DISSERTATION

THE ROLE OF DOMESTIC DUCKS IN THE MAINTENANCE AND SPREAD OF AVIAN INFLUENZA VIRUSES IN INDONESIA

Submitted by

Kristy L. Pabilonia

Department of Microbiology, Immunology and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2012

Doctoral Committee:

Advisor: Richard Bowen

Tawfik Aboellail
Doreene Hyatt
Anthony Knight

ABSTRACT

THE ROLE OF DOMESTIC DUCKS IN THE MAINTENANCE AND SPREAD OF AVIAN INFLUENZA VIRUSES IN INDONESIA

Wild waterfowl and aquatic birds serve as the natural reservoir host for influenza A viruses. As the reservoir, wild waterfowl play an important role in the persistence and transmission of influenza viruses among bird populations and to other mammalian species. In many Asian countries, domestic ducks are raised for meat and egg production. Some of these domestic ducks are ranged on rice paddies or post-harvest rice fields. The ducks provide service to the rice fields by fertilizing the field with feces and aerating the field by swimming and walking through the ground cover. Additionally, the ducks serve as a form of insect control through their natural grazing behaviors. The role that domestic ducks play in the ecology of influenza viruses is poorly understood.

Highly pathogenic avian influenza H5N1 virus (HPAI H5N1) originated in Guangdong Province, China in 1996, which was followed by global dissemination of the virus that began in 2003. This virus is unprecedented in geographical spread, economic consequences and public health significance. At the present time, HPAI H5N1 virus is endemic six countries, including Indonesia. Indonesia has experienced the highest incidence of human infections with HPAI H5N1 virus and one of the highest case fatality rates. Control of the virus in Indonesia has proven extremely challenging, due to its diverse and complex poultry and domestic duck production systems.

HPAI H5N1 virus is highly virulent in chickens and turkeys and causes severe systemic disease. Outbreaks of HPAI H5N1 in poultry populations are accompanied by high mortality. In contrast, HPAI H5N1 virus is typically nonpathogenic or mildly pathogenic in ducks and mortality in duck flocks during outbreaks of the virus is absent or limited. This allows ducks to serve as silent carriers of the virus, as they may shed large quantities of virus without displaying clinical signs of illness allowing infected ducks to evade detection by flock owners or government livestock officials.

Domestic duck production is common in Southeast Asia. Indonesia has a large domestic duck population, estimated at more than 34 million ducks. Because HPAI H5N1 induces only mild disease in domestic ducks, outbreaks of the virus are difficult to detect and are rarely reported by domestic duck flock owners. Thus, domestic duck flocks have been left out of government HPAI H5N1 surveillance and control programs. While a number of studies have demonstrated that the presence of domestic ducks in a country or at a specific location may be a risk factor for the presence of HPAI H5N1 virus, few studies have been conducted evaluating the role that domestic ducks play in the ecology of HPAI H5N1 virus.

The objectives of the studies described in this dissertation were to elucidate the role of domestic ducks in the maintenance and spread of avian influenza viruses, particularly HPAI H5N1 virus, by evaluating domestic duck flock characteristics and behaviors, estimating the prevalence and incidence of avian influenza viruses in these flocks and characterizing HPAI H5N1 viruses detected in the field. To meet the objectives, two studies were conducted in West Java, Indonesia. The first study was a cross-sectional study aimed at characterizing domestic duck flocks and estimating the point prevalence and seroprevalence of avian influenza viruses, particularly HPAI H5N1 virus. This study was followed by a 7 month longitudinal study, aimed

at estimating the incidence of avian influenza viruses, particularly HPAI H5N1 virus, in domestic duck flocks and evaluating flock illness and mortality during avian influenza virus outbreaks. A subset of samples from each of the studies was transported to the United States for virus characterization.

The findings of the studies conducted demonstrate that domestic duck flocks are raised in complex production systems, are highly mobile, have significant contact with wild and domestic birds and mammals, are frequently ill and are provided with little formal veterinary care. The prevalence and incidence of avian influenza virus, including HPAI H5N1 virus, are high in domestic duck flocks in Indonesia. Clinical signs of illness and increased mortality did not correlate with the presence of avian influenza virus in the flock. Interestingly, there was also no correlation between increased flock mortality and the presence of HPAI H5N1 virus in the flock, demonstrating that domestic duck flocks can be asymptomatically infected with HPAI H5N1 virus while shedding high quantities of virus. Characterization of some of the viruses isolated from domestic duck flocks demonstrated that the flocks can be infected with more than one avian influenza virus at one time, as demonstrated by one flock that was positive for HPAI H5N1, as well as H3 and H7 avian influenza viruses. These situations are concerning, as domestic duck flocks may serve as mixing vessels for avian influenza viruses and co-infections in these flocks may result in the emergence of novel influenza viruses that may have capabilities for human-to-human transmission.

It is likely that domestic ducks play an important role in the maintenance and spread of avian influenza viruses, including HPAI H5N1 virus. A number of domestic duck flock practices, including extensive flock movement, frequent introduction and sale of ducks, free-ranging of ducks in areas where they have contact with wild birds and animals and continual

contact of duck flocks with other duck and poultry flocks, increasingly adds to the difficulty of control of HPAI H5N1 virus within this production system and makes eradication of the virus within a country extremely challenging.

TABLE OF CONTENTS

Chapter 1

Introduction

Domestic Ducks and Avian Influenza Viruses

Influenza A viruses are globally important human and animal pathogens. Waterfowl and shorebirds serve as the reservoir for influenza A viruses. As the natural host, these aquatic birds are asymptomatic or show mild clinical disease when infected with influenza viruses. In 1996, a novel highly pathogenic avian influenza H5N1 virus (HPAI H5N1) emerged in China. This virus is highly lethal in poultry; however, infection of most waterfowl species is characterized by mild clinical illness and limited mortality.

Domestic duck production is common in Asia. Domestic duck flocks are used for meat and egg production and these food products are widely distributed throughout Asia and other parts of the world. Additionally, domestic duck flocks may be used to service rice paddies. As they free range on the post-harvest rice fields, they eat the insects and left behind rice grains, fertilize the fields with fecal material and aerate the fields through walking and swimming on the ground cover.

