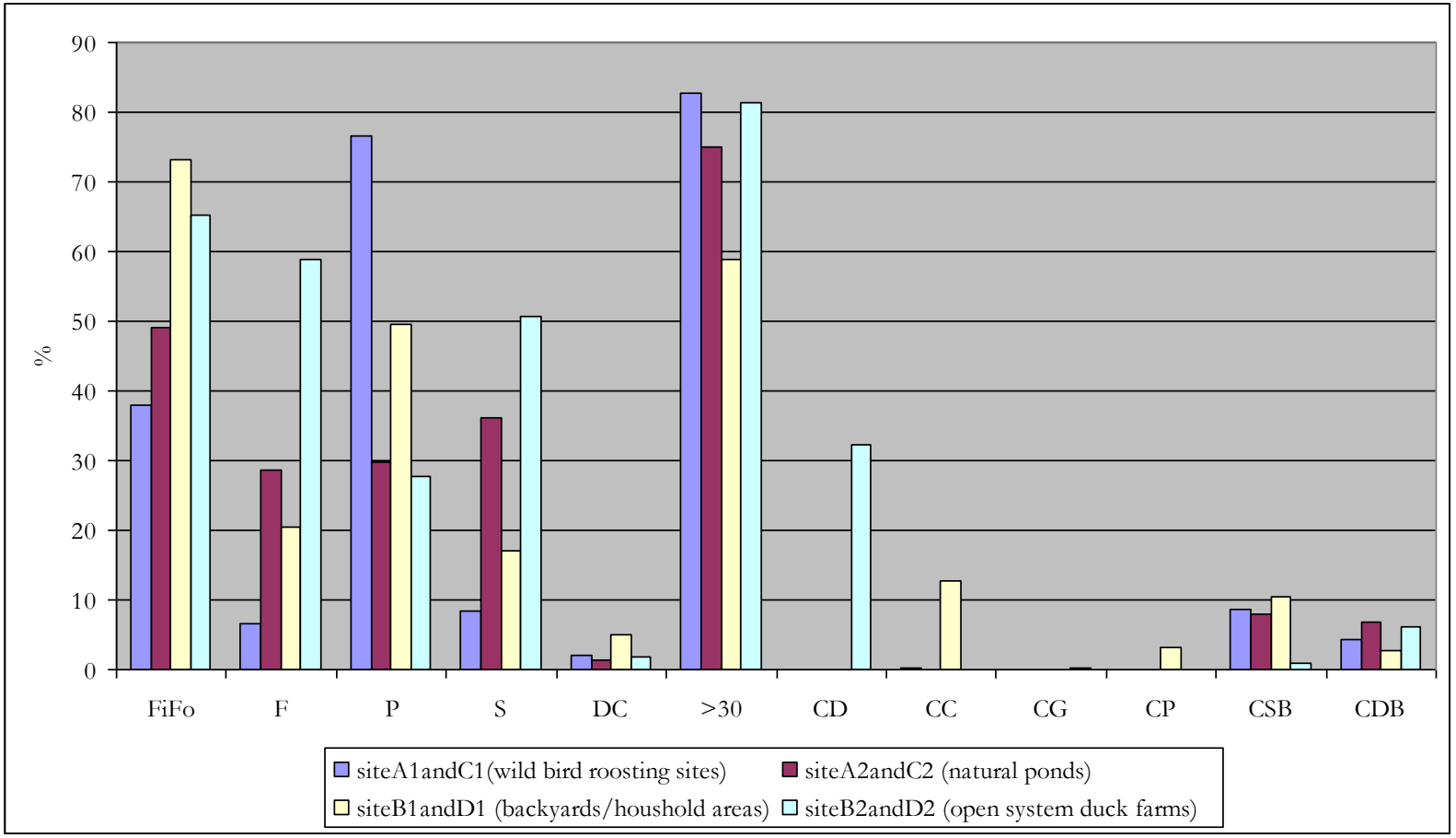
Common name	n	Behaviour*												Close contact/ living close approximately less than 1 metre*							
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CD	%CD	CG	%CG	CSB	%CSB	CDB	%CDB
Asian koel	2	1	50	-	-	1	50	-	-	-	-	1	50	-	-	-	-	-	-	-	-
Asian open bill stork	141	40	28	1	1	100	71	-	-	-	-	119	84	-	-	-	-	-	-	-	-
Asian pied starling	73	39	53	28	38	20	27	22	30	4	5	49	67	3	4	2	3	10	14	16	22
Black bittern	1	1	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Black drongo	26	16	62	2	8	18	69	-	-	-	-	18	69	-	-	-	-	-	-	2	8
Bulbul	57	36	63	3	5	36	63	6	11	2	4	41	72	4	7	-	-	7	12	-	-
Cattle egret	3	1	33	1	33	-	-	2	67	-	-	2	67	-	-	-	-	-	-	-	-
Common myna	197	80	41	131	66	48	24	105	53	1	1	174	88	58	29	4	2	31	16	51	26
Common tailorbird	1	-	-	1	100	-	-	1	100	-	-	1	100	1	100	-	-	-	-	-	-
Egret	40	31	78	8	20	1	3	10	25	-	-	12	30	1	3	-	-	2	5	2	5
Golden weaver	23	6	26	-	-	18	78	-	-	2	9	19	83	-	-	-	-	3	13	-	-
Greater coucal	6	2	33	3	50	2	33	3	50	-	-	5	83	-	-	-	-	2	33	-	-
Lesser cormorant	87	85	98	-	-	1	1	1	1	-	-	2	2	-	-	-	-	-	-	1	1
Lesser whistling duck	93	93	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Little egret	10	9	90	-	-	1	10	1	10	-	-	2	20	1	10	-	-	-	-	-	-
Little grebe	4	-	-	2	50	-	-	4	100	-	-	4	100	-	-	-	-	-	-	-	-
Night heron	4	4	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Common name	n	Behaviour*												Close contact/ living close approximately less than 1 metre*							
		FiFo	%FiFo	F	%F	P	%P	S	%S	DC	%DC	>30	>30%	CD	%CD	CG	%CG	CSB	%CSB	CDB	%CDB
Olive-backed sunbird	1	1	100	-	-	1	100	-	-	-	-	1	100	-	-	-	-	-	-	-	-
Oriental magpie-robin	17	5	29	5	29	7	41	4	24	2	12	13	76	1	6	-	-	1	6	4	24
Pied fantail	40	24	60	16	40	30	75	17	43	2	5	35	88	3	8	-	-	3	8	4	10
Pigeon	2021	1367	68	1467	73	373	18	1248	62	7	0	1710	85	1079	53	-	-	84	4	93	5
Plain-backed Sparrow	3	2	67			3	100	-	-	-	-	2	67	-	-	-	-	-	-	-	-
Pond heron	74	46	62	19	26	23	31	26	35	2	3	45	61	8	11			2	3	5	7
Prinia	13	5	38	1	8	8	62	-	-	-	-	8	62	-	-	-	-	-	-	-	-
Red turtle dove	246	99	40	74	30	142	58	76	31	14	6	209	85	58	24	-	-	49	20	18	7
Red wattle lapwings	5	4	80	1	20	2	40	1	20	-	-	5	100	-	-	-	-	-	-	-	-
Scaly breast munia	7	1	14	4	57	3	43	-	-	-	-	7	100	-	-	-	-	-	-	-	
Sparrow	2136	1531	72	1558	73	593	28	1346	63	64	3	1931	90	638	30	5	0	313	15	133	6
Spotted dove	45	9	20	8	18	28	62	12	27	2	4	39	87	-	-	-	-	2	4	5	11
Swallow	176	128	73	12	7	52	30	3	2	-	-	99	56	1	1	-	-	7	4	-	-
Tailorbird	5	4	80	-	-	5	100	-	-	-	-	3	60	-	-	-	-	-	-	1	20
Weaver	2	-	-	-	-	2	100	-	-	-	-	2	100	-	-	-	-	-	-	-	-
White breast waterhen	2	-	-	2	100	-	-	1	50	-	-	2	100	-	-	-	-	-	-	-	-
White vented myna	255	155	61	71	28	99	39	63	25	2	1	170	67	29	11	2	1	37	15	19	7
Zebra dove	86	20	23	50	58	26	30	44	51	-	-	76	88	25	29	-	-	7	8	8	9
Total	**5902**	**3845**	**65**	**3468**	**59**	**1643**	**28**	**2996**	**51**	**104**	**2**	**4806**	**81**	**1910**	**32**	**13**	**0**	**560**	**9**	**362**	**6**

*a bird can perform more than one behaviour and/or close contact

FiFo = Fly in Fly out, F = Feeding, P = Perching, S = standing/landed on the ground or water bodies, DC = direct contact or close to (<1 metre) other wild and/or domestic birds, >30 = spending more than 30 seconds at the site, CD = close to and/or feeding together (<1 metre) with ducks, CG = close to and/or feeding together (<1 metre) with geese, CSB = close to and/or feeding together (<1 metre) with the same species of wild bird, and CDB = close to (<1 metre) and/or feeding together with different species of wild birds

Figures 6.9; Comparison of behaviours and activities of wild birds observed at the sites



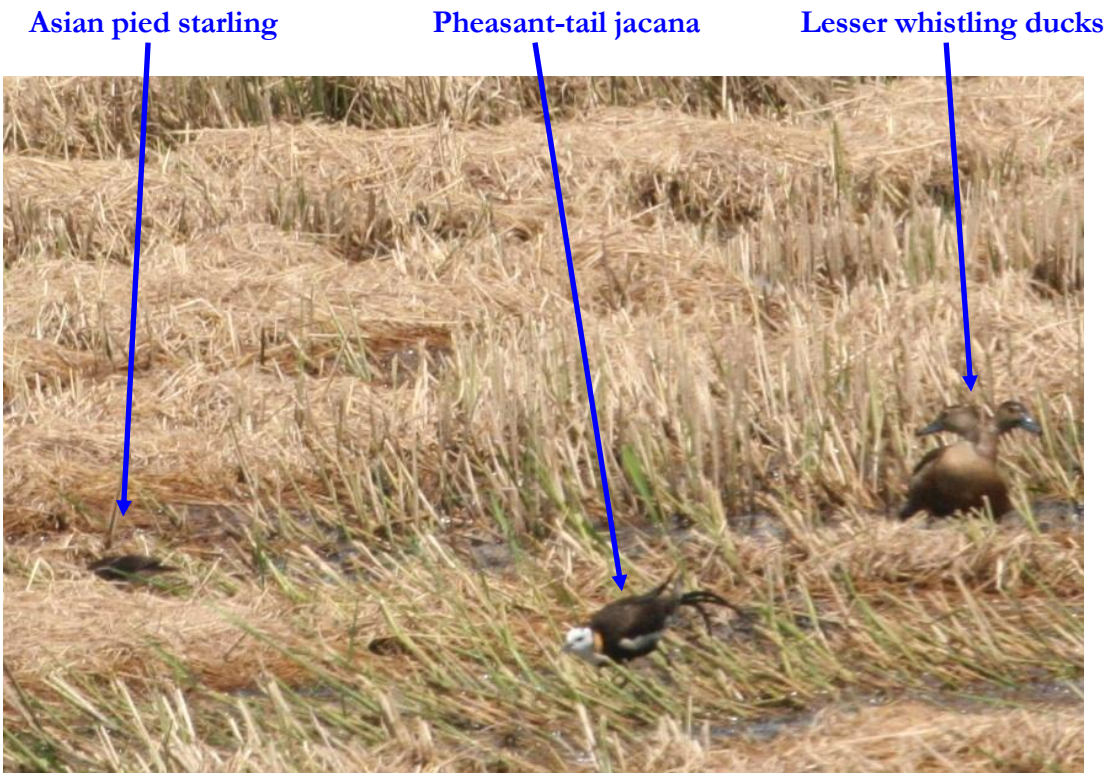
FiFo = Fly in Fly out, F = Feeding, P = Perching off the ground, S = standing/landed on the ground or water bodies, DC = direct contacting or observed close contact (<1 metre) to other wild and/or domestic birds, >30 = spending more than 30 seconds at the site, CD = observed close contact and/or feeding together (<1 metre) with ducks, CC = observed close contact and/or feeding together (<1 metre) with chicken, CG = observed close contact and/or feeding together (<1 metre) with geese, CP = observed close contact and/or feeding together (<1 metre) with pigs, CSB = observed close contact and/or feeding together (<1 metre) with the same species of wild birds, and CDB = observed close contact (<1 metre) and/or feeding together with different species of wild birds

6.4 Discussion

The wild birds that were observed in this study were common wild birds and included both terrestrial and water birds. The majority of the birds were non-migratory birds (Lekagul and Round, 1991). Some migratory birds, such as the Asian open bill stork, lesser whistling duck, and garganey, were also observed in the study areas; however, the number of migratory birds was small when compared to the number of local/non-migratory birds. Water birds were found clustering at water sources where some of the birds nested and fed. Unlike scavenging or feral birds, wild water birds are less frequently affected by human exposure and disturbances (Gill, 2007; Rees et al., 2005). Water birds were not often observed in households, backyards, and/or farms.

The feeding grounds of water birds, terrestrial birds, and domestic birds overlapped and these birds were observed to have a range of contacts depending upon the habitat. For example, water birds and terrestrial birds were seen sharing rice paddy fields (Figures 6.9 and 10). The method of rice growing operated in the study site was the direct seeding technique, where rice seeds are broadcasted by hand and the rice is generally left to grow in the prepared paddy fields (Azmi and Baki, 2002). After harvesting some rice seed remains in the paddy fields. In the paddy fields water birds feed on snails and/or small freshwater fish and crustaceans, while common terrestrial wild birds such as pigeons, white vented mynas, and sparrows feed on the leftover rice grain. Domestic poultry, such as grazing ducks and/or backyard chickens and ducks, would sometimes share the rice paddy fields with wild birds (Gilbert et al., 2006). In the case of influenza outbreaks, direct transmission, such as direct contacts between birds, and/or indirect transmission through contact with contaminated feed and water sources or fomites can occur.

Figures 6.9 and 6.10; Water birds and terrestrial birds sharing a rice paddy field



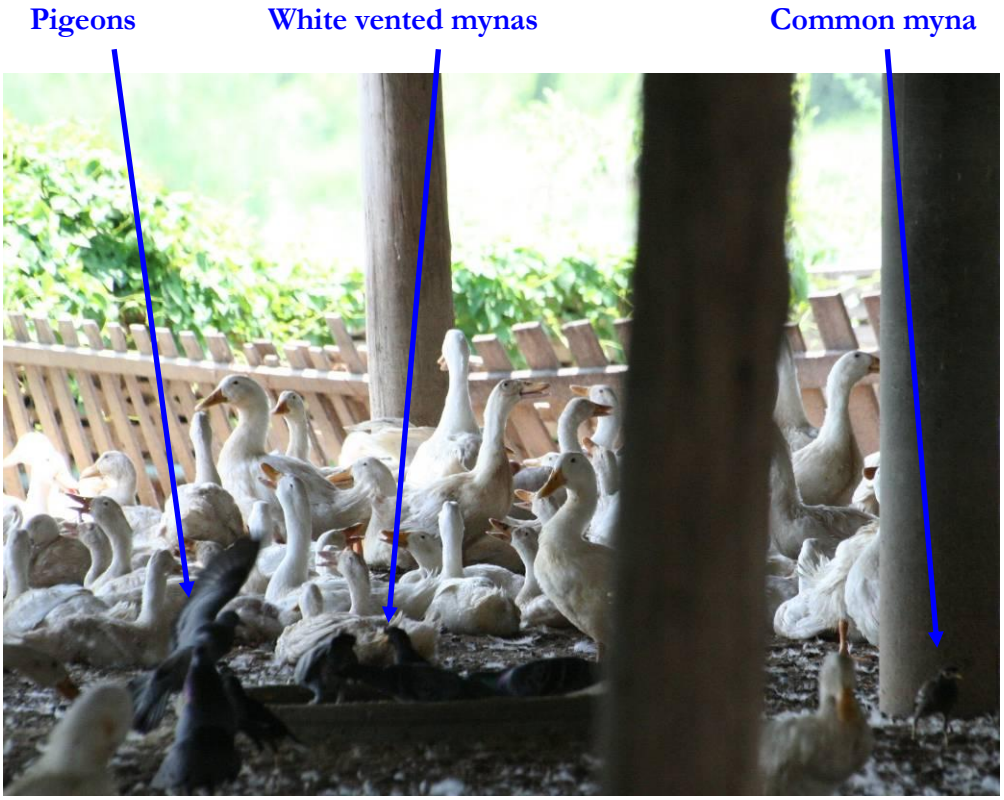
Even though the total number of observed birds at the wild bird roosting sites (A1 and C1) was higher than that at the other sites, the birds were mostly perching and staying off the ground with little contact with other wild birds or domestic poultry. The risk of spreading HPAI among bird species is likely to be low at these habitats as there were low interspecies interactions. However, roosts and nests are places where the birds raise their young which are known to be susceptible to avian influenza viruses (Munster et al., 2007; Stallknecht, 2003). Additionally, large numbers of birds may congregate at nesting sites during the breeding season and their behaviour may increase the risk of disease transmission. For example, Asian open bill storks generally nest in colonies where one tree may accommodate a large number of nests (Figure 6.11). This behaviour will increase the risk of disease transmission in the case of an avian influenza outbreak as manure from a high nest can easily drop down onto other lower nests. Thus, the behaviour of wild birds and the ecology of the habitat should be consolidated in epidemiological studies of avian influenza infection in wild birds.