Because ducks may show only mild clinical illness when infected with avian influenza viruses, including HPAI H5N1, infected flocks may evade detection by flock owners and government livestock officials. Consequently, domestic duck flocks are often neglected by government avian influenza prevention and control programs. In addition, domestic duck flocks and their food products are moved extensively in many parts of Asia, providing opportunities for dissemination of avian influenza viruses and hindering global control of avian influenza.

HPAI H5N1has remained endemic in parts of Southeast Asia, including Indonesia, for more than a decade. The role that domestic ducks play in the maintenance and spread of avian influenza viruses, particularly HPAI H5N1, is poorly understood. The aims of this dissertation are to characterize domestic duck flocks in Indonesia, determine the prevalence and seroprevalence of avian influenza viruses in these flocks, and elucidate the role of domestic duck flocks in the maintenance and spread of avian influenza viruses, including HPAI H5N1.

Influenza A Virus

Classification and Nomenclature

Influenza viruses belong to the family Orthomyxoviridae. This family of viruses is composed of three influenza genera, classified as Influenza A, B and C, and two non-influenza genera. Influenza A viruses can infect humans, birds and mammals. Influenza B and C viruses are primarily human pathogens and rarely infect avian and non-human mammalian species.

Influenza A viruses possess a negative-sense, single-stranded, segmented RNA genome. The virus is composed of eight gene segments that encode ten known viral proteins – hemagglutinin (HA) and neuraminidase (NA), which serve as surface proteins, matrix (M, also known as M1) protein, nucleoprotein (NP), two nonstructural proteins (NS1 and NS2), membrane ion channel proteins (M2) and the polymerase complex, which is composed of polymerase basic proteins (PB1, PB2) and the polymerase acidic protein (PA) (47).

Influenza A viruses are subtyped based on their HA and NA surface proteins. Currently, there are 16 known HA subtypes and 9 known NA subtypes, providing 144 possible HA and NA subtype combinations. Many diverse virus strains exist within each subtype classification and thousands of genetically distinct viruses have been characterized. The virus subtype is included

as part of the descriptive nomenclature for all influenza viruses, along with the influenza virus type (A, B or C), host animal (omitted for humans), location of origin of the isolate, laboratory reference number and year of isolation (102).

Antigenic Drift and Shift

Antigenic shift is the result of the reassortment of two influenza virus gene segments infecting the same cell. This type of significant change can result in the generation of a novel virus and was involved in the generation of the influenza viruses that caused the pandemics of 1957 and 1968. Antigenic drift is the result of point mutations in the genome occurring over time and results in slow, progressive virus evolution. It is theorized that antigenic drift, in the face of selective pressures, may generate viruses with a genetic advantage (53).

Molecular Determinants of Pathogenesis

The hemagglutinin gene of influenza A viruses is the primary determinant of pathogenicity in avian and mammalian species (7). The hemagglutinin surface glycoprotein is responsible for binding to host cell receptors containing sialic acid bound to glycoproteins. Avian influenza viruses preferentially bind to α-2,3 linkage receptor types present in avian respiratory epithelial cells, while human influenza viruses preferentially bind to the predominant α-2,6 linkage receptor types present in human respiratory epithelial cells. Recently, studies have demonstrated that humans also have small amounts of α-2,3 linkage receptor types, making them susceptible to certain avian influenza viruses, including HPAI H5N1 virus (61, 62). Swine influenza viruses will bind to both receptor types. Swine have both receptor types in significant

numbers so are susceptible to infection with not only swine influenza viruses but also human and avian influenza viruses (57).

Cleavage of the HA protein into HA1 and HA2 is essential for fusion of the virus with the endosome and, thus, viral infectivity. For low pathogenic avian influenza viruses, the HA protein is cleaved by trypsin-like proteases, which are located in intestinal and respiratory epithelial cells, resulting in specific tissue tropism for these viruses. Highly pathogenic avian influenza viruses are cleaved by the trypsin-like proteases, as well as ubiquitous furin-like proteases that are present throughout the cells of the visceral organs, cardiovascular system and nervous system, allowing for systemic spread of these viruses (74, 80).

Alterations of the hemagglutinin structure, particularly at the HA cleavage site, may confer a change in virulence. The substitution of non-basic with multiple basic amino acids of the cleavage site of an H5 or H7 virus can result in increased virulence and transformation of the virus from low pathogenicity to high pathogenicity for poultry (74, 84). A number of recent studies have demonstrated that a motif of multibasic amino acids around the hemagglutinin cleavage site in HPAI H5N1 viruses also can act as a virulence factor in mammals. The substitution of even one amino acid can result in a significant increase in virulence (83, 101, 120).

Host Range

Influenza A viruses can infect humans, birds and a wide variety of mammalian species, including pigs, horses, dogs, mink, dogs, cats, whales and seals. Influenza A viruses have been isolated from a wide variety of avian species, including chickens, turkeys, upland game birds, caged pet birds and wild birds representing most of the major Families of birds throughout the

world. Waterfowl and other aquatic birds serve as the viral reservoir and the influenza viruses isolated from this group of birds are extremely diverse and are globally distributed (3). In a review of influenza virus prevalence in aquatic birds, virus was isolated from 15.2% of birds of the Order Anseriformes (waterfowl), followed by the Passeriformes (perching birds) and Charadriiformes (shorebirds and gulls), with isolation rates of 2.9% and 2.2%, respectively (96).

Pathobiology in Poultry

Introduction of Avian Influenza Viruses to Poultry

Wild birds serve as the source of primary introduction of avian influenza viruses to poultry. After introduction, the virus may become poultry-adapted, improving its capabilities for replication and transmission in poultry. Poultry are exposed to avian influenza viruses via a number of routes, including direct and indirect contact with wild birds, consumption of contaminated water or feed and introduction of the virus through fomites, particularly introduction of the virus through mechanical movement by humans (3, 90).

Avian Influenza Virus Pathotyping

Avian influenza viruses are classified as one of two pathotypes – highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI) – based on pathogenicity in an experimental setting and molecular analysis of the virus. HPAI viruses have an intravenous pathogenicity index (IVPI) of 1.2 or greater in 6 week old birds or cause mortality of 75% or greater in 4 to 8 week old chickens. HPAI viruses also have multiple basic amino acids at the hemagglutinin cleavage site, providing a different template for cleavage by proteases. This motif allows for cleavage by systemic furin-like proteases, resulting in systemic replication of the

virus. To date, only viruses within the H5 and H7 subtypes have been classified as HPAI viruses; however, not all H5 and H7 viruses are highly pathogenic in nature, as some are classified as low pathogenic viruses. Interestingly, low pathogenic H5 and H7 viruses have the potential to mutate to become highly pathogenic (3, 80).

Pathogenesis in Poultry

HPAI viruses cause severe systemic disease accompanied by high mortality in chickens, turkeys and other gallinaceous birds. Infection occurs via the respiratory tract, where the virus initially replicates in epithelial cells. The virus then disseminates through the vascular and lymphatic systems to multiple organs. The virus may replicate in the parenchymal cells of various organs, resulting in necrotic and apoptotic cellular death, followed by inflammation and multi-organ failure. Conversely, the virus may instead replicate in the endothelial cells, resulting in damage to the blood vessels and increased permeability with edema, hemorrhage, microthrombosis and multiorgan failure (49, 102).

Highly Pathogenic Avian Influenza H5N1 Virus

Origin

The precursor to the Asian-origin HPAI H5N1 virus that circulates today emerged in Guangdong Province, China in 1996. The virus was atypical in that it caused a mortality rate of 40% in geese, as previously identified avian influenza viruses are not associated with high mortality in waterfowl (117). Guangdong Province is adjacent to Hong Kong and produces considerable numbers of poultry for the Hong Kong live bird markets, which were the location of HPAI H5N1 virus emergence in 1997. On initial detection, the virus was isolated from 20% of

the chickens sampled in the live bird market, indicating that the virus had circulated for some time prior to detection. With a mortality rate varying between 75 - 100% in chickens, this virus is one of the most lethal avian influenza viruses ever detected (85-87). The outbreak was controlled through the unprecedented mass depopulation of more than 1.5 million birds in the live bird markets (87).

Global Dissemination

In the five years following the successful control of HPAI H5N1 virus in the Hong Kong live bird markets, circulation of the virus was limited, likely to domestic duck and goose flocks in Southern China. Due to the asymptomatic nature of the virus infection in domestic waterfowl and due lack of disease surveillance in China during that period, few detections of HPAI H5N1 virus were recorded from 1998-2003. Then, from December 2003 to January 2004, almost simultaneous outbreaks of HPAI H5N1 virus were reported in the eight neighboring Asian countries of China, Cambodia, PDR Laos, Thailand, Vietnam, Korea, Japan and Indonesia (86). This event was the start of the most globally significant avian influenza virus outbreak identified to date. HPAI H5N1 virus is unprecedented in geographic dissemination, public health significance and economic consequences. To date, sixty three countries in Asia, Africa and Europe have reported outbreaks of HPAI H5N1 virus in poultry and wild birds since the beginning of the epizootic in 2003. The virus remains endemic in China, Indonesia, Egypt, Vietnam, India and Bangladesh (23).

Unlike other avian influenza viruses, direct transmission of HPAI H5N1 virus from birds to mammals (other than pigs) has been demonstrated, both naturally and experimentally,

including transmission to felids (domestic cats and large zoo cats), dogs, ferrets, mice and other small mammals (42, 114).

Public Health Impacts

The 1997 outbreak of HPAI H5N1 virus accounted for the first detection of direct transmission from birds to humans, without prior reassortment with a human virus. In total, there were 18 human cases with 6 deaths (16, 68, 100). HPAI H5N1 virus is unique, as compared to other avian influenza viruses, based on its ability to infect certain mammalian species, including humans, without adaptation (75). The HPAI H5N1 virus outbreak in Hong Kong in 1997 led to research that demonstrated that while humans have predominantly α-2,6 receptor linkages in respiratory epithelium, α-2,3 linkages are also present, although in low numbers. The presence of these avian-type receptor linkages allows for infection of human airway epithelium, although virus replication is limited by less than optimal cellular tropism (61, 62).

The World Health Organization reports 603 human cases with 356 deaths from 2003 to May 2012 (116). Based on these case numbers, the current case-fatality rate of HPAI H5N1 is approximately 60%. However, this may be an overrepresentation, as subclinical human infections go undetected. A number of studies have been conducted in Asian countries that assess the level of subclinical infections by evaluating seroconversion to HPAI H5N1 virus. In these studies, overall seroprevalence was low – 2.61% in poultry workers in China (36), 2.6%, 1% and 0% in three studies of villagers in Cambodia (12, 109, 110) and 3.5% of villagers in Thailand (43). The results of these studies indicate that zoonotic transmission of HPAI H5N1 virus is infrequent; although any level of human infection with HPAI H5N1 should be

considered significant. Interestingly, direct contact with poultry or working with poultry did not consistently correlate with a higher risk for infection, which is consistent with current information on documented human cases, indicating that many human exposures may result from indirect contact with poultry or contact with contaminated environments or fomites. To date, HPAI H5N1 virus has not developed the capacity for sustained human-to-human transmission, as the virus has not acquired the ability to efficiently bind to the predominant α-2,6 receptor types of human respiratory epithelial cells (61, 62).

With the exception of HPAI H5N1 virus, transmission of avian influenza viruses to mammalian species other than pigs is rare. Viral replication is usually limited and does not result in overt disease in mammalian hosts.