Some water birds had a lower chance of having contact with or sharing habitats with domestic poultry. Bridge species, which share habitats with both wild and domestic birds, can play an important role as a vector for spill back and/or spill over of any infectious diseases (Dent et al., 2008; Pfeiffer, 2006). Bridge species identified in this study included Asian pied starling, bulbul, common myna, egrets, oriental magpie-robin, pied fantail, pigeons, pond herons, red turtle dove, sparrows, swallows, tailorbird, white breast waterhen, white vented myna, and zebra dove. As these birds were observed in a wide range of habitat types and they shared habitats with other wild and domestic birds, there is opportunity for interactions and/or close contacts between these species and domestic poultry, as was observed commonly in open system farms and backyards in this study. These birds scavenged poultry and/or pig food from enclosures. The degree of close contact with domestic poultry was high with sparrows, pigeons, starlings, mynas, and doves in particular having close contact (Figure 6.12). Moreover, close contacts between sparrows and pigs were observed in the

backyard sites. Sparrows also spent a longer time feeding at the open system farms and backyards than at other sites. Thus, poultry keeping areas, where poultry and/or animal food is easily accessed by wild and domestic birds and other animals, has an increased risk of spill over and/or spill back if avian influenza viruses are present in the area.

Figure 6.11; The roosting site of an Asian open bill stork colony with nests and young chicks visible

Figure 6.12; Close contact between wild birds and farm ducks



Even though nocturnal birds of prey, such as owls, have also been reported to be affected in a HPAI H5N1 outbreak (Artois et al., 2009), this study involved only collection of data on birds during the daylight hours due to difficulties in observing birds at night. Consequently the role of nocturnal species or the activities of birds at night could not be observed. Data were collected over a one year period, however behaviours of wild birds in an area can change from time to time (Robinson and Holmes, 1982) because of a range of factors such as disturbances by humans and/or domestic animals (Blumstein et al., 2005; Klein, 1993). In this study some species were rarely observed and as the percentages of behaviours were calculated at the individual species level some percentages need to be interpreted with caution. If the same individual birds flew in and out of the sites several times, the numbers of this species may be overestimated as it was impossible to identify individual birds. Consequently interpretation of the results should be done with caution.

The risk of disease transmissions between wild birds and domesticated poultry could be high in some habitats due to the degree of contact between the species. To evaluate the risk of disease transmission between species risk assessments using mathematical models are required and this forms the basis for the following chapter.

Chapter 7

RISK ASSESSMENT FOR THE TRANSMISSION OF H5N1 VIRUS FROM WILD BIRDS TO DOMESTIC POULTRY IN THAILAND

7.1 Introduction

To generate an effective emerging infectious disease control and prevention program, multi-disciplinary techniques are applied to create and provide useful outcomes and information for policy and/or decision makers. Risk analysis is one technique that is well known and recommended by many scientific organizations, including the FAO and the OIE. Risk assessment is used in broad areas including biological, environmental, and economic settings as a method to evaluate the likelihood of an event occurring and its consequences (Murray et al., 2004). Risk assessment is also a technique to identify possible factors that may be involved in disease transmission and its epidemiology (Pfeiffer, 2007).

Previous studies suggested that free flying birds (also called feral birds or common terrestrial birds) living near poultry could spread and cause infection with avian influenza in wild bird populations (Gauthier-Clerc et al., 2007; Gilchrista, 2005; Kwon et al., 2005). Boon et al. (2007) stated that terrestrial wild birds that wandered close to wild and domesticated bird populations were potentially important hosts of influenza viruses. Feare (2007) reported that most HPAI H5N1 outbreaks in wild birds occurred close to a source of infection. Domestic poultry and captive birds can be primarily infected depending on the degree of contact with feral species and secondarily through contamination or indirect contact (Alexander, 2007). High risk and/or bridge species (Chapter 1) were included in this study as these species

represent important transmission risks from wild birds and need to be considered in avian influenza control policy. Risk management and risk communication were not evaluated in this study.

According to the OIE guidelines on a risk analysis framework, a risk analysis combines four steps: hazard identification, risk assessment, risk management, and risk communication (Murray et al., 2004). The OIE framework was adopted for this study. Epidemiological and experimental data used for the qualitative and quantitative assessment were primarily generated from previous studies (Chapters 3, 4, 5, and 6), as well as expert opinions. Expert opinion and/or literature reviews from previous research studies were included in the assessment to fill some knowledge gaps not covered in Chapters 3 to 6. The gaps included the sensitivity of the national wild bird surveillance program, the likelihood of symptomatic infection and viral titres shed by infected birds. The studies were used for judging the probable transmission pathways and risk estimates. Qualitative assessment was undertaken, where possible, in order to clarify outcomes from the quantitative assessments. If outcomes of the qualitative assessment showed more than "negligible risk" of disease transmission from wild birds to poultry, it is important to assess the risk quantitatively. A quantitative risk assessment was conducted to estimate the likelihood of involvement of particular higher risk wild bird species (Chapter 4) in the transmission of H5N1 viruses between wild birds and poultry in central Thailand. It targeted areas including households/backyards and/or farms where species interactions are likely to occur. Before risks were estimated, all terminologies used in the assessments were clarified, as well as the scope of the assessments. As existing knowledge may not be complete leading to uncertainty (Pfeiffer, 2007), measurement of uncertainty was included in the assessment.

7.2 Materials and Methods

The assessment processes began with a literature review and information gathering in order to identify hazards and their ecology (Chapter 1). The possible transmission pathways of HPAI H5N1 virus were generated and experts were asked to comment on the pathways. Information and data were collected from a variety of organizations under the collaboration formed by the School of Veterinary and Biomedical Sciences, Murdoch University; Australian Biosecurity CRC; VSMU; Faculty of Veterinary Medicine, Kasetsart University; and the DNWPC. Hazard identification was the same for both the qualitative and quantitative assessments, except for the release and exposure assessments.

Hazard identification includes defining the hazard, risk questions, and biological pathways. In this study, the hazard of interest was HPAI H5N1 virus infection. Risk questions were identified and could be separated into two aspects; questions of release and questions on exposure pathways. A biological pathway of the disease transmission was defined based on risk questions, biological characterization of the HPAI H5N1 virus, and known routes of transmission. The pathway combined two consequence sub-pathways which represented release and exposure pathways. A description of the pathways was commented on by experts and then the pathways were revised. Release and exposure assessments were evaluated separately. To estimate possible risks of transmission of HPAI H5N1 virus from wild birds, data and outcomes of Chapters 3 (prevalence of the infection in wild birds), 4 (risk factors for the disease outbreak), 5 (molecular study of the virus isolated from wild birds), and 6 (ecology and behaviour of wild birds) were applied. For qualitative assessment, the likelihood of each pathway was justified step by step, as well as estimation of uncertainty based on a literature review of previous experiments and research and expert opinions, if available. Results from all steps were then combined to generate an outcome of the whole pathway.

According to the outcomes of Chapter 4, the presence of lesser whistling ducks (*Dendrocygna javanica*) in farms was identified as a risk factor for disease occurrence. With actual data being available the risk of transmission by this particular wild bird species was assessed quantitatively. Risk was assessed at the individual level of the duck then time was taken into account with the number of birds found per day and per year included in order to evaluate the risk per day and year respectively. Webster and others (1978) reported that in experimental studies Muscovy ducks (*Cairina moschate*) infected with LPAI virus produced 6.4 g of faeces per hour with a viral titre of 6.3×10^7 EID_{50} or a total of 10^{10} EID_{50} over a 24 hour period. Wild birds normally visit poultry-keeping areas and scavenge food. Most wild birds are not likely to stay in a poultry keeping area permanently, except for some species that nest in household areas such as under the roof (Chapter 6). As there were no data available on the amount of faeces produced by wild birds, this assessment assumed that birds that stayed in an area longer than 30 seconds defaecated at least once.

Software packages including Microsoft Excel (version 2003), SPSS 17.0 for Windows, and @risk 5.0 for Microsoft Excel (student version) were used to assess the risk of infection. The prevalence of infection and the exact 95% CI were calculated in Microsoft Excel. The probability of each event (or node) in the scenario trees were calculated using the appropriate distribution. The distributions were selected based on the type of data with a minimum of 1,000 iterations calculated. The distributions used in this chapter were based on the OIE's Handbook on Risk Analysis (Murray et al., 2004) and included the Beta distribution for calculation of probability of successes (prevalence), the Negative binomial distribution for the number of sample collected, the Binomial distribution for the number of successes (positive birds), and the Uniform distribution for events containing equally probabilities. The probabilities for each node were then combined to calculate an overall probability. The outcome of the assessment was to determine the probability of infected wild birds shedding

sufficient doses of virus (>$10^{3.5}$ EID_{50} - Dr Trevor Ellis 2009; personal communication) close to (<1 metre) domesticated poultry per year.

7.3 Results

7.3.1 Hazard identification

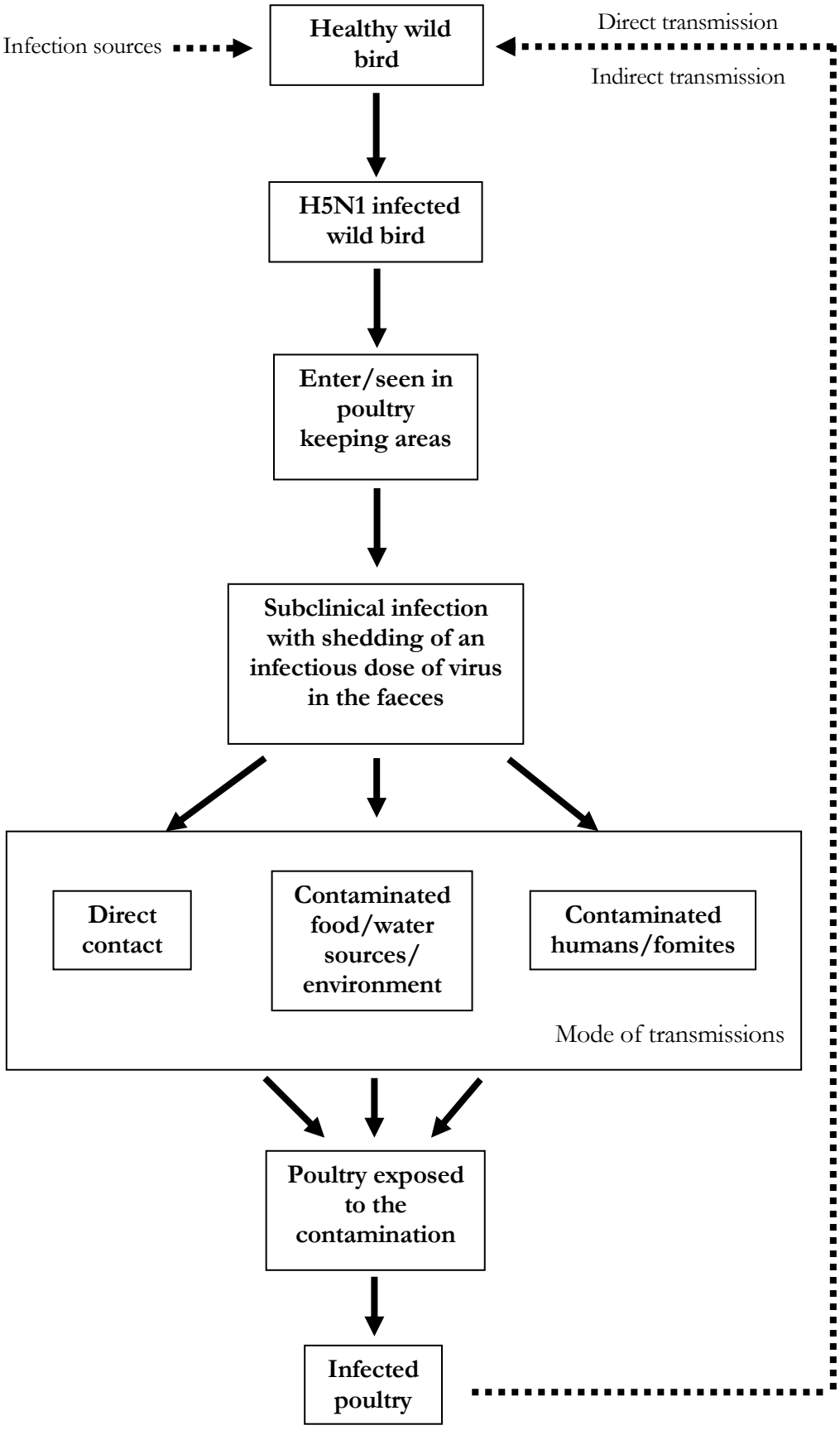
7.3.1.1 Hazard of interest

The hazard targeted was transmission of H5N1 HPAI virus infection between wild bird species and poultry.

7.3.1.2 Biological pathway for disease transmission

The risk or biological pathways for disease transmission of avian influenza described in this study only explained potential transmission pathways of the disease in general. Thus, the risk pathway was applied for each risk question individually. The pathway was generated based on low and medium bio-security small farms and/or household practises. The core physical pathway of the disease transmission is shown in Figure 7.1. The pathway combined release and exposure sections with each section containing an individual likelihood or probability. The release pathway included likelihood of a healthy wild bird being infected by H5N1 (prevalence) which was considered to be the first step of the pathway.