Evolution of Asian-Origin Highly Pathogenic Avian Influenza H5N1 Virus

The progenitor virus for currently circulating Asian-Origin HPAI H5N1 viruses is A/goose/Guangdong/1/1996. Since the emergence of this virus in 1996, HPAI H5N1 viruses have significantly evolved and continue to evolve over time. So far, 20 distinct clades of the HPAI H5N1 virus have been identified. Of the 20 clades, 13 have become inactive and are no longer detected. Due to significant divergence of the HA gene, second-, third- and fourth-order clades have been assigned to further differentiate viruses. The currently circulating clades include clade 1 in the Mekong River Delta, 2.1.3 in Indonesia, 2.2 in India and Bangladesh, 2.2.1 in Egypt and clades 2.3.2, 2.3.4 and 7 in Asia (27).

Figure 1.1 represents the phylogenetic relationships of H5N1 viruses diverging from A/goose/Guangdong/1/1996, based on HA genotype. The system of nomenclature for HPAI H5N1 viruses was developed by the WHO/OIE/FAO H5N1 Evolution Working Group. The

phylogenetic tree was developed by this working group and updated in 2011 (27, 28). The inactive clades are collapsed on the phylogram.

Indonesian lineage HPAI H5N1 viruses belong to clade 2.1.3 (52, 66). Evidence suggests that this lineage originated from a single introduction of HPAI H5N1 virus into East Java between November 2002 and October 2003. The initial introduction was followed by a rapid expansion of viral genetic diversity then steady state genetic evolution (52). Significantly divergent Indonesian lineage viruses have not been recently detected.

Because HPAI H5N1 viruses are very dynamic and continually changing, continual evolution and emergence of new virus clades is expected. Monitoring of viruses is critical in order to detect antigenic drift and shift and the emergence of viruses with pandemic potential. In a number of countries including China, Egypt and Vietnam, HPAI H5N1 viruses have evolved considerably and newly emerged reassortant HPAI H5N1 viruses are increasingly virulent in ducks and other mammalian species (27, 56).

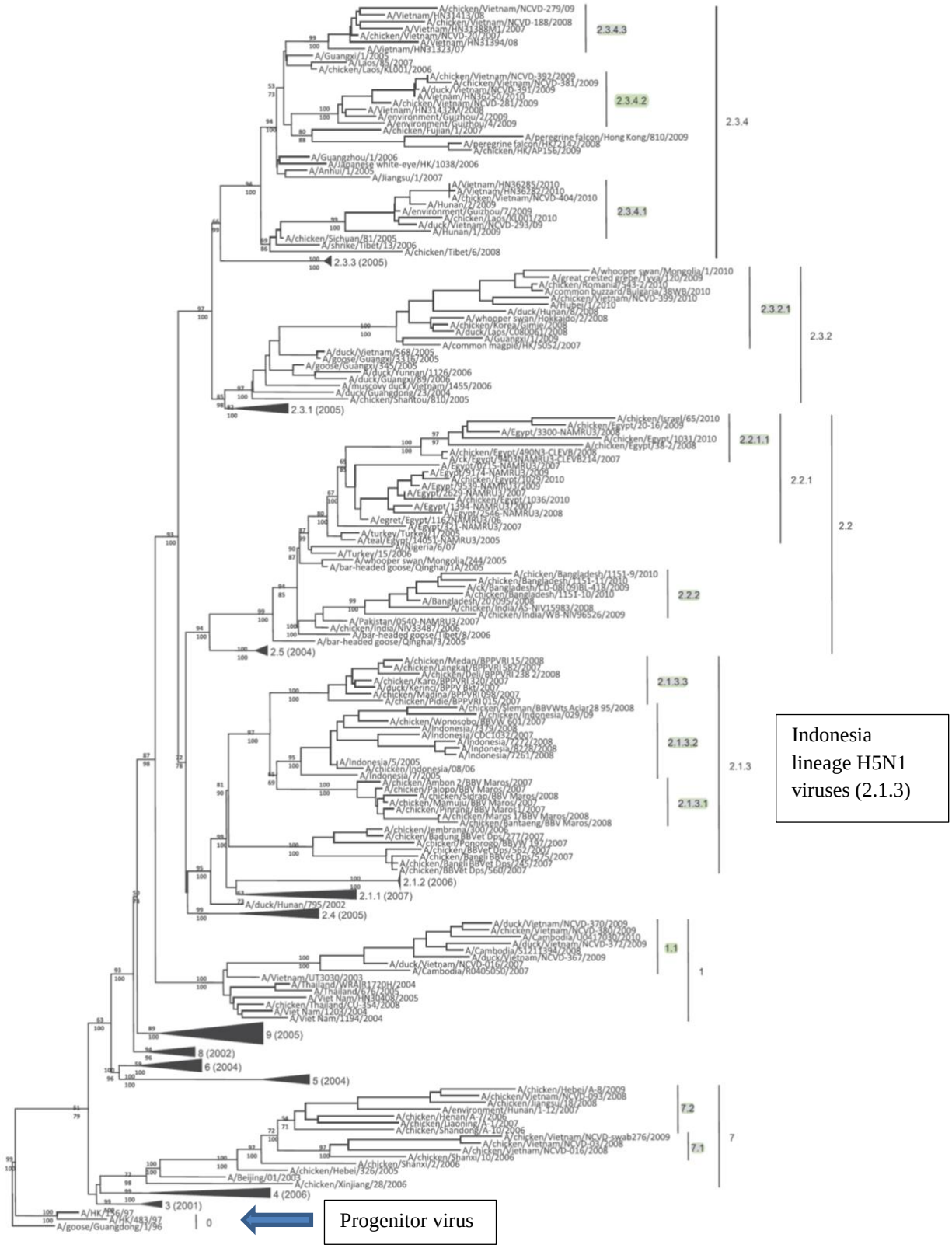

Figure 1.1 – Phylogenetic relationships of Asian-origin HPAI H5N1 virus

Pathobiology of Avian Influenza Viruses in Ducks

Ecology in Ducks

Ducks and other aquatic birds are the natural reservoir for influenza A viruses. As such, influenza A viruses are typically nonpathogenic or mildly pathogenic in these birds and usually do not cause death (40, 78, 91). Other than HPAI H5N1, avian influenza viruses primarily replicate within the intestinal tract of ducks, are shed in high levels in the feces and are transmitted through the fecal-oral route, although some replication in the respiratory epithelium and shedding from the respiratory tract may occur (34, 115). However, despite the virus replication in the respiratory and intestinal epithelia of infected ducks, the virus is minimally virulent in the reservoir host and does not cause appreciable gross or histologic lesions (17).

Pathobiology of Asian-Origin Highly Pathogenic Avian Influenza H5N1 Virus in Ducks

Prior to the emergence of HPAI H5N1 virus, the only documented avian influenza virus reported to cause significant clinical illness and mortality in ducks was an HPAI H7N1 virus outbreak in Italy in 1990-2000. During this outbreak, neurologic illness and death were reported in Muscovy ducks and geese in a backyard flock (9). In the few years following the initial HPAI H5N1 virus outbreak in Hong Kong in 1997, the virus produced only mild respiratory infection in ducks. The pathobiology of the virus began to change in 2001, when the virus reassorted with other aquatic avian influenza viruses and become more virulent in ducks (29). In 2002, two Hong Kong bird parks reported mortality in waterfowl and other wild avian species caused by the HPAI H5N1 virus (21, 98). At one of the parks, approximately 40% of the waterfowl displayed clinical signs of neurological illness, including paresis, paralysis, tremors and head tilt. Overall, almost 45% of the ducks died during the outbreak (21).

As the HPAI H5N1 virus evolved, many of the virus strains became increasingly pathogenic in ducks, with some producing severe systemic disease and high mortality. Clinically, HPAI H5N1 viruses can cause severe neurological disease and depression in ducks. Experimental studies have demonstrated that the virus has a pantropic potential, primarily targeting the respiratory tract, brain, heart, pancreas and adrenal glands (70, 71, 75). Virus replicates to a lesser extent and with less consistency in the liver, kidneys and skeletal muscle (5, 71). Within these organs, the HPAI H5N1-infected cells undergo necrosis and apoptosis, resulting in severe organ dysfunction and failure (102). The intestinal tract, the site of replication of low pathogenic avian influenza viruses (34, 115), is not consistently affected (71). Vascular endothelial replication and vascular damage, resulting in pulmonary edema, congestion, hemorrhage and microthrombosis of capillaries, is typical of HPAI infections of gallinaceous species but is limited or not observed in ducks (71). The capability of the virus to effectively replicate in the brain is likely the most important element in induction of morbidity and mortality in ducks and other avian species (75, 98). Pathogenesis is likely the result of a combination of a number of factors, including neurologic dysfunction, multiorgan failure, myocardial conduction disruption or insufficiency and systemic cytokine release (70). Other factors, such as flock environment, amount of viral exposure and concurrent infections, likely influence clinical presentation and mortality (71).

In experimental studies, ducks shed HPAI H5N1 virus primarily from the oropharynx, and to a lesser extent, the cloaca, for up to 17 days post infection (6, 35, 70, 77, 98). Peak viral shedding typically occurred around days 2 to 3 post infection (46, 50). HPAI H5N1 virus is shed at high titers and for a longer duration than LPAI viruses and other HPAI viruses. Ducks are more susceptible to infection with HPAI H5N1 via respiratory exposure, which requires a lower

infectious dose as compared to alimentary exposure (50). Interestingly, replication of HPAI H5N1 virus in the feather epidermal cells of ducks has also been demonstrated, suggesting that feathers are potential sources of virus transmission and environmental contamination (118, 119).

There is significant variation in pathogenicity, from nonpathogenic to highly lethal, of the circulating HPAI H5N1 viruses in ducks. Many reports of asymptomatically infected domestic ducks have demonstrated that HPAI H5N1 viruses are not always highly pathogenic in a field situation (35, 99). In 2004, surveillance programs in live bird markets in Southern China revealed that approximately 25% of clinically normal ducks in the markets were positive for HPAI H5N1 virus (54). However, recent studies have shown that some of the strains of HPAI H5N1 virus, including the Vietnamese strain and Egyptian strain, are becoming progressively more virulent in ducks over time, causing severe morbidity and mortality in ducks (6, 77, 112). On the contrary, the Indonesian strain remains mildly pathogenic in ducks, causing mild neurologic disease and minimal mortality. In experimental studies, there was less viral antigen detected in tissues, including brain, heart, lung and pancreas, in ducks infected with Indonesian strains versus ducks infected with Vietnamese strains (6). These data suggest that the Vietnamese and Egyptian strains of HPAI H5N1 virus may be more adapted to ducks than the Indonesian strain.

A number of studies have demonstrated that the age at infection affects the pathogenicity of the virus, with older ducks experiencing progressively milder clinical illness and less mortality. Three studies by Pantin-Jackwood, et al. demonstrated that the difference in age at inoculation with four different HPAI H5N1 viruses between 2 week and 5 week old ducks was significant, with the 2 week old ducks experiencing severe neurologic illness and depression with high mortality and the 5 week old ducks experiencing moderate neurological illness and

mortality. Additionally, the length and amount of viral shedding from the oropharynx of younger ducks was greater than older ducks. The variation in age-related outcomes may be due to a number of factors, including increasing maturation of the immune system with age and an association between host cell maturation and capabilities for viral replication (69-71).

Highly Pathogenic Avian Influenza H5N1 Virus in Domestic and Wild Bird Populations

Village Poultry

Poultry production is an extremely common and rapidly growing food production system throughout the world, particularly in developing countries. Because poultry species, including chickens, turkeys, ducks, geese, pheasants and quail, are cost-effective food production systems, many countries rely on poultry as a primary protein source. Poultry species are often kept in small holder settings (village-based production) and are sold through complex movement and marketing systems. This type of production system promotes the spread of infectious diseases and makes disease control challenging. The frequent introduction of and/or persistence of HPAI H5N1 virus in village poultry populations has been demonstrated in many countries in Asia and Africa (19, 58, 109, 110). Movement of poultry through legal and illegal trade plays a key role in virus dissemination (86) and movement of poultry has been shown to be a risk factor for HPAI H5N1 virus introduction (19). Endemnicity of HPAI H5N1 virus in Indonesia has been demonstrated in a number of surveys, including a survey of village poultry in Indonesia during 2006 and 2007, which determined that 8.8% of 2310 villages surveyed were positive for HPAI H5N1 virus (58).