Figure 7.1; Physical pathway of HPAI H5N1 transmission



The next step considered the likelihood of infected wild birds having subclinical infection, or being in the incubatory stages of infection, enabling the birds to still be capable of flying and feeding. Infection of wild birds with H5N1 is dependent upon the species of bird (Isoda et al., 2006; Perkins and Swayne, 2001, 2002, 2003b). Individual species are likely to show differences between infection and shedding patterns (Brown et al., 2006; Brown et al., 2008). Risk assessments were evaluated separately for each individual wild bird species in this study. Virus needs to be shed in the bird's faeces or secretions to a degree that would effectively cause disease in poultry if they were exposed to the contaminated source. In order for infection to develop, poultry are required to be exposed to a certain amount of virus in the environment or on a fomite. Comparison of doses that were used in challenge studies with A/chicken/Hong Kong/97 (H5N1) and other A/Goose/ Guangdong/96-like H5N1 viruses (Liu et al., 2003; Swayne et al., 2001) suggested that a 50% lethal dose (LD_{50}) for chickens was of the order of $10^{3.5}$ EID_{50} and challenge studies with a Vietnamese H5N1 HPAI virus in ducks conducted in Hong Kong suggested a similar LD_{50} was likely for ducks (Dr Trevor Ellis 2009; personal communication). Thus, the second step in the release pathway was to determine the likelihood of a bird having a subclinical infection or incubating the disease and shedding more than $10^{3.5}$ EID_{50} of virus in their cloaca or trachea. As well, for the infection to pass from an infected wild bird to poultry, the bird should present or have been seen in areas where domestic poultry are kept. Thus, the probability of an asymptomatic infected wild bird entering a poultry keeping area was the third step of the release pathway.

The exposure pathway started with the likelihood of an infected wild bird having close contact with or feeding together with domestic poultry. Direct contacts between a wild bird and domestic poultry are uncommon in open spaces such as backyards and natural habitats (Chapter 6). However, infection through contact with a contaminated environment (Brown et al., 2007; Halvorson et al., 1985; Webster et al., 1992) or fomite (Hayden and Croisier, 2005) is possible. The rate of survival of the virus in the environment depends upon many

factors including the type of environment, temperature, moisture and time (Brown et al., 2007; Songserm et al., 2005; Webster et al., 1992).This assessment did not include the routes of transmission as there were insufficient data on indirect transmission. The exposure pathway in this study ended with an assessment of the likelihood of a wild bird getting close enough to poultry to effect exposure, either by being close to, having direct contact with, and/or feeding with domesticated poultry.

7.3.2 Risk questions

The outcomes of the risk assessments conducted in this study represent an estimation of the likelihood of an H5N1 infected wild bird dropping a sufficient dose of H5N1 close to domestic poultry (< 1 metre) in a poultry keeping area. The questions for both qualitative and quantitative risk assessments were divided into two separate sections for both release and exposure assessments.

7.3.2.1 Release assessment

- What is the probability of a wild bird being infected with HPAI H5N1?
- What is the probability of an infected wild bird that shows no clinical signs but sheds sufficient virus to cause infection, coming into close contact with or feeding together with domestic poultry in a backyard?
- What is the probability of an infected wild bird that shows no clinical signs but sheds sufficient virus to cause infection, coming into close contact with or feeding together with domestic poultry in an open-system poultry farm?
- What is the probability of the introduction of a H5N1 infected wild bird into a backyard/household?

- What is the probability of the introduction of a H5N1 infected wild bird into an open-system poultry farm?

7.3.2.2 Exposure assessments

- What is the probability of an infected wild bird having close contact with or feeding together with domestic poultry in a backyard/household?
- What is the probability of an infected wild bird having close contact with or feeding together with domestic poultry in an open-system poultry farm?

7.3.3 Release and Exposure assessments

In this section, the core pathway was divided into two pathways (Figure 7.2) which were specific to release and exposure risk questions. Pathways were simplified and did not include modes of transmission for these assessments. To assess risk qualitatively, scores and a list of categories were used to represent degrees of likelihoods (or probabilities of events) and degrees of uncertainties. Interpretation of scores and terminologies used in this section are described in Tables 7.1 and 7.2. Each step of the pathway was considered individually in which all scores were averaged and interpreted at the end of the qualitative assessment. A risk ranking matrix was used to combine risks and their impacts to finalize severity of the risk (Table 7.3). Steps were assessed qualitatively and quantitatively one by one in parallel. For the quantitative assessment, the probability (p; Figure 7.2) of each step in the pathway was estimated from quantitative data from studies undertaken in Chapters 3-6 and from expert opinion (Section 7.3.4.5). The distributions used to calculate each probability are shown in Table 7.4.

Figure 7.2; Release and exposure pathways for risk assessments (*p*=probability)

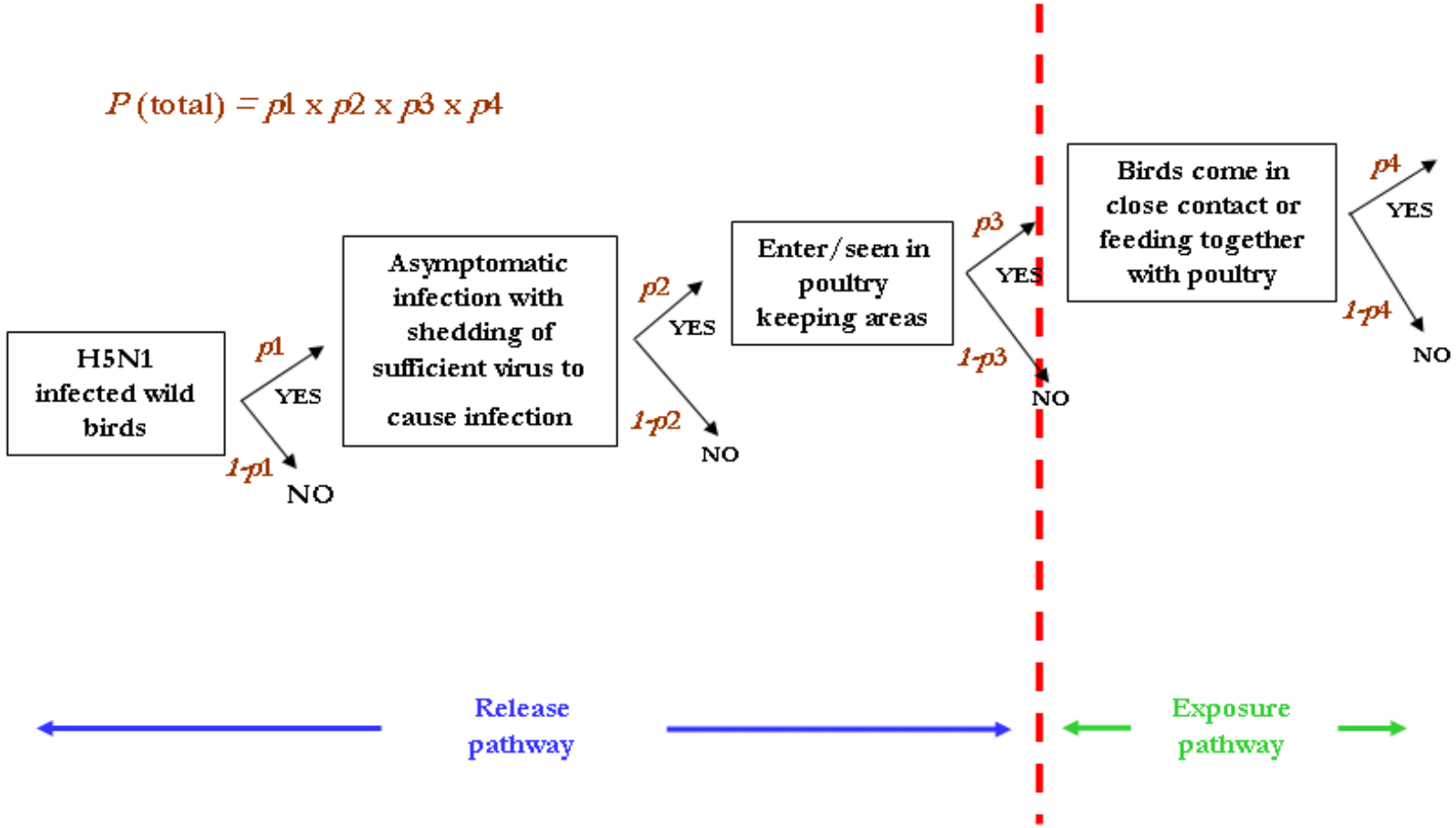


Table 7.1; Definition of scores used in the qualitative assessment

Modified from Pfeiffer et al. (2006)

Probability scores	Definition	Percentage
Negligible	Very rare event which can be excluded	<1%
Very low	Rare event but cannot be excluded	1-10%
Low	Rare event but does occur sometimes	11-30%
Moderate	Regularly occurring event	31-70%
High	Event that occurs quite often	71-90%
Very high	Event that usually occurs	>90%

Table 7.2; Definition of categories for uncertainty for qualitative assessment

(Pfeiffer et al., 2006)

Degree of uncertainty	Definition
Low	Complete data and/or strong evidence to support the events. Various references report similar outcomes and conclusions.
Moderate	Incomplete data but previous studies have been done. There are a small number of references to support the events.
High	No published reference or available data but observations, unpublished works, and/or personal communications can be used with caution.

Table 7.3; Risk ranking matrix [modified from (Vose, 2008)]

Impact \ Probability	Very low	Low	Moderate	High	Very High
Very High	6	7	8	9	10
High	5	6	7	8	9
Moderate	4	5	6	7	8
Low	3	4	5	6	7
Very low	2	3	4	5	6

8-10	High severity
5-7	Medium severity
1-4	Low severity

Table 7.4; Distributions used in @risk® in the quantitative risk assessment

Section	Probability*	Data source	Distributions
7.4.2.5	$p1$	Experts' opinion and results of the surveillance program (Chapter 3)	Uniform, Negative Binomial, and Beta
7.4.2.6	$p2$	Literature review and/or unpublished work	Pert
7.4.2.7	$p3$	Experts' answers (Questionnaire; Chapter 4)	Beta
7.4.2.8	$p4$	Observational study (Chapter 6)	Beta

* Probabilities from the risk pathways in Figure 7.2

7.3.4 Qualitative risk assessment

7.3.4.1 Probability of wild birds being infected with H5N1 ($p1$)

Figure 7.3 details pathways for the interactions between avian species in the study sites. From this diagram it is evident that bridge species are important in terms of disease transmission as their behaviour involves multiple species interactions. Wild migratory birds were included in the transmission pathway as possible carriers of the virus in this study.

The prevalence for each high risk family varied and the overall probability of the detection of disease in these families was classified as "Very low" (Table 7.5). The sensitivity of the survey should also be taken into account and results should incorporate the uncertainty of measurement. The survey was conducted in areas where poultry outbreaks had occurred and/or target wild migratory bird species were present. There were possibilities that the H5N1 virus prevalence, in some areas where the survey was applied, may be over-estimated and, in other areas where the surveillance was not conducted H5N1 infections may have been missed or the prevalence under-estimated. The level of uncertainty for this data would be considered "High".

Figure 7.3; Potential pathways for spread of H5N1 between bridge species and other avian species from one area (A) to another area (B)

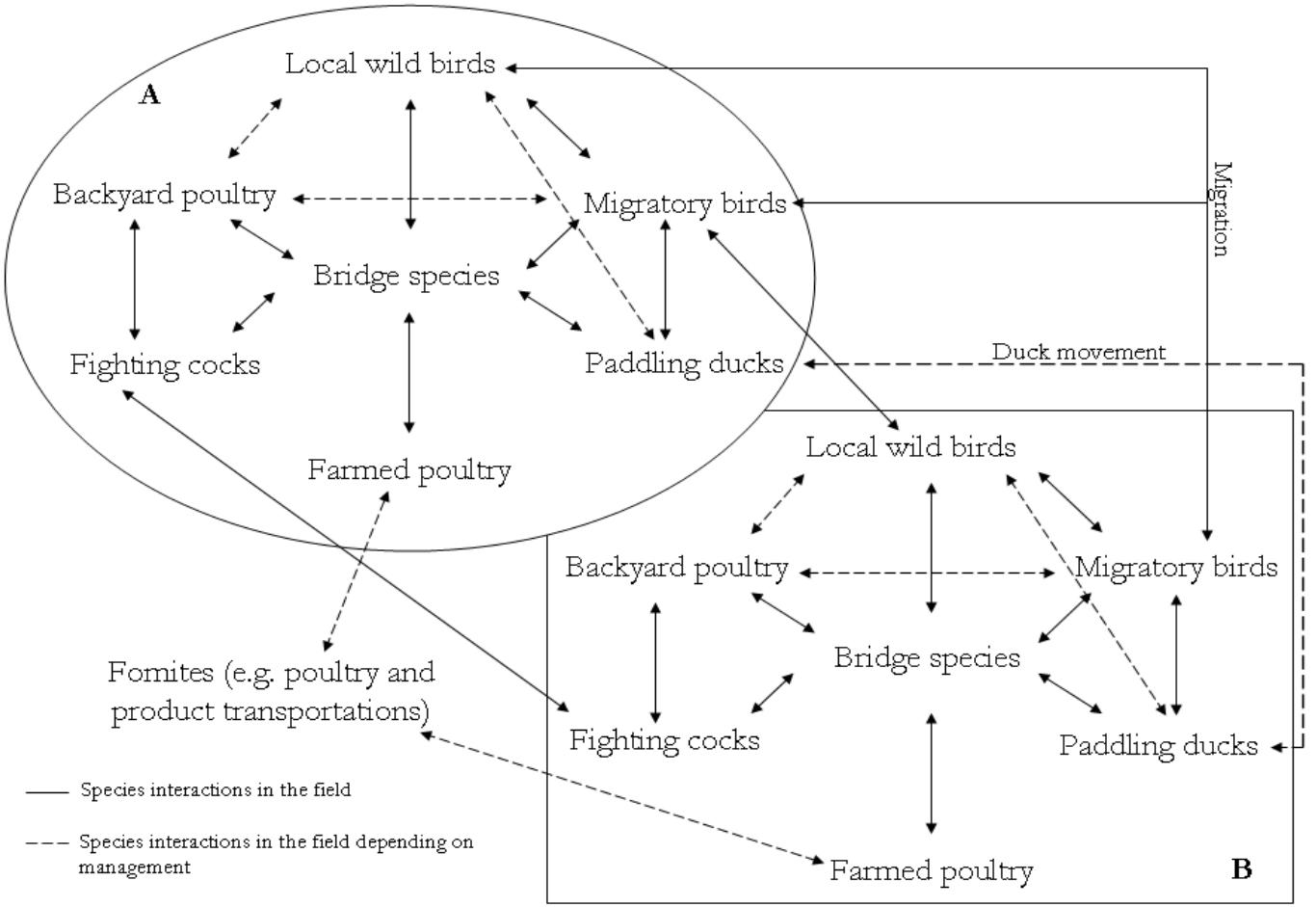


Table 7.5: Probability of infection with H5N1 in wild birds belonging to a range of high risk families (Data summarized from Table 3.1)

Family	**Positive samples**	**Total samples**	**Prevalence (%)**	**95% CI**	***p*1***
Anatidae	1	85	1.2	0.0, 3.5	Very low
Ardeidae	3	744	0.4	0.0, 0.9	Negligible
Charadriidae	1	83	1.2	0.0, 3.6	Very low
Ciconiidae	17	1,036	1.6	0.9, 2.4	Very low
Columbidae	20	1,594	1.3	0.7, 1.8	Very low
Cuculidae	1	8	12.5	0.0, 35.4	Low
Dicruridae	1	11	9.1	0.0, 26.1	Very low
Emberizidae	6	813	0.7	0.1, 1.3	Negligible
Estrildidae	1	89	1.1	0.0, 3.3	Very low
Sturnidae	7	568	1.2	0.3, 2.1	Very low
Overall	**58**	**5,031**	**1.2**	**0.9, 1.4**	**Very low**

*The *p*1 estimation using the criteria listed in Table 7.1

7.3.4.2 Probability of subclinical infection with sufficient virus shedding to cause infection (*p2*)

The probability of subclinical infection with viral shedding was estimated using values from previous research and data from the national surveillance study (Chapter 3). In 1978, Webster et al. (Webster et al., 1978) reported that ducks were asymptomatically infected with AIV and could shed virus in their faeces. Some studies have reported that terrestrial birds, such as feral pigeons (*Columba livia*), peregrine falcons (*Falco peregrinus*) (Li et al., 2004), jungle crows (*Corvus macrorhynchos*) (Kanai, 2004), magpies (*Pica pica sericea*) (Kwon et al., 2005), large-billed crows (*Corvus macrorhynchos*) (Tanimura et al., 2006), and tree sparrows (*Passer montanus*) (Kou et al., 2005; Li et al., 2004), have been infected with H5N1 viruses with varying levels of virus shedding from these birds. Kou and others (2005) also reported natural subclinical infection in sparrows. These findings are consistent with those from the national surveillance program for wild birds in Thailand where subclinical infection was also reported (Chapter 3).