Poultry Products

Duck eggs and duck meat are a popular commodity in many countries, including many parts of Asia, where duck eggs and meat are favored over chicken eggs and meat and sell for a higher price in live bird markets. As potential asymptomatic carriers of the HPAI H5N1 virus, ducks should be a particular focus for surveillance programs; however, they are unfortunately often excluded from programs as their HPAI H5N1 virus infections often go unnoticed and undetected.

The legal and illegal trade of poultry food products has been implicated as a source of disease introduction and potential route of zoonotic transmission to humans. In 2006, HPAI H5N1 virus was detected in shell washes from waterfowl eggs confiscated from Vietnamese travelers (55). Experimental studies in ducks have demonstrated that HPAI H5N1 viruses can be recovered from duck skeletal muscle post-experimental infection (71, 104). In a natural setting, HPAI H5N1 virus was isolated from duck meat imported from China to South Korea (104).

Live Bird Markets

Many developing countries have extensive live animal marketing systems which provide inexpensive, easy to produce, readily available protein products to local populations. These marketing systems are found throughout Asia and are particularly widespread in Southeast Asia. Significant efforts have been focused on evaluating the live bird markets, also known as wet markets, due the unique environment they provide for disease introduction and maintenance. A number of their production practices have been implicated as a source of virus introduction and persistence, including the daily introduction of birds and other mammals, carryover of animals from one day to the next, housing of animals for long periods of time, poor cleanliness and

sanitation, overcrowding of cages, housing of various bird and mammal species in close proximity or in the same cages, congregation of animals in a small area, and general poor health of animals in the markets (24, 86, 113).

HPAI H5N1 virus has been detected in live bird markets in China (15, 111), Egypt (1), Indonesia (37), Vietnam (18) and Thailand (4). In Egypt, 12.4% of the live bird markets examined in one study were positive for HPAI H5N1 virus. When broken down by market type based on species sold, live bird markets that only sold waterfowl had a prevalence of 70.4%, demonstrating that domestic waterfowl play an important role in the persistence of HPAI H5N1 virus in the market system (1). In China, one study elucidated the link between human infection with HPAI H5N1 virus and live poultry markets by demonstrating that the viruses detected in markets were genetically highly similar to the viruses detected in HPAI H5N1 positive human patients (111). This study established that live bird markets are an important source of human exposure to the HPAI H5N1 virus. In Indonesia, a recent study determined that nearly 50% of the live bird markets were positive for HPAI H5N1 virus. Risk factors were analyzed and markets with Muscovy ducks (heavy-bodied meat ducks) and/or more than 200 ducks other than Muscovys had a significantly higher risk for environmental contamination with HPAI H5N1 virus. Daily removal of waste was a protective factor, demonstrating the importance of market cleanliness (37).

Wild Birds

The first large-scale outbreak of HPAI H5N1 occurred at Qinghai Lake in western China in 2005 (14). Prior to this outbreak, detections of HPAI viruses in migratory birds were limited. Over the course of the outbreak, 6184 dead gulls, geese, cormorants, shelducks, swans and

cranes were discovered at Qinghai Lake. This geographical area serves as an aggregation and breeding site for many bird species (13). This magnitude of this outbreak was of great concern due to the potential for spread of HPAI H5N1 through migratory birds. Additionally, this outbreak demonstrated the capabilities of the circulating HPAI H5N1 virus to cause severe clinical disease in the aquatic bird viral reservoir. Since this outbreak, HPAI H5N1 virus has been detected in wild birds in Asia, Africa and Europe and the dissemination of the virus to poultry populations in a number of outbreaks has been linked to movement of infected wild birds (26, 81, 82, 97).

Domestic Ducks

Studies in Vietnam, Indonesia and Thailand have demonstrated HPAI H5N1 prevalence and seroprevalence in domestic duck populations, which varied based on country, H5N1 outbreak status and duck production systems (25, 32, 33, 89). In Thailand, 45.9% of free-ranging duck flocks sampled during an HPAI H5N1 outbreak were positive (89). In Vietnam, domestic duck flocks were sampled during a period when there were no reported outbreaks of HPAI H5N1 in the country. In this study, flock-level virus prevalence was low (0.7%) but flock-level seroprevalence indicating past exposure to HPAI H5N1 with recovery was high, with 43% of ducks testing positive for antibodies to HPAI H5N1 virus (32). In Indonesia, 2.5% of domestic duck flocks sampled were positive for H5-subtype virus. The overall flock-level seroprevalence to HPAI H5N1 was 19.5% (33). These results demonstrate that domestic duck flocks may be important in the maintenance of HPAI H5N1 virus and may serve as a source of viral dissemination.

Avian Influenza Virus Diagnostic Assays

Virus Isolation

Virus isolation in embyronated eggs is considered the gold standard method for detection of avian influenza viruses (67). Specific-pathogen-free embyronated chicken eggs are used to propagate virus, followed by a viral detection assay (48, 64). Hemagglutination assays are often used to detect hemagglutinating virus present in the amniotic allantoic fluid or yolk sac fluid harvested from the inoculated eggs. However, these assays are not specific to avian influenza viruses as other infectious agents also have hemagglutinating capabilities (45).

Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR)

Over the past decade, the rRT-PCR has become the test of choice for the rapid and accurate detection of avian influenza viruses. The advantages of rRT-PCR over virus isolation include faster turnaround time (4 hours versus 3-10 days) and ease of performance. Studies evaluating rRT-PCR performance as compared to virus isolation have demonstrated a high level of correlation between the assays (92, 93). Influenza A virus rRT-PCR assays can be designed to detect highly conserved sections of the genome, such as the matrix gene, in order to detect all influenza A viruses with a high level of sensitivity and specificity (95). Subtype-specific assays can be used to differentiate viruses based on hemagglutinin or neuraminidase subtype (93, 94) but genetic variation must be monitored to ensure that the assay is detecting the target viruses.

Sampling of Ducks for rRT-PCR

Individual and pooled cloacal and oropharyngeal swab samples have been used as the primary sample type for detection of avian influenza virus by rRT-PCR (44, 51). The optimal sample type for testing ducks, cloacal or oropharyngeal swab, has been the subject of much debate. Traditionally, cloacal swab samples have been collected from ducks, based on tropism of avian influenza viruses for the gastrointestinal tract (34, 88, 115). However, HPAI H5N1 virus has been detected at higher viral titers in oropharyngeal swab samples, as this virus has an increased tropism for the respiratory system (46, 70, 71).

A number of studies have been conducted to determine the best sample type for detection of avian influenza viruses in ducks and have yielded different conclusions. Jindal, et al. detected a large number of LPAI viruses in oropharyngeal samples alone (39), while Ellstrom, et al. found that detection of LPAI viruses was higher in cloacal samples than oropharyngeal samples (22). The work performed by Ip, et al. demonstrated that a combined cloacal and oropharyngeal sample yielded the highest recovery of LPAI viruses (38). These results demonstrate that while LPAI viruses have a predilection for the gastrointestinal tract, viral replication and shedding in the respiratory system does occur, making the collection of both cloacal and oropharyngeal swab samples an important consideration for detection of LPAI viruses. Furthermore, as most surveillance programs are designed to lead to the rapid detection of HPAI viruses, the significance of collecting oropharyngeal samples should not be disregarded.

Serological Assays

Three serological assays, hemagglutination-inhibition (HI), enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID), are commonly used

serological assays for the detection of influenza virus antibodies in avian serum. The HI test is the classical laboratory technique for the detection of subtype-specific antibodies. The HI assay is rapid and sensitive; however, it requires a panel of optimized reference antigens that are not widely available outside of OIE avian influenza virus reference laboratories (72). Conversely, homologous virus can be used for antibody detection for specific outbreaks. The AGID assay is widely used in the United States for poultry surveillance programs but the assay is unreliable in ducks for two reasons. First, the AGID reagents produced in the United States are made in embryonated chicken eggs and duck antibodies bind less efficiently to chicken-adapted avian influenza viruses. Second, the AGID assay targets antibodies to influenza nucleoprotein (NP) and ducks do not produce significant levels of antibodies to NP (80, 102). Commercially available blocking ELISAs are newly available and recent studies have demonstrated that these assays are similar in sensitivity to the HI assay when used for testing duck sera but have the advantage of practicality and ease of use (2, 92).

Zoonotic Transmission of Avian Influenza Virus to Humans

Prior to 1997, there were three documented cases of zoonotic transmission of avian influenza virus to humans, with limited illness. Starting with the HPAI H5N1 outbreak in 1997, an increase in zoonotic transmission of avian influenza viruses was detected. Five viral subtypes, HPAI H5N1, HPAI and LPAI H7N3, HPAI and LPAI H7N7, LPAI H9N2 and LPAI H10N7, have been documented to cause sporadic human infection via direct transmission of the virus from birds to humans. Only HPAI H5N1 and HPAI H7N7 have been documented to cause death, with HPAI H7N7 resulting in only one death to date (41, 60). Subclinical infection with seroconversion to a number of avian influenza virus subtypes, including LPAI subtypes, has also

been demonstrated (36, 43, 65). While only HPAI H5N1 virus has been a significant cause of mortality, all avian influenza viruses have the potential evolve to become readily transmissible to and highly virulent for humans.

Control of Asian-Origin Highly Pathogenic Avian Influenza H5N1 Virus

Control Strategies

The primary strategies used for control of HPAI viruses involve depopulation of affected and at-risk flocks, movement controls and increased biosecurity practices. These strategies have been effective in controlling HPAI outbreaks in countries with industrialized poultry populations and significant government outbreak response capabilities (31, 59, 73, 103, 108). However, their use in village poultry flocks in developing counties is limited due to the decreased ability to locate flocks, control movement and enforce biosecurity procedures, as well as diverse poultry populations and insufficient government capacity for disease response. These issues make control of HPAI H5N1 virus in developing countries a significant challenge.

Vaccination

Vaccination programs have been used effectively in a number of countries to aid in the control of outbreaks of HPAI virus (10, 107). However, these vaccination campaigns have been directed at controlling HPAI virus in chickens and turkeys. The efficacy and applicability of vaccination use in domestic ducks for control of HPAI has only been minimally evaluated. Experimental studies evaluating the efficacy of vaccines against HPAI H5N1 virus in domestic ducks have provided inconsistent results and are highly dependent on the vaccine developed, the challenge virus used and age of the ducks at vaccination and inoculation. Some studies have

demonstrated that vaccination of domestic ducks against HPAI H5N1 virus is highly efficacious, resulting in significantly decreased to absent morbidity, mortality, clinical illness and viral shedding (46, 63, 106). Others have demonstrated only moderate efficacy, without significant decreases in viral shedding (20, 76, 79, 105). One study demonstrated that different domestic duck species respond significantly differently to the same vaccine, making analysis of a vaccine in an experimental setting only partially applicable to a field situation where many different species of domestic ducks are produced (8). These mixed results confound the assessment of the use of vaccination in domestic ducks in the field. Additionally, in a field situation, many other factors exist that may impede vaccine efficacy.