Results of previous experiments on shedding of H5N1 by wild birds are displayed in Table 7.5. An experiment involving inoculation of guinea fowls, pheasants and partridges with A/chicken/Hong Kong/220/97 revealed a 100% mortality in guinea fowls and pheasants compared with 75% in partridges (Perkins and Swayne, 2001). A similar experiment with the A/chicken/Hong Kong/220/97 virus was conducted in emus and pigeons which revealed that pigeons, unlike emus, did not develop clinical signs or were virus positive (Perkins and Swayne, 2002). In 2003, inoculation of the same strain into zebra finches (*Taeniopygia guttata*), house finches (*Carpodacus mexicanus*), budgerigars (*Melopsittacus undulatus*), house sparrows (*Passer domesticus*), and European starlings (*Sternus vulgaris*) resulted in morbidities of 8%, 64%, 60%, 43% and 0%, respectively (Perkins and Swayne, 2003b).

Sparrows and starlings were not killed by the infection (Perkins and Swayne, 2003a), however, there was no report on viral shedding in that experiment.

An experiment involving inoculation of H5N1 [A/whooper Swan/ Mongolia/244/05 (H5N1) and A/Duck Meat/Anyang/01 (H5N1)] into ducks [mallard (*Anas platyrhynchos*), northern pintail (*Anas acuta*), blue-winged teal (*Anas crecca*), redhead (*Aythya americana*), and wood duck (*Aix sponsa*)] and gulls [laughing gulls (*Larus atricilla*)] showed that the viruses were more likely to be shed through oronasal cavities than via the cloaca, 1 to 10 days post inoculation (Brown et al., 2006). Virus was shed by all ducks and gulls; however higher viral titres were detected in species that developed clinical signs (Brown et al 2006). Experiments with swans [whooper swan (*C. cygnus*), black swan (*C. atratus*), trumpeter swan (*C. buccinator*), and mute swan (*Cygnus olor*)] and geese [bar-headed geese (*Anser indicus*) and cackling geese (*B. hutchinsii*)] inoculated with A/whooper swan/Mongolia/244/2005, demonstrated the onset of the disease 1 to 7 days after challenge (Brown et al., 2008). Clinical signs were detected within 0-9 days with 100% mortality in swans and 40-75% in geese. Virus shedding was also detected one day post-inoculation of every inoculated and in-contact bird (except for one bar-headed goose) with the average duration of shedding being 2-6 days from the oropharynx and 2-4 days from the cloaca (Brown et al., 2008).

An experiment conducted by Boon and others (2007) revealed that wild house sparrows (*Passer domesticus*) inoculated with H5N1 (A/duck/Thailand/144/2005, A/quail/ Thailand/551/2005, A/common magpie/Hong Kong/ 645/2006, and A/Japanese white-eye/Hong Kong/1038/2006) had a mortality of 66-100% within 4.2-6.3 days. None of the challenged European starlings (*Sturnus vulgaris*) and white Carneux pigeons (*Colomba* spp.) died. Virus was shed by the sparrows and starlings through the oropharynx and cloaca two days after challenge and via the oropharynx and cloaca in pigeons on days 3 and 5, respectively (Boon et al., 2007). Different patterns of shedding were observed for different

viral strains (Figure 7.4). Only starlings showed transmission of the A/common magpie/Hong Kong/645/2006 (H5N1) virus from infected to contact birds of the same species (Boon et al., 2007).

As indicated in Table 7.5, passerines and columbiforms are less susceptible to infection with H5N1, and show mild or no clinical signs and shed lower viral titres in their secretions than do waterfowl. Even though infected wild birds that displayed clinical signs shed higher viral titres, the observational studies suggested it was less likely that sick birds of these species moved into poultry keeping areas to shed viruses. The estimated probabilities of subclinical infection with high titre viral shedding ($>10^{3.5}$ EID_{50}) for wild bird species in the high risk group are summarised in Table 7.6. As data on shedding patterns are available for only some wild bird species, the level of uncertainty for this data was categorised as "High".

Table 7.5; The relative pathogenic effects of inoculating avian influenza virus into different wild birds

H5N1 strain (Inoculation dose EID_{50})	**Species/ total number of birds inoculated**	**Clinical signs (DPI; Day Post Inoculation)**	**Viral shedding (log10 EID_{50}/ml)**		**References**
			Oropharyn	**Cloacae**	
A/chicken/Hong Kong/ 220/97 ($10^{5.8}$ - $10^{6.2}$)	Pearl guineafowl (*Numida meleagris*)/ 11	Depression to death (6-8 hours)	N/A	N/A	Perkins and Swayne 2001 Note: Mortalities of guineafowls and pheasants were 100% and 75% in chukars.
	Ring-necked pheasant (*Phasianus colchicus*)/ 15	Depression, mucoid diarrhoea, and neurological signs (3)	N/A	N/A	
	Chukar partridges (*Alectoris chukar*)/ 11	Depression, mucoid diarrhoea and, neurological signs (4)	N/A	N/A	
A/chicken/Hong Kong/220/97 (10^{6})	Emus (*Dramaius novaehollandiae*)/ 2	Depression to neurological dysfunction including torticollis, hyperexcitability, and incoordination (8)	0.9 - 4.9	1.2 - 1.5	Perkins and Swayne 2002 Note: The virus was isolated from Emus and pigeons at 2, 4, 5, 7, 10, and 14 DPI in range (min-max).
	Pigeons (*Columba livia*)/ 10	No clinical sign	Not detected	Not detected	
A/chicken/Hong Kong/220/97 (10^{6})	Zebra finches (*Taeniopygia guttata*)/ 9	Depression and neurological sign	N/A	N/A	Perkins and Swayne 2003
	House finches (*Carpodacus mexicanus*)/ 11	depression, ruffled feathers, neurologic signs, and tremors	N/A	N/A	
	Budgerigars (*Melopsittacus undulatus*)/ 10	depression and neurologic signs	N/A	N/A	

H5N1 strain (Inoculation dose EID_{50})	Species/ total number of birds inoculated	Clinical signs (DPI; Day Post Inoculation)	Viral shedding (log10 EID_{50}/ml)		References
			Oropharyn	Cloacae	
	Sparrows (*Passer domesticus*) / 7	depressed, anorexic, and ruffled feathers	N/A	N/A	
	European starlings (*Sternus vulgaris*) / 4	No clinical signs	N/A	N/A	
A/whooper swan/Mongolia/244 /05 (10^6)	Blue-winged teal (*Anas crecca*)/ 3	No clinical signs	3.8	1.0	Brown et al 2006 Note: quantity of the virus calculated from average maximum titres. The virus was first detected 1 DPI. However, titres of the virus in cloacal swabs were low.
	Redhead (*Aythya americana*)/3	No clinical signs	2.8	1.2	
	Wood duck (*Aix sponsa*)/3	Cloudy eyes, ruffled feathers, rhythmic dilation and constriction of the pupils, severe weakness, incoordination, tremors, and seizures(N/A)	4.6	3.8	
	Northern pintail (*Anas acuta*)/ 3	No clinical signs	1.5	1.0	
	Laughing gulls (*Larus atricilla*)/ 3	Cloudy eyes, ruffled feathers, weakness, and incoordination (N/A)	4.2	2.6	
A/Duck Meat/Anyang/01 (10^6)	Blue-winged teal (*Anas crecca*)/ 3	No clinical signs	2.0	-	Brown et al 2006 Note: quantity of the virus calculated from the average maximum titres. The virus was first detected 1 DPI. However, titres of the virus in cloacal swabs were low.
	Redhead (*Aythya americana*)/ 3	No clinical signs	4.0	-	
	Wood duck (*Aix sponsa*)/ 3	Cloudy eyes, ruffled feathers, rhythmic dilation and constriction of the pupils, severe weakness,	5.0	2.8	

H5N1 strain (Inoculation dose EID_{50})	Species/ total number of birds inoculated	Clinical signs (DPI; Day Post Inoculation)	Viral shedding (log10 EID_{50}/ml)		References
			Oropharyn	Cloacae	
		incoordination, tremors, and seizures(N/A)			
	Mallard (*Anas platyrhynchos*)/3	No clinical signs	5.0	1.0	
A/Duck Meat/Anyang/01 (10^6)	Northern pintail (*Anas acuta*)/3	No clinical signs	2.1	-	
	Laughing gulls (*Larus atricilla*)/3	Cloudy eyes, ruffled feathers, weakness (N/A)	1.1	2.0	
A/whooper swan/Mongolia/244 /2005 (10^6)	Black swan (*Cygnus atratus*)/5	Severe listlessness and neurological dysfunction consisting of seizures, tremors, and marked incoordination (1-2)	6.5	4.9	Brown, Stallknecht, and Swayne 2008 Note: quantity of the virus calculated from the average maximum titres. The virus was first detected 1 DPI
	Trumpeter swan (*Cygnus buccinator*)/ 5	Listlessness and neurological signs(2)	6.1	3.2	
	Whooper swan *(Cygnus cygnus*)/ 4	Listlessness and neurological signs (2-4)	6.3	4.3	
	Mute swan (*Cygnus olor*)/ 5	listlessness and neurological signs (5-7)	5.6	4.5	
	Cackling goose (*Branta hutchinsii*)/ 4	Listlessness and neurological signs (3-7)	5.3	3.0	
	Bar-headed goose (*Anser indicus*)/ 5	depressed with transiently cloudy eyes (3-7)	5.1	2.6	

H5N1 strain (Inoculation dose EID_{50})	Species/ total number of birds inoculated	Clinical signs (DPI; Day Post Inoculation)	Viral shedding (log10 EID_{50}/ml)		References
			Oropharyn	Cloacae	
A/duck/Thailand/144/2005 (10^6)	Wild house sparrows (*Passer domesticus*)/3	N/A	2.4 - 4.7	1.7 – 4.1	Boon et al 2007 Note: quantity of the virus detected from swabs collected in 2, 4, and 6 DPI for sparrows and starlings and 3, 5, and 7 DPI for pigeons. No starlings and pigeons were died as a result of the infection in the experiment.
	European starlings (*Sturnus vulgaris*)/1	N/A	2.0 - 3.8	<1-0.8	
	White Carneux pigeons (*Colomba* spp.)/3	N/A	<1	<1	
A/quail/Thailand/551/2005 (10^6)	Wild house sparrows (*Passer domesticus*)/3	N/A	1.0- 3.1	<1 – 1.3	Boon et al 2007 Note: quantity of the virus detected from swabs collected in 2, 4, and 6 DPI for sparrows and starlings and 3, 5, and 7 DPI for pigeons. No starlings or pigeons died from the infection.
	White Carneux pigeons (*Colomba* spp.)/3	N/A	<1 – 0.8	<1 – 0.5	
A/common magpie/Hong Kong/645/2006 (10^6)	Wild house sparrows (*Passer domesticus*)/3	N/A	1.6 – 2.6	0.8 - 2.1	Boon et al 2007 Note: quantity of the virus detected from swabs collected in 2, 4, and 6 DPI for sparrows and starlings and 3, 5, and 7 DPI for pigeon. No starlings or pigeons died from the infection.
	European starlings (*Sturnus vulgaris*)/3	N/A	1.7 – 3.6	0.8 - 1.5	
	White Carneux pigeons (*Colomba* spp.)/3	N/A	<1 – 1.9	<1	
A/Japanese white-eye/Hong Kong/1038/2006 (10^6)	Wild house sparrows (*Passer domesticus*)/3	N/A	2.1 – 2.7	<1 – 3.3	Boon et al 2007 Note: quantity of the virus detected from swabs collected in 2, 4, and 6 DPI for sparrows and starlings and 3, 5, and 7 DPI for pigeons. No starlings or pigeons died from the infection.
	European starlings (*Sturnus vulgaris*)/2	N/A	1.8 – 2.5	<1 – 1	

H5N1 strain (Inoculation dose EID_{50})	Species/ total number of birds inoculated	Clinical signs (DPI; Day Post Inoculation)	Viral shedding (log10 EID_{50}/ml)		References
			Oropharyn	Cloacae	
	White Carneux pigeons (*Colomba* spp.)/3	N/A	<1 – 0.5	<1	

Table 7.6; Assessment of the probability of subclinical infection with shedding of sufficient virus to cause infection (*p*2) [*If B=N/A, B=1]

Family	Data sources	Infected birds show a healthy appearance			Shed infectious dose			Probability of AxB*	*p*2	Uncertainty
		Total number of birds	Positive samples from birds with a healthy appearance	Percent A	Total number of birds	Number of birds shedding > $10^{3.5}TCID_{50}$	Percent B			
Anatidae	Literature	55	21	38.2	55	43	78.2	0.3	Low	Moderate
Ardeidae	The national survey	3	2	66.7	N/A	N/A	N/A	0.7	Moderate	Very high
Charadriidae	The national survey	1	1	100.0	N/A	N/A	N/A	1.0	Very high	Very high
Ciconiidae	The national survey	17	12	70.6	N/A	N/A	N/A	0.7	Moderate	Very high
Columbidae	The national survey/ Literature	20	8	40.0	12	0	0.0	0.0	Negligible	Moderate
Cuculidae	The national survey	1	0	0.0	N/A	N/A	N/A	0.0	Negligible	Very high
Dicruridae	The national survey	1	1	100.0	N/A	N/A	N/A	1.0	Very high	Very high
Emberizidae	The national survey / Literature	6	6	100.0	12	3	25.0	0.3	Low	Moderate
Estrildidae	Literature	9	0	0.0	N/A	N/A	N/A	0.0	Negligible	Very high

Family	Data sources	Infected birds show a healthy appearance			Shed infectious dose			Probability of AxB*	*p*2	Uncertainty
		Total number of birds	Positive samples from birds with a healthy appearance	Percent A	Total number of birds	Number of birds shedding > $10^{3.5}$TCID$_{50}$	Percent B			
Sturnidae	Literature	4	4	100.0	6	4	66.7	0.7	Moderate	Moderate
	Overall	117	55	64.7	85	50	58.8	0.4	Low	High

7.3.4.3 Probability of the presence of wild birds in poultry keeping areas (*p*3)

The results of the questionnaire study reported in Chapter 4 of wild birds identified by villagers were used for this probability assessment. For *p*3 qualitative assessment, probabilities generally represent the likelihood of a particular wild bird species presenting in poultry keeping areas including backyards, households and farms. Probabilities of families were determined individually and are displayed in Table 7.7. These probabilities were averaged to obtain an overall probability for the high risk and/or bridge species entering the study site and having some contact with poultry. Qualitative data were based on the answers provided by villagers, which may contain some bias due to errors in recall and/or poor species identification. Photographs of wild birds were provided during the interview to reduce the biases. Uncertainly of this probability assessment (*p*3) was considered to be "Low".

7.3.4.4 Probability of wild birds being in close proximity to and/or feeding together with domestic poultry (*p*4)

Assessment of the probability of wild birds being close to and/or feeding together with domestic poultry (*p*4) was based on the questionnaire survey (Chapter 4). Data from the observational studies were applied if that bird family was not reported as being present in the questionnaire survey. The qualitative assessment was analysed using results from Questions 5.1 and 5.2 in the questionnaire survey (Chapter 4). The probability *p4* was estimated from the ratio of villagers who had observed a wild bird species having close contact and/or feeding together with their chickens and/or ducks compared to the total number of villagers who had seen these birds. The probability of a wild bird species being close to and/or feeding together with domestic poultry was estimated individually and represents the likelihood of the event occurring in a poultry-keeping area in general, including backyards/households and open system poultry farms (Table 7.7). Biases from field data

collection were similar to those reported in Section 7.3.2.3. Many owners would not be able to accurately observe interactions between wild bird species and backyard poultry under a system of free-range management. Thus, the level of uncertainty of $p4$ was categorised as "Moderate".

The overall probability of an infected wild bird shedding an infectious dose of the virus close to poultry was classified as "Low" while the uncertainty was classed as "Moderate" (Table 7.8). It needs to be taken into account that the probability assessed in this study represented the likelihood per single bird. The probability of infected wild birds entering poultry keeping areas and shedding an infectious dose of virus close to poultry will increase as the number of wild birds visiting the areas within a time period increases. Even though the overall probability was "Low" in this study, the impact following an occurrence of the risk was considered "Very high" due to the contagious nature of H5N1. Using the risk ranking matrix (Table 7.3), a "Low" probability of occurrence with a "Very high" impact, gives an overall risk ranking of "Medium severity". The quantitative assessment of the risk factor (lesser whistling ducks seen in farms; section 7.2) was performed in the next section.

Table 7.7; Assessments of *p*3 and *p*4

Family	Average percentage of villagers who saw birds enter the poultry keeping areas (N=217)	*p3*	Average percentage of villagers who saw birds in close contact to poultry (N=234)	*p4*
Anatidae	7.1	Very low	0.4	Negligible
Ardeidae	7.0	Very low	6.4	Very low
Charadriidae	1.7	Very low	0.4	Negligible
Ciconiidae	1.9	Very low	2.6	Very low
Columbidae	53.3	Moderate	39.3	Moderate
Cuculidae	29.3	Low	0.9	Very low
Dicruridae	32.7	Moderate	50.0*	Moderate
Emberizidae	88.0	High	65.0	Moderate
Estrildidae	6.0	Very low	10.0*	Very low
Sturnidae	45.0	Moderate	14.1	Low
Overall	27.2	Low	16.1	Low

* Data from the observational study

Table 7.8; Qualitative risk assessment of an infected wild bird shedding an infectious dose of virus close to poultry

Pathway	Risk	Uncertainty
Probability of a wild bird infected by avian influenza H5N1 virus	Very low	High
Probability of a sub-clinically infected wild bird shedding virus with a titre $>10^{3.5}$ $TCID_{50}$	Low	High
Probability of a wild bird present in poultry keeping areas	Low	Low
Probability of a wild bird feeding together and/ or having close contact with domestic poultry	Low	Moderate
Overall probability	**Low**	**Moderate**

7.3.5Quantitative risk assessment

7.3.5.1 Probability of lesser whistling ducks infected with HPAI H5N1 virus ($p1$)

Quantitative risk methodology was used to analyse the risk of transmission of H5N1 viruses from wild lesser whistling ducks and wild pigeons to poultry. Unlike the qualitative assessment, the sensitivity of the National Surveillance Program in wild birds was taken into account in order to assess the risk quantitatively. Thus, the sensitivities of the survey were estimated by Ms. Duangrat Pothieng, a government authority who was responsible for the National Surveillance Program for H5N1 in wild birds. The estimated sensitivities of the wild bird surveillance program, which represent the likelihood of detecting the disease in the populations of interest, were 100% maximum (Se_{max}) and 90% minimum (Se_{min}). The sensitivities from the experts were put into the Uniform distribution (Continuous) in order to calculate a suitable sensitivity using the following equation.

$$Se_{survey} = \text{RiskUniform}(Se_{min},Se_{max})$$

The sensitivity of the wild bird surveillance (Se_{survey}) was 95.0%. Even though there were reports of HPAI H5N1 infected lesser whistling ducks (Chantratita et al., 2008; Pothieng and Jamjomroon, 2006), there were no positive samples from 29 sampled during the survey. The number of wild birds that were considered to be false negatives was calculated from the sensitivity of the survey and the total bird number tested in the survey using a Negative Binomial distribution (for discrete numbers) with the following equations (Wongsathapornchai et al., 2008):

$$\text{False negative} = \text{RiskNegBin}(s+1, Se_{survey})$$

s= number of positives successfully detected in the survey.

The number of false negatives in the survey for lesser whistling ducks was zero. The true prevalence of the disease or probability of a wild bird infected by HPAI H5N1 virus ($p1$) was calculated using the Beta distribution with the following equations (Wongsathapornchai et al., 2008). True prevalence was 0.005% for lesser whistling ducks (Table 7.9 and Figure 7.4).

$$\text{True prevalence} = \text{Risk}\beta\ ([s_{true}+1], [N- s_{true} +1])$$

$$\text{True positive } (s_{true}) = \text{false negatives} + s$$

N=Total number of the birds tested in the surveillance

7.3.5.2 Probability of subclinical infection with shedding sufficient virus to cause infection in poultry ($p2$)

For assessment of the lesser whistling duck, the probability of subclinical infection with sufficient virus shedding to result in infection in poultry was based on unpublished data of an inoculation experiment in lesser whistling ducks from Dr. Wittawat Wiriyarat, Faculty of Veterinary Science, Mahidol University (Wiriyarat, 2009) where highly pathogenic H5N1 virus (A/Chicken/Thailand (Bangkok)/vsmu-3/2004) was inoculated into 26 lesser whistling ducks. Groups of three to four ducks were inoculated with different doses (10^6, 10^5, 10^4, 10^3, 10^2, 10, and 0 $TCID_{50}$) of virus. The probability of infected ducks not displaying clinical signs (pAsym) and the probability that the ducks shed a sufficient viral titre to infect poultry (pShed) was assessed separately and then combined together as $p2$ (Figure 7.5). Both pAsym and pShed were calculated with a Beta distribution using the following equations.

$$p\text{Asym} = \text{Risk}\beta\ ([s_{asym}+1], [N- s_{asym} +1])$$

$$p\text{Shed} = \text{Risk}\beta\ ([s_{shed}+1], [N- s_{shed} +1])$$

s_{asym} = Number of birds in the experiment infected by HPAI H5N1 without evidence of clinical signs

s_{shed} = Number of the birds in the experiment infected by HPAI H5N1 shedding a viral titre in their cloaca and/or trachea >$10^{3.5}$ EID_{50})

N=Total number of birds tested in the experiment

Eight of the ducks showed no clinical signs in the experiment. However the virus was detected in the cloacal and/or tracheal swabs of all 16 ducks with titres higher than $10^{3.5}$ EID_{50}. For the ducks, *p*Asym was 0.393 and *p*Shed 0.683 (*p*2 = 0.25; Table 7.9).

Figure 7.4; Cumulative probability distribution of prevalence of lesser whistling ducks

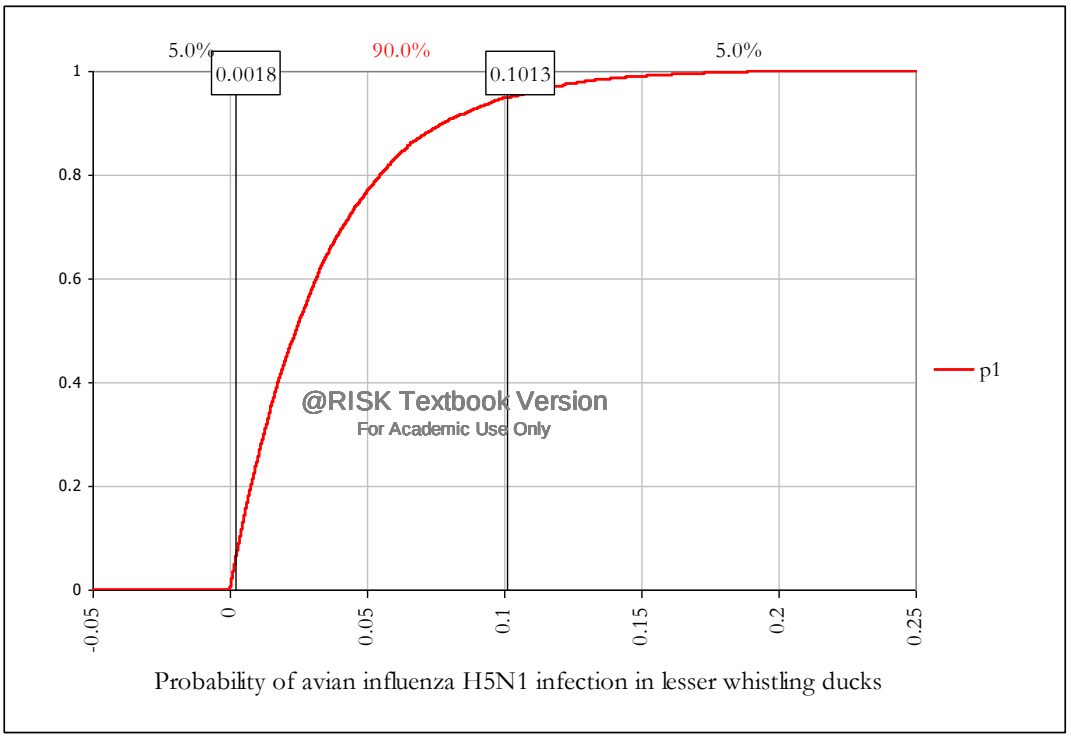
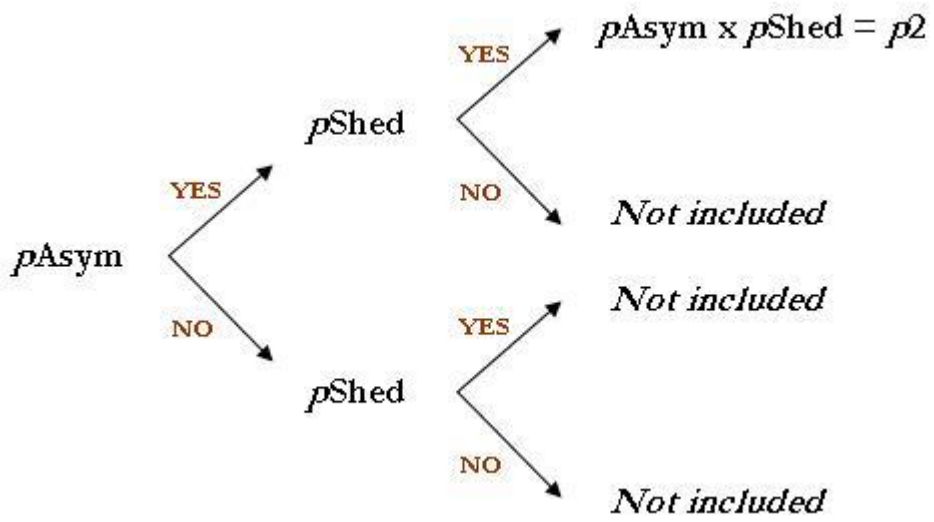


Figure 7.5; The process of calculating *p*2



7.3.5.3 Probability of lesser whistling ducks present in farms (*p*3)

The results of the wild bird observational study (Chapter 6) were used to estimate this probability by comparing the total number of times that the ducks were observed in all habitat types (N) with the number of times that the ducks were observed in farms (s_{seen}). A Beta distribution was used with the following equation.

$$p3 = \text{Risk}\beta\ ([s_{seen}+1], [N- s_{seen} +1])$$

Lesser whistling ducks were seen in the observed open system duck farms 12 out of the total of 133 times that the ducks were observed in this study. Thus, *p*3 for the ducks was 0.096 (Table 7.9).

7.3.5.4 Probability of lesser whistling ducks living close to and/or feeding together with domestic poultry ($p4$)

The total number of times that lesser whistling ducks were seen in farms compared with the number of times that the ducks had direct or close contact with domestic poultry (less than 1 metre) and/or were feeding together with domestic poultry was calculated as the probability of the lesser whistling ducks being close to and/or feeding together with domestic ducks in farms. A Beta distribution was then used to calculate $p4$ (using the following equation).

$$p4 = \text{Risk}\beta\ ([s_{feed}+1], [N- s_{feed} +1])$$

s_{feed} = Number of observed occasions that the bird(s) were seen feeding together and/or having close contact to domestic poultry

N=Total number of wild bird observation times

However, the outcomes of the observational study showed that from the 12 times that the ducks were seen in farms, no close contact and/or feeding with domestic ducks was observed. The probability of lesser whistling ducks being close to and/or feeding together with domestic poultry was 0.071 (Table 7.9).

Table 7.9; Summary of the probabilities calculated by @risk

Variable	*p*1	*p*2	*p*3	*p*4	Overall (P1xP2xP3xP4)	The risk per year
Minimum	1.40×10^{-5}	7.10×10^{-2}	3.10×10^{-2}	4.20×10^{-5}	9.50×10^{-9}	4.03×10^{-5}
Maximum	2.10×10^{-1}	5.30×10^{-1}	2.00×10^{-1}	4.70×10^{-1}	1.40×10^{-3}	1
Mean	3.40×10^{-2}	2.50×10^{-1}	9.60×10^{-2}	7.10×10^{-2}	5.80×10^{-5}	2.46×10^{-1}
SD	3.30×10^{-2}	7.40×10^{-2}	2.50×10^{-2}	6.70×10^{-2}	1.10×10^{-4}	4.67×10^{-1}
Variance	1.10×10^{-3}	5.50×10^{-3}	6.40×10^{-4}	4.40×10^{-3}	1.10×10^{-8}	4.67×10^{-5}
Median	2.40×10^{-2}	2.40×10^{-1}	9.40×10^{-2}	5.20×10^{-2}	2.10×10^{-5}	8.91×10^{-2}
Mode	3.50×10^{-3}	2.20×10^{-1}	9.50×10^{-2}	3.80×10^{-4}	1.30×10^{-7}	5.52×10^{-4}
5%	1.80×10^{-3}	1.30×10^{-1}	5.80×10^{-2}	3.90×10^{-3}	6.20×10^{-7}	2.63×10^{-3}
25%	1.00×10^{-2}	1.50×10^{-1}	7.80×10^{-2}	2.20×10^{-2}	5.70×10^{-6}	2.42×10^{-2}
75%	4.70×10^{-2}	1.70×10^{-1}	1.10×10^{-1}	1.00×10^{-1}	6.40×10^{-5}	2.72×10^{-1}
95%	1.00×10^{-1}	1.80×10^{-1}	1.40×10^{-1}	2.00×10^{-1}	2.20×10^{-4}	9.33×10^{-1}

The overall probability of a H5N1 infected wild bird excreting an infectious dose of the virus close to domestic poultry was generated by multipling $p1$, $p2$, $p3$, and $p4$. Thus, the mean risk of transmission for a lesser whistling duck was 5.8×10^{-6} (Table 7.9). The total number of birds found in the area per year was multiplied with the overall risk for a lesser whistling duck to estimate the overall risk per year. Based on outcomes of the observational study (Chapter 6 - section 6.3), there was an average of 12 lesser whistling ducks seen at an open system farm per day which was extrapolated to 4,243 per year (12 x 365 days). Thus, the overall risk of transmission from the ducks per year was 2.5×10^{-1} (Table 7.9). Moreover, the probability of at least one overall event ($p1xp2xp3xp4$) occuring in a year (4,243 ducks/year) was 0.024 (using the following equation).

$$\text{Probability at least one event occurs} = 1-(1-p)^n$$

p = Probability of an event occuring

n = Total number of birds

7.4 Discussion

The risk assessments undertaken in this study were based on the release and exposure pathway (Section 7.3 and Figure 7.2) which did not cover all the possible transmission pathways for HPAI H5N1 virus. The complete transmission pathway would include both direct and indirect pathways, which would involve many factors including environmental factors (e.g. temperature, humidity, and infrastructures of poultry keeping areas), biological factors (e.g. infectious agents and hosts), and movements of fomites. Movement of domestic poultry, such as paddling ducks (Gilbert et al., 2006) and fighting cocks (Sims et al., 2005), are also considered to play a role in spreading the infection which should be included in the complete transmission pathway. Unfortunately there were not enough available data to assess the complete risk pathway at the time of this study due to insufficient information on the likelihood of viral intake in poultry and limitations of the surveillance programs for H5N1 in

wild birds due to the difficulty of trapping birds and the lack of a true random sampling procedure. For example, trapping techniques used to survey birds may cause bias (Feare and Yasué, 2006). Further studies involving both field and challenge studies are required to estimate the likelihood of viral intake by poultry.

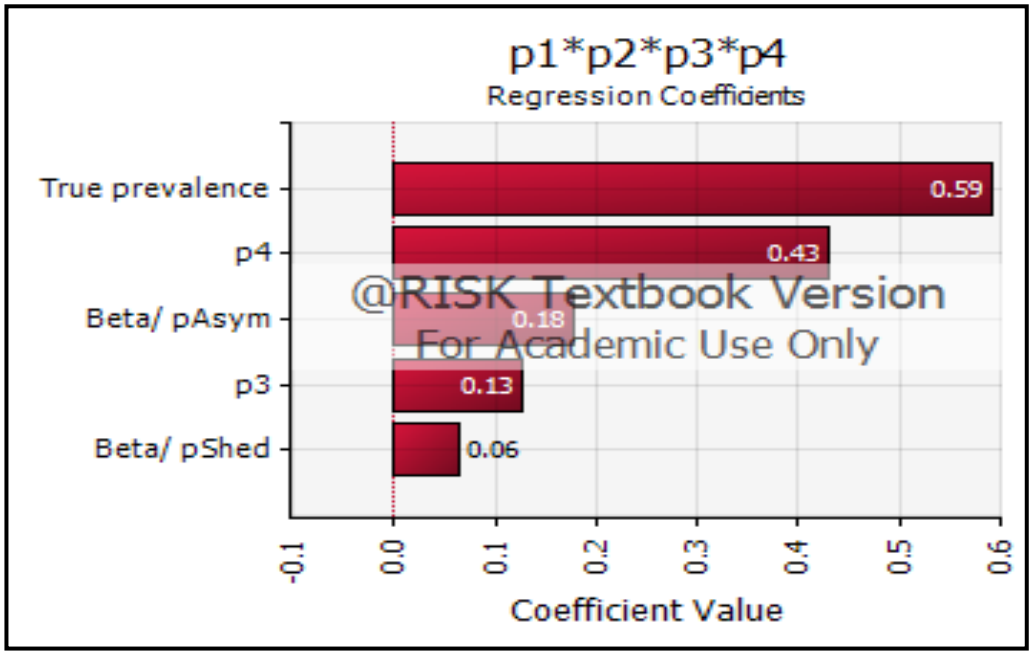
Spatial-temporal studies of the H5N1 outbreaks by Kilpatrick and others (2006) indicated that wild migratory bird movements were likely to have introduced the infection to a number of Asian countries. As well, the qualitative risk assessment done by Pfeiffer (2007) reported that the exposure of migratory birds to free-range or backyard flocks was "High". In contrast the qualitative assessment performed in the current study found that the exposure of backyard and open-house poultry to wild birds was "Low". However, the qualitative assessment undertaken in this chapter focussed on common wild birds (bridge species) in general, instead of specific species. Exposure of domestic poultry to wild birds is more likely to occur for species which are terrestrial and non-migratory such as pigeons, sparrows, doves, and/or starlings (Section 6.3). Free range or backyard poultry in the Banglane district are mainly native chickens/fighting cocks (Section 4.3) which are unlikely to share water sources with migratory waterfowls. Thus, the average probability of wild birds in close proximity or being exposed to domestic poultry in all bird groups was low. Probabilities ($p1$, $p3$, and $p4$) in this qualitative risk assessment were estimated based on the outcomes of the wild bird surveillance in Thailand and the questionnaire study in Banglane district, which were specific to the wild bird species and disease situation of the area. The outcomes consequently need to be interpreted carefully based on those specific criteria.

In the quantitative assessment, the true prevalence of infection with H5N1 had the greatest impact on the risk model (Figure 7.6). A higher prevalence of H5N1 in lesser whistling duck species will increase the risk of viral contamination close to domestic poultry in open system farms. Because a low sensitivity surveillance program can lead to a high number of false

negatives which will reduce test prevalence (Figure 7.7), the sensitivity of the wild bird surveillance program can also affect the overall risk of the lesser whistling ducks. In this assessment, the sensitivity of the survey provided by the government authority was high (90-100%; see Section 7.3.5.1). A simulation demonstrated that if a surveillance program has a lower sensitivity for detecting the virus, the overall risk will increase (Figure 7.8). For example, if a survey had a sensitivity of 20 to 40%, the overall risk of transmission from ducks would be higher (x10) compared to that reported in this study.

The second variable that had a significant impact on the risk model was the probability of lesser whistling ducks being close to and/or feeding together with domestic poultry ($p4$). Lesser whistling ducks are strongly gregarious, often seen in large groups, and are commonly observed in lakes, marshes, and wetlands (Robson, 2004). Open system duck farms normally have a duck pond, however the size of the ponds are relatively small with no vegetation (Figure 7.9) which may not be suitable for a group of lesser whistling ducks to feed and/or hide in. The observational study (Section 6.3) revealed that the lesser whistling ducks observed at farms were flying past only without stopping or landing in the farm area. In Section 7.3.5.4 of the quantitative risk assessment, the number of close contacts and/or feeding together events between the lesser whistling ducks and domestic ducks was "zero". However, the figures used in this assessment were from observational data collected from only several open system duck farms. Other open system farms may contain different conditions and environments resulting in different estimates of risk.

Figure 7.6; Sensitivity analysis of the p1xp2xp3xp4 model*



*True prevalence = *p*1, Beta/pAsym = *p*Asym, Beta/pShed = *p*shed

Figure 7.7; Impact of the sensitivity of the wild bird surveillance program on the true prevalence of avian influenza H5N1 infection in lesser whistling ducks

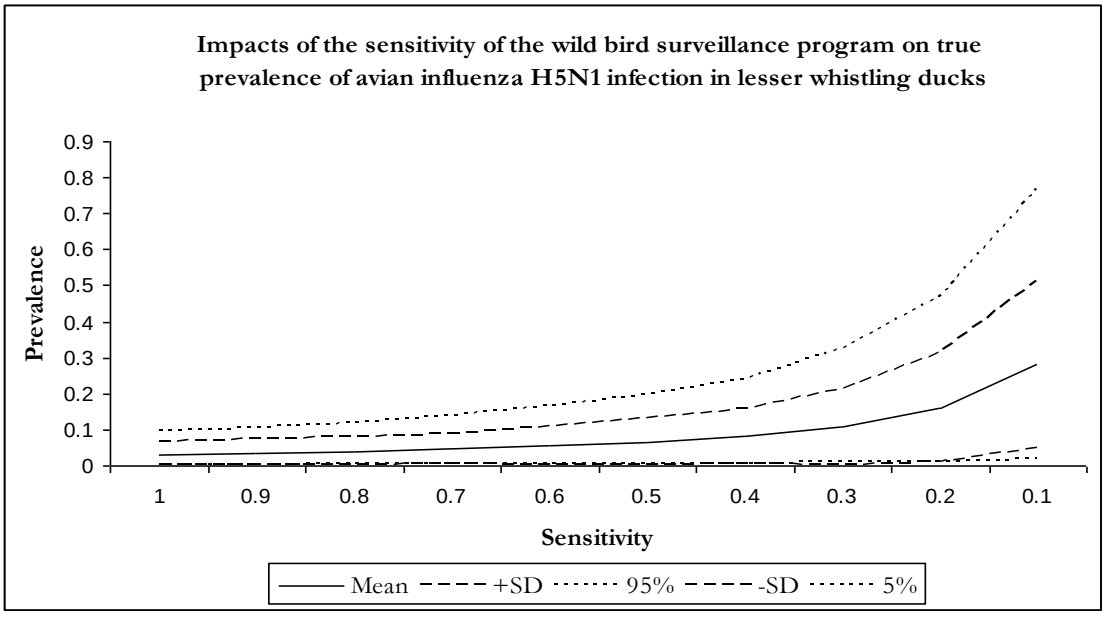


Figure 7.8; Impact of the sensitivity of the wild bird surveillance program on the lesser whistling ducks' overall probability (risk); Mean (solid line) and Standard Deviation (+/- SD; dash lines)

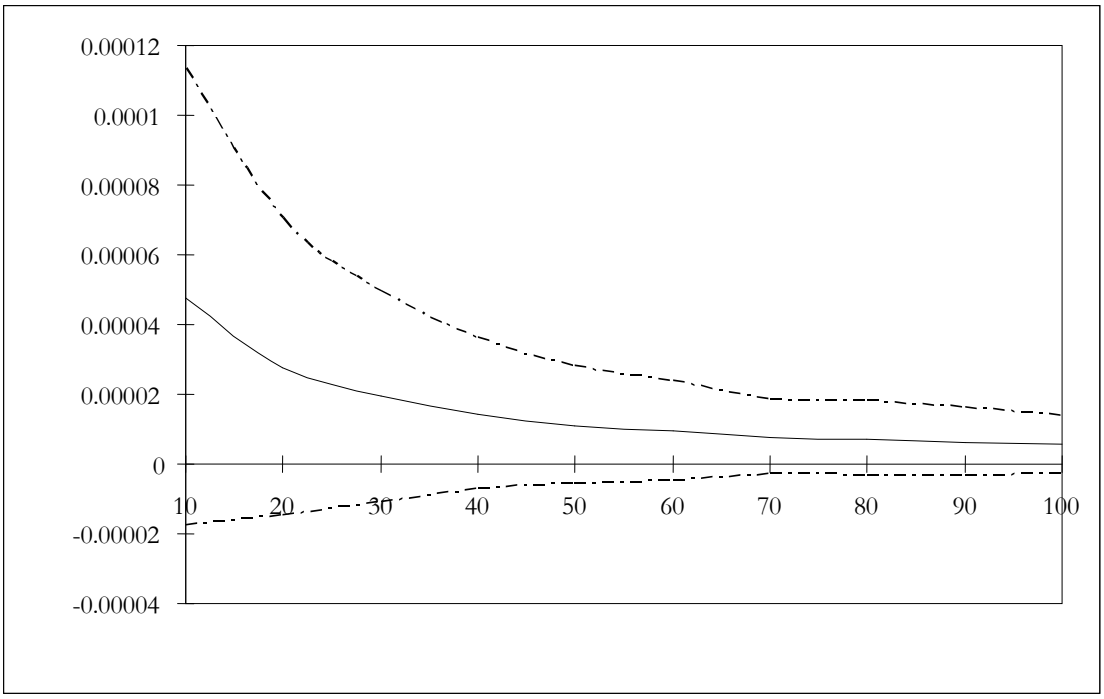


Figure 7.9; An open system duck farm with a duck pond between duck houses

The numbers used to estimate risks in this assessment are only a general indication of the situation. For example, the probability of the presence of lesser whistling ducks in farms was estimated from the number of observed times that the ducks were seen on farms. Data on how often the ducks were observed per day on a farm and the number of ducks present per day was not included in the assessment. Even though in this study the risk per lesser whistling duck was low, the overall risk increases with time and with the number of ducks present. Habitat types would have a significant effect on the total number of ducks per group that visit. For example, the number of lesser whistling ducks observed in natural ponds can exceed 200, however the number observed in the poultry keeping areas/farms was generally small and they were unlikely to stop over (Section 6.3).

Various factors can affect the risk model and the assessments in this study. For example, the production rate and volume of faeces are species dependent and are influenced by diet, body size and physical structure of the bird (Klasing, 2005). The data on viral shedding from the experimental research (unpublished) that were used in the quantitative assessment of $p2$ was determined from viral titres from cloacal and tracheal swabs. Diarrhoea is one of the clinical signs of avian influenza H5N1 infected birds (Liu et al., 2005) and has been observed in lesser whistling ducks challenged with HPAI H5N1 (Dr Wittawat Wiriyarat (2009); Personal communication). However, clinical signs in HPAI H5N1 infected birds are species specific (Perkins and Swayne, 2001, 2002, 2003b; Songserm et al., 2006c), for example in chickens which die rapidly, diarrhoea is not a feature of infection (Perkins and Swayne, 2001). Consequently some species of infected birds may produce more faeces than non-infected birds. The longer an infected bird spends in an area, the higher the chance that it would shed virus into that area. The risk assessments reported in this chapter took into account the virus load shed and the duration of potential contact time in proximity to poultry in the area. However, the results need to be interpreted with caution as they only represent the risk of

transmission between targeted species and backyard or free-grazing poultry under conditions existing in central Thailand at that time.

After virus is shed, the titre of virus at the site of exposure will decrease over time. The H5N1 virus can be inactivated completely soon after exposure to direct sunlight or to high temperatures (Songserm et al., 2005) and different environmental conditions in the areas where poultry are kept can affect the rate of reduction of the viral load. For example, earthen floors in duck houses can be wet and shaded allowing for the virus to survive for longer periods. In the current risk assessment a reduction in the viral load with time was not included in the analysis. The likelihood of poultry being exposed to faeces from wild birds would be influenced by the density of poultry. In some backyards there may be between 1 and 200 chickens (Chapter 4, section 4.3) while an open system duck farm may have 3,000 to 5,000 layer ducks (Songserm et al., 2006c). Each area type was considered separately in this study. In areas where the density of poultry is low, such as backyard areas (Figure 7.10), there would be a smaller likelihood of direct exposure by domesticated poultry to fresh faeces from wild birds. In areas where the density of poultry is high, such as with an open system poultry farm (Figure 7.11), the likelihood of direct exposure to faeces from an infected wild bird would increase giving a moderate chance of exposure of poultry to the virus. However, even though the number of wild birds feeding in an open farm may be high, when compared to the number of ducks that may be present, the chance of direct exposure of an individual duck to fresh faeces from an infected wild bird may be only low to moderate. It would be advantageous to consider the ecology and behaviour of the virus and wild birds, as well as management and husbandry practices on farms, when undertaking further risk assessment studies.

Figure 7.10; A backyard chicken, six white vented mynas and a pigeon sharing the same habitat

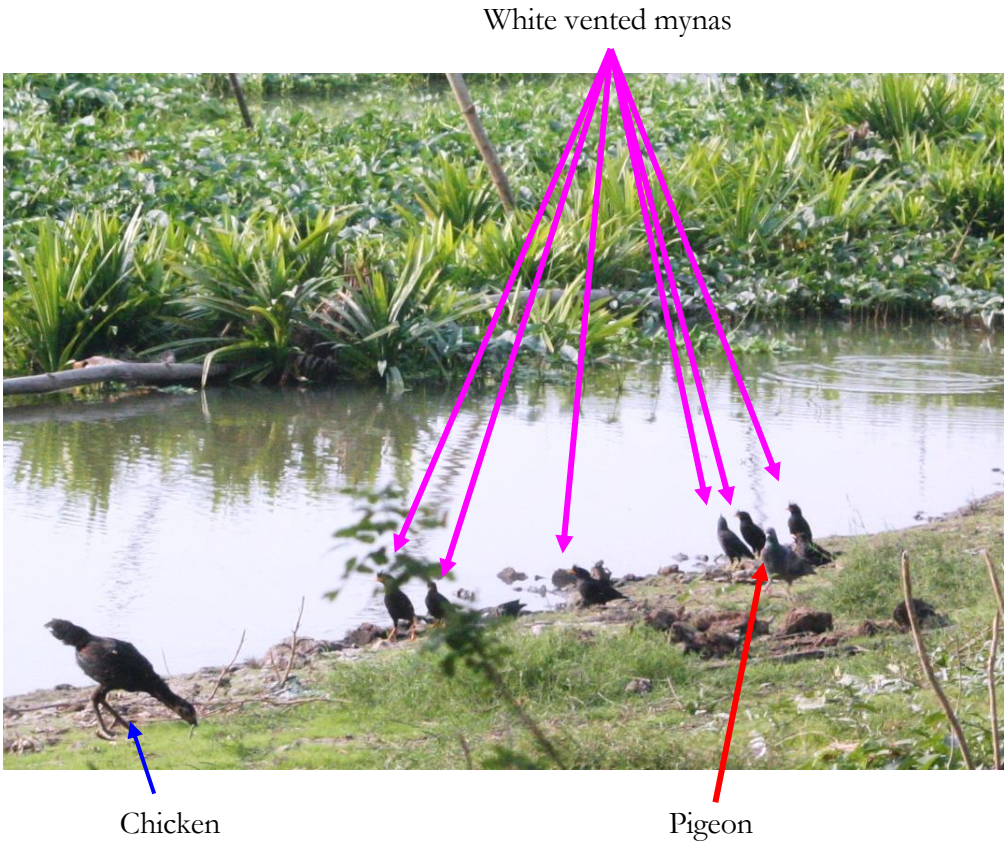


Figure 7.11; Pigeons feeding in a layer duck farm

Chapter 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 Introduction

This series of studies set out to answer the research questions on the prevalence of AI H5N1 in the wild bird population, the likelihood of H5N1 virus transmission between wild birds and domestic poultry, as well as the likelihood of spread of H5N1 infection from an infected wild bird to domestic poultry. Clark and Hall (2006) stated that the risk of transmission of avian influenza virus depended upon a combination of the prevalence of the virus in reservoirs, the susceptibility of hosts, the degree of contact between reservoirs and hosts, and the level of biosecurity. Thus, this project has involved the collection of samples from wild birds and field data from areas where low biosecurity poultry enterprises are present. Such areas are considered to be high risk areas for HPAI H5N1. These data were used to estimate the risks of a wild bird being involved in the transmission of HPAI H5N1 to domestic poultry.

8.2 General discussion and conclusions

The outcomes of the wild bird surveillance programs conducted in this report found that HPAI H5N1 infections were detected in wild bird populations in Thailand between 2004 and 2007 (details in Chapter 3 and 5). However, the serological and virological prevalence in wild birds was low and some wild birds were infected with HPAI H5N1 virus without displaying any obvious clinical signs. Also, there were a number of healthy wild birds that had neutralizing antibody to H5 avian influenza viruses indicative of either subclinical infection with H5 avian influenza viruses or infection with subsequent recovery. Most positive samples

were from common terrestrial wild birds, which are known to share habitats with domestic poultry. As Guan et al (2004) mentioned that interspecies transmission causes viral re-assortment, the viruses isolated from wild birds in this study (in 2007) were typed and found to be closely related to viruses previously isolated from poultry in Northern and Central Thailand (in 2004 and 2006). This provides additional evidence that HPAI H5N1 outbreaks in poultry could spill over to and persist in wild bird populations in Thailand and potentially these viruses could be transmitted back to poultry from wild birds.

High risk areas for influenza viral transmission included poultry keeping areas, especially open system poultry farms, where interaction between wild birds and domestic poultry was observed most often (Chapter 6). As was reported in the observational study, backyard poultry have less close contact and interaction with free ranging birds compared to poultry in an open farming system, due to the lower population density of poultry in backyard systems. Backyard areas normally accommodate pet and backyard animals, including pigs which are known to be mixing vessels for influenza viruses (Webster, 1998) and interspecies transmission may occur if the virus is present in the areas. Munster et al. (2007) stated that surface water contaminated with influenza A virus may be a source of transmission to other hosts. This project also revealed that wild birds that are commonly present in open system poultry farms were also observed in natural ponds feeding with other wild local and migratory birds. In wild bird feeding grounds where domestic poultry were not observed, such as natural ponds and water bodies, bridge species may transmit the infection to other wild birds directly or indirectly. Viral contamination of the environment may also act as a source of infection. Thus, habitats where contact between wild and domestic birds occurs frequently could be considered as high risk areas. Habitats where the level of contamination from wild bird faeces and secretions and where environmental conditions facilitate virus survival should also be considered as risk areas. Moreover, Tiensin and others (2009) reported that ecological risk factors for clustering of avian influenza H5N1 infection in

central Thailand included high densities of backyard chickens and fighting cocks, a high human population, presence of quail flocks and free grazing duck flocks, and the presence of poultry slaughterhouses. In high risk areas and areas that contain these risk factors, close monitoring of the disease status should be applied.

The risk factor study (Chapter 4) showed that poor farm biosecurity practises in the presence of observed wild birds increased the risk of having HPAI H5N1 infection. The observational study (Chapter 6) revealed that overlap of wild and domestic bird habitats and close contact between common terrestrial wild birds and domesticated poultry were frequent in the field in the study sites. Since 2006, the DLD has encouraged all poultry holders (commercial and small farms) to improve farm biosecurity, using methods such as applying a strict disinfection scheme, covering poultry enclosures to stop wild birds and/or rodents entering the enclosure, and introducing a compartmentalisation system (DLD, 2006). In the case of small poultry holders, where poultry may have less economic value compared to commercial farms, the holders are less likely to implement biosecurity measures. In order to introduce biosecurity into small poultry holders, it is important to make them appreciate the benefits of biosecurity, as well as the consequences of having disease in their poultry.

Kilpatrick and others (2006) reported that half of the H5N1 introductions in Asia were most likely through the movement of poultry. Moreover, Nguyen et at (2008) reported that the H5N1 viruses clade 1, which were previously common in Northern Vietnam, were replaced by clade 2 viruses introduced from China through the movement of poultry. Unlike Vietnam, the HPAI H5N1 viruses isolated in Thailand were closely related to each other and clustered in the same clades and genotype (details in Chapter 5). Even though Chen and others (2006) reported that H5N1 virus was isolated from some healthy migratory birds in southern China, there was no evidence of infection in migratory birds in the current study. The conclusion could be made that a single introduction of the virus in 2004 caused outbreaks of HPAI

H5N1 in Thailand. No evidence of the introduction of a new strain from neighbouring countries into Thailand was apparent at the time of this study.

Agricultural areas in central Thailand, where poultry farms were clustered (Tiensin et al., 2009), may not be suitable as a stop-over site for migratory waterfowls. This was confirmed by the results of the observational study (details in Chapter 6). The risk of introduction of a new influenza strain from migratory waterfowls would appear to be very low in these areas from national surveillance data and from the risk analysis findings from this study. Similar to the current project outcomes, the probability of transmission of the virus from migratory birds to backyard and free range poultry in the European Union was assessed by the European Food Safety Authority (EFSA) as low but with a high level of uncertainty (Pfeiffer, 2007). A phylogenetic study performed by Uchida and others (2008) demonstrated that even though there was no evidence of involvement of wild birds in HPAI transmission to poultry, the viruses could have been maintained in wild bird populations for a certain period. As was seen from the outcomes of Chapter 7, the risk of infected wild birds dropping an infectious dose of the virus close to poultry was low in central Thailand. However, if the virus is present in these areas, infected wild birds are likely to help maintain the virus in the area by persistent circulation through wild bird populations. Smith et al. (2009) stated that wild birds may also disperse the virus for at least tens if not up to hundreds of kilometres. This is consistent with finding viruses in wild birds in the Banglane District in 2007 that are closely related to viruses isolated from the Pichit province in Thailand in 2006.

8.3 Limitations of the project and requirements for further study

In interpreting the results of the current study one must also make allowance for potential limitations from the study design. As the project targeted a high risk area, outcomes of the study can only be used to explain the risk involving wild birds in the area where habitats were

similar to the study site. The outcomes may not be suitable to explain risk in different birds or habitats. This project targeted common terrestrial wild birds which were in high numbers and obviously shared habitats with domesticated poultry in the central part of Thailand where HPAI H5N1 had previously resulted in severe outbreaks in poultry. Migratory birds may play a larger role in other areas, as has been seen in Europe (Burgos and Burgos, 2008) where the wild bird population is larger and lives closely to domestic poultry. The current project used a multiple species approach resulting in bias towards species present in high numbers. For example, as the number of pigeons and sparrows were high in the study site, samples and data collected in this project were dominated by these species. Identifying wild bird species using binoculars, which requires expertise, can lead to human errors but to lessen this field staff conducting the questionnaire and observational studies had some experience with wild bird surveillance and were provided relevant well illustrated bird guides (A guide to the Birds of Thailand; (Lekagul and Round, 1991)). With birds where the species could not be fully identified, the birds could be identified to the level of genus or family and this allowed analysis to be conducted at the level of bird family. Alternatively, in future wild bird ecology studies, additional funding for ornithologists could be sought to include them in field data collection teams. Another limitation of the risk analysis study related to the risk assessments for the transmission of HPAI H5N1 from wild birds to domestic poultry.

It is important to control outbreaks of HPAI H5N1 viruses as the viruses not only impact on public health and economies but also impact on wild bird populations (Roberton et al., 2006). In order to control and prevent outbreaks of HPAI H5N1, a multidisciplinary approach which involves all stakeholders should be applied. Smith and others (2009) suggested that both passive and active surveillance in wild birds are useful to monitor the presence of the virus. Even though application of biosecurity measures in farms and households with poultry is known to reduce the risk of introducing diseases, some farmers are not in a position to implement such measures (Sims, 2008). The cost effectiveness of such measures should be

investigated in the future with the aim of developing effective but economic monitoring and control strategies. As well, collaboration between local and international governments and non-government organizations needs to be encouraged to ensure there is information sharing, sufficient technical and academic support, and funding to fight against HPAI and other emerging infectious diseases.

In summary, outcomes of this project revealed that there is evidence for persistence of H5N1 infection in local non-migratory wild birds in areas of central Thailand where multiple poultry outbreaks have occurred previously. This showed that wild birds can play a role in the HPAI H5N1 viral persistence and possibly transmission; however, poultry trade and movement are more likely to be involved in spreading H5N1 HPAI viruses in Thailand. However, the risk analysis study conducted into transmission pathways of H5N1 HPAI viruses did not clearly identify high risk species or pathways that could explain how and if these viruses will spread between wild birds and domestic poultry. It is important to conduct targeted surveillance programs in wild birds and domestic poultry, as well as study wild bird ecology and behaviour in order to gain more understanding of the disease's epidemiology.

Appendix I

Field sample collection form

The monitoring and surveillance center for zoonotic diseases in wildlife and exotic animals, Faculty of Veterinary Science , Mahidol University

Date(DD/MM/YY)..... Sample collector..... Phone No.....

Location.....GPS.....

Establish of flock.....Morbidity rate(%)..... Mortality rate(%).....

Environmental description/surronding area.....Type of animals in the areas.....

Outbreak situation of the areas.....

No.	Species (type of animals)	No. of animal	Sex		Age		Health status			Type of samples				Lab No.	Quick test	Comments
			♂	♀	Adult	Young	Healthy	Sick	Dead	Blood	Tracheal/ Choana swab	Faeces/ Cloacal swab	Carcass			

Appendix II

Reagent preparation protocols

1. Earle's minimal essential medium (EMEM)

1.1. Stock solution 10X

EMEM powder	95.3 grams

Add sterile distilled deionized water to 1,000 ml and then filtrated by 0.45 μm millipore membrane. Aliquot 100ml/tube and kept frozen at -20°C.

1.2. Working solution 1X

EMEM 10X	10 ml
1M HEPES	1 ml
Penicillin 40,000 U/ml	0.5 ml
Gentamycin 4 mg/ml	0.5 ml
Fungizone 1 mg/ml	0.1 ml
5% $NaHCO_3$	4 ml

Add sterile distilled deionized water to 100 ml and kept at 4°C.

1.3. Growth media (10%FBS in EMEM) kept at 4°C

EMEM (working solution 1X)	90 ml
Foetal Bovine serum	10 ml

1.4. Maintenance media for influenza virus infection

EMEM (working solution 1X)	100 ml
Trypsin-TPCK 500 μg/ml	0.4 ml

2. Trypsin-TPCK 500 µg/ml

TPCK-trypsin	10 mg
MEM 1X	20 ml

Mixed solution was filtrated by 0.45 µm millipore membrane. Aliquot 200 µl/tube and kept at -20°C

3. 1M HEPES

HEPES	23.83 g

Sterile distilled deionized water was added to 100 ml and then filtrated by 0.45 µm millipore membrane (kept at 4°C).

Appendix III

Questionnaire for villages

Interviewer:	Date:

This questionnaire is being used to gain information on wild birds in this village and basic information about village farm structure. The information will only be used for the risk assessment of disease transmission. None of your details will be released to people outside the project.

1. Respondent/General Information

1.1 Name:_____

1.2 Age:_____1.3 Gender: [ ] Female [ ] Male

1.4 What is your main occupation? : _____

1.5 What is your highest level of education? :

[ ] None [ ] Primary school [ ] Secondary school

[ ] High school [ ] Voluntary school/college/university [ ] Others

1.6 Address:_____

_____GPS:_____,_____

1.7 How many people live in your household?_____

2. Animals in household and/or farm areas (*if have avian species*)

2.1. Do you own birds?

[ ] Yes [ ] No

2.1.1. If yes, what kinds and number of birds do you have? (*can answer more than one*)

2.1.1.1.[ ]Chicken; please identify below

[ ] Native breed total_____

[ ] Broiler total_____

[ ] Layer total_____

[ ] Breedertotal_____

[ ] Fighting cock total_____

2.1.1.2.[ ]Duck; please specify below

[ ] eggs total_____, Breed _____

[ ] meat total_____, Breed _____

2.1.1.3. [ ]Pet or song birds; please specify types

Species;_____total_____

Species;_____total_____

2.1.1.4.[ ]Other poultry ; please specify types

Species;_____total_____

Species;_____total_____

2.1.1.5.[ ]Other animals

[ ] Dog total_____

[ ] Cat total_____

[ ] Pig total_____

[ ] Cattle; please specify types

Species;_____ total _____

Species;_____ total _____

2.2. Why do you keep poultry? (*can answer more than one*)

[ ] Self consumption [ ] Sell to local market

[ ] Sell as breeder [ ] Sell to commercial market

[ ] As pet [ ] Other (please give detail);_____

2.3. *If Fighting cock in Q2.1.1.1 was ticked, please answer this question.*

2.3.1. Have your fighting cocks competed in any competitions?
Yes [ ] No [ ] Don't know [ ]

2.3.2. Are your cocks involve in fighting competitions?
Once every_____days/weeks/months

2.3.3. Do you know how often fighting cock competitions are held?
Once every_____days/weeks/months

2.3.4. Do you know in which subdistrict or village are competitions usually held, please give detail?_____

2.4. If Pet/Song bird in Q2.1.1.3 was ticked, please answer this question.

2.4.1. Have your pet/song birds competed in any competitions?
Yes [ ] No [ ] Don't know [ ]

2.4.2. Are your birds involve in pet/song bird competitions?
Once every_____days/weeks/months

2.4.3. Do you know how often pet/song bird competitions are held?
Once every_____days/weeks/months

2.4.4. Do you know in which subdistrict or village are competitions usually held, please give detail?_____

3. Husbandry and Management

May I have a look at the area where you keep your poultry?

Interviewer: as you observe the areas of the household/farm where poultry are kept, please answer Q3.1 and Q3.2 yourself.

3.1. How do they keep poultry, please tick an appropriate category(s) below?

Chicken	**Duck**	**Pet birds**	**Other poultry; please specify** _____
Free ranging [ ] Cage/coop [ ] Housing [ ] Other [ ]; please specify _____	Free ranging [ ] Cage/coop [ ] Housing [ ] Paddy field [ ] Other [ ]; please specify _____	Free ranging [ ] Cage/coop [ ] Housing [ ] Other [ ]; please specify _____	Free ranging [ ] Cage/coop [ ] Housing [ ] Other [ ]; please specify _____

3.2. If one answer above is Housing, please identify the material the house is constructed from

Roof [ ]No[ ]Yes, please give detail; _____
Solid wall [ ]No[ ]Yes, please give detail; _____
Non-solid wall (e.g. net)[ ]No [ ]Yes, please give detail; _____
Solid floor [ ]No[ ]Yes, please give detail; _____
Bedding [ ]No[ ]Yes, please give detail; _____
How big is the housing? _____m x_____m
How many animals per house?_____

3.3. What do you feed your chickens or duck? (*Tick all ingredients*)

Type of food	**chicken**	**duck**	**Other poultry; specify……**
Premixed commercial feed			
Self-mixed feed or purchase ingredients			
Kitchen leftovers/Let them find own feed			
Graze paddy fields			
Other: please specify: _____ _____			

3.4. If you graze ducks, please describe

3.4.1. How often do you graze ducks? (e.g. daily) _____
for how long? _____months

3.4.2. When do you graze ducks? (e.g. whole year or certain months)
_____

3.4.3. What are the name(s) of village(s) where you graze ducks:
_____

3.4.4. How do you bring your ducks to the paddy?
a. Walk my ducks [ ]; b. Transport my ducks by vehicle [ ]

3.4.5. Do ducks from other households or villages usually graze in the same paddy area?
Yes [ ] No [ ]

3.4.5.1. If yes, how often?
[ ] Most of the time, other ducks graze in same paddy area as my ducks
[ ] Sometimes, other ducks graze in same area or in nearby paddy within metres
[ ] Never, I always graze my ducks in areas far away from other ducks

3.5. Water. What is the source of drinking water for your poultry?
a. Pond or lake [ ]
b. River water [ ]
c. Own well [ ]
d. Community well [ ]
e. Collected rain water [ ]
f. Piped or tap water [ ]
g. Other source: please specify:_____[ ]

3.6. How often do you sell, offer or give away your poultry or pet birds?
a. Never, I only keep them for our own eating [ ]
b. Every (please specify):_____days [ ]
c. Every (please specify):_____weeks [ ]
d. Every (please specify):_____months [ ]
e. Others (please specify):_____ [ ]

3.7. Where do you sell, offer or give away your poultry/birds? (*Can be more than one tick*)

a. Market [ ] Please specify where:_____
b. Slaughterhouse [ ] Please specify where: _____
c. Wholesaler or dealer [ ]
d. Household in same village [ ]
e. Household in other villages [ ]
f. Temple(s) [ ]
g. Others: please specify [ ] _____

3.8. Who collects or transports the poultry/birds to these places? _____

_____

3.9. Approximately what age do you sell/ offer/ give away poultry/birds? And on average how many poultry are sold each time?

Chickens	Broiler/Layer (Chicken)	Ducks	Other birds: _____
Age: days	Age: days	Age: days	Age: days
total	total	total	total

3.10. Do you usually sell/ offer/ give away your poultry during festivals or religious ceremonies?

Yes [ ] No [ ]

3.10.1. If yes, what are these (major) festivals?_____ When (which month)? _____

3.11. Where do you usually buy or acquire new chickens, ducks or other birds? (*please tick an appropriate category(s) for each species*)

Places	**Chickens**	**Ducks**	**Other birds: _____**
Breed myself			
Market			
Wholesaler/ dealer			
Commercial hatchery			
Same village house/farm			
Other village house/farm			
Others:_____			

3.12. Approximately how often do you buy new poultry/birds? _____

_____

3.13. At what age do you usually buy, receive, and/ or get new poultry/birds into your farm? And on average how many poultry do you buy each time?

Chickens	Broiler/Layer (Chicken)	Ducks	Other birds: _____
Age: days	Age: days	Age: days	Age: days
total	total	total	total

3.14. Do you do anything to ensure that these new birds are free from disease?

Yes [ ] No [ ]

3.14.1. If yes, how do you make sure these new poultry/birds are free of disease?

[ ] a. I know the seller and trust him/her
[ ] b. I check the birds are healthy
[ ] c. I buy from safe places e.g. standardized company
[ ] d. My concern is only the price but not disease
[ ] e. Others: please specify:_____

3.15. Describe how you usually bring in new poultry into your farm? :

[ ] a. Replace all-in-all-out (whole farm)
[ ] b. Replace birds by house
[ ] c. Replace birds in small batches
[ ] d. Replace birds individually
[ ] e. Others; please explain_____

3.16. Do you separate newly arrived birds from your other birds?
Yes [ ] No [ ]
If yes, for how long? _____

3.17. If you keep poultry for eggs, what are these eggs used for?
[ ] Self consumption
[ ] Hatching
[ ] Selling

3.17.1. If you sell eggs; please give detail
3.17.1.1. Where do you *sell* these eggs? _____
3.17.1.2. How often do you sell these eggs? _____
3.17.1.3. How many eggs do you usually sell each time?_____

3.18. Do you vaccinate your poultry?
Yes [ ] No [ ]

3.18.1. If yes, vaccine for …… by injection/ oral/ drop
If yes, vaccine for …… by injection/ oral/ drop
If yes, vaccine for …… by injection/ oral/ drop

3.19. Please describe what you do with poultry manure and litter?
[ ] a. Throw outside house; please specify where_____
distance from house: _____metre/s
[ ] b. Bury or compost distance from house: _____metre/s
[ ] c. Burn on a pile distance from house: _____metre/s
[ ] d. Spread onto fields distance from house: _____metre/s
[ ] e. Spread around house garden distance from house: _____metre/s

[ ] f. Leave where it is
[ ] g. Others; please explain_____

3.20. How often are poultry cages / sheds / backyards where chicken are kept in your household cleaned or washed?
[ ] a. Every day
[ ] b. Every 2-3 days
[ ] c. Once a week
[ ] d. Once a month
[ ] e. Others; please explain_____

3.21. Please specify if any chemicals are used for cleaning or washing?
_____

3.22. Do you grow crops (e.g. rice, maize)?
Yes [ ] No [ ]
If yes, please indicate type of crop you grow:_____

3.23. If you grow rice, please explain
3.23.1. How many times do you grow rice per year? _____times/yrs
3.23.2. Which month(s) do you start to grow rice?_____
3.23.3. How many months before you harvest?_____months
3.23.4. Which month do you harvest rice?_____

4. Animal diseases

4.1. Have your poultry/birds ever been sick in the last three years?
[ ] Yes [ ] No [ ] Don't know

4.1.1. If Yes, Please explain what species were sick? What signs did they have? If possible please recall when it occurred?

Species;_____signs;_____when;_____
Species;_____signs;_____when;_____
Species;_____signs;_____when;_____

4.1.2. Did you know what the disease(s) was? How did you know? _____

_____

4.2. Did your poultry/birds have any of these signs in the last three years?

Sudden death [ ] Yes [ ] No[ ] Don't know
Blue comb [ ] Yes [ ] No[ ] Don't know
Swollen wattles and joints [ ] Yes [ ] No[ ] Don't know
Breathing difficulty/nasal discharge [ ] Yes [ ] No [ ] Don't know
Trembling [ ] Yes [ ] No [ ] Don't know
Diarrhoea [ ] Yes [ ] No [ ] Don't know
Other; please specify…….

4.2.1. If yes, can you recall?

4.2.1.1. When it occurred?

Year_____Month_____Date_____

4.2.1.2. What kinds of poultry/birds were affected? (Please specify types)___

_____

4.2.1.3. How many poultry/birds affected? Sick_____Dead_____

4.2.1.4. How many poultry/birds survived?_____

4.2.1.5. What did you do with the sick poultry/birds? Please explain

_____

4.2.1.6. What did you do with the dead poultry/birds? Please explain

_____

____

4.2.1.7. What did you do with poultry/birds that survived? Please explain

_____

4.2.1.8. During that time, did any of your other animals (e.g. dog, cat, pig, cattle, etc) have health problems/sick/dead?

[ ] Yes [ ] No [ ] Don't know If yes, please specify species and symptoms? Species_____

Symptoms_____ Has your district (Bang-lane) been affected by bird flu?

[ ] Yes [ ] No [ ] Don't know

4.2.2. If Yes, can you recall? When it happened? Year__Month_____Date_____

___

4.2.2.1. If you know, can you tell me the address of the outbreak area?

_____

4.2.2.2. If you know, what kinds of animals were affected (sick/dead)? please specify species_____

4.2.2.3. How many animals affected?_____

5. Wild birds around your household/farm

5.1. What kind of wild birds do you normally see in your backyard/ household, farm, and/or paddy areas? How often do you see them? And if possible could you estimate number of each species found in the areas?

Interviewer show picture of wild birds then write down the number of each species and the estimated population.

Species No.	Backyard/household	Farm	Paddy field
___________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________
___________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________
___________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________
___________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________
___________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________	estimated number________ [ ] Everyday [ ] 2-3 times a week [ ] Once a week [ ] once a month [ ] 2-3 times a year; when? ____________ [ ] once a year; when? __________________ [ ] seasonal; when? __________________

5.2. Have you ever seen wild birds feeding with you backyard/ fighting cock?
Yes [ ] No [ ]
5.2.1. If yes, please specify species; …….
5.3. Have you ever seen wild birds feeding with you backyard/ paddling duck?
Yes [ ] No [ ]
5.3.1. If yes, please specify species; …….
5.4. Could you please show me the areas in your backyard/ household, farm, and/or paddy areas where you have seen those birds? *Interviewer write down description of each area (e.g. poultry feeding area in the backyard)*

_____
_____

5.5. Do you know why they come into those areas? (E.g. to get feeding, scavenge, nesting, etc.)_____

6. Wild bird roosting sites around the village

6.1. Are there wild bird roosting areas close to your household/ or village?
[ ] Yes, What species?_____
[ ] No
[ ] Don't know
6.1.1. If yes, how far is the bird colony from your house/village? Please specify; _____km. and address_____
6.1.2. Do you normally see the birds feed, roost, nest, and/or land in your household, backyard, and/or farm? Please specify
[ ] Yes [ ] No [ ] Don't know
6.1.2.1. If yes, please specify
Species _____ how often?_____
What do the birds do? (circle appropriate choice); Feed / Roost / Nest / Land
Species _____ how often?_____
What do the birds do? (circle appropriate choice); Feed / Roost / Nest / Land
Species _____ how often?_____
What do the birds do? (circle appropriate choice); Feed / Roost / Nest / Land

6.2. Do you or someone you know ever harvest any product from wild birds (including live birds, meat, feathers, and/or eggs)?
[ ] Yes
[ ] No
[ ] Don't know
6.2.1. If yes, what species? _____
What are the products?_____
6.2.2. How do you trap/ catch/ harvest those bird products?_____
_____

7. Attitude and value

7.1. Have you ever heard of bird flu?
[ ] Yes [ ] No

7.2. What do you think poses a risk of introducing Bird flu to your poultry/birds?

Do not read answers. (Can be one or more ticks)
[ ] a. introducing new poultry, birds, and eggs to your household/farm

[ ] b. People, equipment and vehicles entering household/farm
[ ] c. Wild birds near household/farm
[ ] d. Fighting cocks
[ ] e. Paddy ducks
[ ] f. Contaminated feed
[ ] g. Contaminated water sources
[ ] h. Neighbours' poultry
[ ] i. Others (please specify): _____

_____

7.3. What do you see as necessary to prevent or control Bird flu?
Do not read answers. (Can be one or more ticks)
[ ] a. Early bird flu detection in poultry/birds
[ ] b. Higher compensation for culled poultry
[ ] c. Clean feed and water
[ ] d. More education and awareness on disease prevention
[ ] e. Safe source of poultry/birds
[ ] f. Someone to advise me when my birds are sick
[ ] g. Control poultry movement from infected areas
[ ] h. Reduce contact between my poultry and birds from other households
[ ] i. Regular visits from veterinary department
[ ] j. Others (please specify): _____

_____

7.4. How would you recognize Bird flu in Chickens?_____

7.5. How would you recognize Bird flu in Ducks?_____

7.6. Please explain what would you do if you suspect your poultry/birds have Bird flu?

Do not read answers. (Can be one or more ticks)
[ ] a. Treat myself Type of medications used:_____
[ ] b. Throw birds away (please specify where):_____
[ ] c. Eat birds ourselves or share with friends
[ ] d. Feed birds to other animals; which animals: _____
[ ] e. Give away or sell birds
[ ] f. Bury birds
[ ] g. Burn birds
[ ] h. Report immediately to authority
[ ] i. Do nothing
[ ] j. Others: please specify _____

_____

7.7. How are you currently protecting your poultry/birds from getting Bird flu?
Do not read answers. (Can be one or more ticks)
[ ] a. Disinfect household regularly
[ ] b. Not buy poultry/birds from risky sources
[ ] c. Keep poultry in protected or fenced area
[ ] d. Ensure clean water and feed
[ ] e. Discourage casual visitors near poultry
[ ] f. Change clothes and clean shoes after visiting other places
[ ] g. Do nothing
[ ] h. Others: Please specify:_____

7.8. How are you currently protecting yourself and your family from getting Bird flu?

Do not read answers. (Can be one or more tick)

[ ] a. Not eating poultry that fall sick or die
[ ] b. Eat only well-cooked poultry or eggs
[ ] c. Bury or burn dead poultry
[ ] d. Wash hands with soap after handling poultry or manure
[ ] e. Change clothes after handling poultry or manure
[ ] f. Don't let children play with poultry
[ ] g. Disinfect household regularly
[ ] h. Do nothing
[ ] i. Others: Please specify:_____
_____

7.9. Where do you learn most about Bird flu? *Do not read answers.* (*Can be more than one tick*)

[ ] a. Village animal health assistants
[ ] b. Veterinarians or paravets
[ ] c. Village or community leaders
[ ] d. Radio
[ ] e. Television
[ ] f. Newspapers
[ ] g. Pamphlets/brochures/posters
[ ] h. Neighbours, friends or family
[ ] i. Wholesalers or dealers
[ ] j. Others: Please specify:_____

Do you have any other comments which would help our investigation?

_____
_____
_____
_____
_____

Thank you for taking the time to complete this questionnaire.

If you have any questions or further enquiries, please contact;
The Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic animals, Faculty of Veterinary Science, Mahidol University, Sayala campus
Tel/ Fax; 02441 5238 or surveillance_vsmu@yahoo.com

Appendix IV

Observation data collection form

Date_____**Observers**_____

Site Code _____ Start time _____ Finish time _____Temperature ____c, Humidity _____

Habitat description _____

_____

First 5 minutes; Identify species and estimate number

Species	/ no.	Species	/ no.
Species	/ no.	Species	/ no.
Species	/ no.	Species	/ no.
Species	/ no.	Species	/ no.
Species	/ no.	Species	/ no.
Species	/ no.	Species	/ no.
Species	/ no.	Species	/ no.
Species	/ no.	Species	/ no.
Species	/ no.	Species	/ no.
Species	/ no.	Species	/ no.

25 minutes; Observe each individual for 30 seconds (*write species and estimated distance between the observed bird and the closest neighbouring bird, and tick the activities that the observed bird is engaged in*)

Common name	No	Activities					Time*		Proximity distance <1 metre to others			
									Domestic			
		Fly in fly out	Feeding	Perching	Standing	Direct contact	More than 30sec	Less than 30sec	Duck	Chicken	Others; specify	Wild birds; specify

Common name	No	Activities					Time*		Proximity distance <1 metre to others			
									Domestic			Wild birds; specify
		Fly in fly out	Feeding	Perching	Standing	Direct contact	More than 30sec	Less than 30sec	Duck	Chicken	Others; specify	

Note:

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