There are a number of other challenges to the use of vaccinations for the control of HPAI H5N1 virus in domestic ducks. First, the use of antigenically similar vaccines is important to establishing a highly efficacious vaccine, which is difficult with HPAI H5N1 virus due to continual antigenic shift and drift. Second, even with highly efficacious vaccines, such as a homologous virus inactivated vaccines, viral shedding may continue to occur. This makes virus detection difficult, as the ducks may not show clinical signs of illness and experience no or low increases in mortality, in essence concealing the outbreak; however, the ducks continue to shed and transmit virus. Lastly, the use of vaccinations has been implicated in encouraging viral evolution due to selection of escape mutants. A study by Cattoli, et al. evaluated HPAI H5N1 evolutionary dynamics between countries using and not using vaccination programs in poultry. They detected increased HPAI H5N1 virus evolution rates and higher virus populations circulating in countries that had applied vaccination programs (11). Therefore, while the use of vaccination programs may aid in the control of HPAI H5N1 virus, highly efficacious vaccines

should be used and applied appropriately. Additionally, continual monitoring of circulating viruses should be conducted and changes in vaccines used should coincide with virus evolution.

Control of HPAI H5N1 Virus in Domestic Ducks in Indonesia

The current speculation on the link between HPAI H5N1 endemnicity and domestic duck production has gained momentum, as more than 70% of the world's domestic ducks are raised in areas where HPAI H5N1 virus is endemic (30). In Thailand, domestic ducks were identified as the most significant risk factor for outbreaks of HPAI H5N1 virus (25).

Indonesia is recognized by the World Animal Health Organization (OIE) as an HPAI H5N1 virus endemic country, making eradication of the virus in the near future unlikely. Indonesia has experienced the highest incidence of human infections with HPAI H5N1 virus and one of the highest case fatality rates. Unfortunately, control of the virus in Indonesia has proven difficult due to its incredibly diverse and complex poultry and domestic duck production systems. Extensive movement of domestic duck flocks provides opportunities for long-distance spread of HPAI H5N1 virus.

The current outbreak of Asian-origin HPAI H5N1 virus has prevailed for more than 15 years and the virus continues to evolve. While a number of factors have influenced continued maintenance and dissemination this unprecedented virus outbreak, domestic ducks may play a significant role in persistence of this virus, adding to the increasing challenge of control and eradication. As the reservoir host for avian influenza viruses, ducks may serve as a mixing vessel for creating novel virus variants and, potentially, the next pandemic virus. The aim of this dissertation is to elucidate the role of domestic ducks in avian influenza virus, particularly HPAI

H5N1 virus, persistence and spread, in an effort to effectively target and design surveillance, prevention and control programs.

References

1. **Abdelwhab, E. M., A. A. Selim, A. Arafa, S. Galal, W. H. Kilany, M. K. Hassan, M. M. Aly, and M. H. Hafez.** 2010. Circulation of avian influenza H5N1 in live bird markets in Egypt. Avian diseases **54:**911-914.

2. **Achenbach, J. E., and R. A. Bowen.** 2011. Transmission of avian influenza A viruses among species in an artificial barnyard. PloS one **6:**e17643.

3. **Alexander, D. J.** 2007. An overview of the epidemiology of avian influenza. Vaccine **25:**5637-5644.

4. **Amonsin, A., C. Choatrakol, J. Lapkuntod, R. Tantilertcharoen, R. Thanawongnuwech, S. Suradhat, K. Suwannakarn, A. Theamboonlers, and Y. Poovorawan.** 2008. Influenza virus (H5N1) in live bird markets and food markets, Thailand. Emerging infectious diseases **14:**1739-1742.

5. **Antarasena, C., R. Sirimujalin, P. Prommuang, S. D. Blacksell, N. Promkuntod, and P. Prommuang.** 2006. Tissue tropism of a Thailand strain of high-pathogenicity avian influenza virus (H5N1) in tissues of naturally infected native chickens (Gallus gallus), Japanese quail (Coturnix coturnix japonica) and ducks (Anas spp.). Avian pathology **35:**250-253.

6. **Bingham, J., D. J. Green, S. Lowther, J. Klippel, S. Burggraaf, D. E. Anderson, H. Wibawa, D. M. Hoa, N. T. Long, P. P. Vu, D. J. Middleton, and P. W. Daniels.** 2009. Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. Avian pathology **38:**267-278.

7. **Bosch, F. X., M. Orlich, H. D. Klenk, and R. Rott.** 1979. The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology **95:**197-207.

8. **Cagle, C., T. L. To, T. Nguyen, J. Wasilenko, S. C. Adams, C. J. Cardona, E. Spackman, D. L. Suarez, and M. J. Pantin-Jackwood.** 2011. Pekin and Muscovy ducks respond differently to vaccination with a H5N1 highly pathogenic avian influenza (HPAI) commercial inactivated vaccine. Vaccine **29:**6549-6557.

9. **Capua, I., and F. Mutinelli.** 2001. Mortality in Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var. domestica) associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. Avian pathology **30:**179-183.

10. **Capua, I., A. Schmitz, V. Jestin, G. Koch, and S. Marangon.** 2009. Vaccination as a tool to combat introductions of notifiable avian influenza viruses in Europe, 2000 to 2006. Revue scientifique et technique **28:**245-259.

11. **Cattoli, G., A. Fusaro, I. Monne, F. Coven, T. Joannis, H. S. El-Hamid, A. A. Hussein, C. Cornelius, N. M. Amarin, M. Mancin, E. C. Holmes, and I. Capua.** 2011. Evidence for differing evolutionary dynamics of A/H5N1 viruses among countries applying or not applying avian influenza vaccination in poultry. Vaccine **29:**9368-9375.

12. **Cavailler, P., S. Chu, S. Ly, J. M. Garcia, Q. Ha do, I. Bergeri, L. Som, S. Ly, T. Sok, S. Vong, and P. Buchy.** 2010. Seroprevalence of anti-H5 antibody in rural Cambodia, 2007. Journal of clinical virology **48:**123-126.

13. **Chen, H., Y. Li, Z. Li, J. Shi, K. Shinya, G. Deng, Q. Qi, G. Tian, S. Fan, H. Zhao, Y. Sun, and Y. Kawaoka.** 2006. Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. Journal of virology **80:**5976-5983.

14. **Chen, H., G. J. Smith, S. Y. Zhang, K. Qin, J. Wang, K. S. Li, R. G. Webster, J. S. Peiris, and Y. Guan.** 2005. Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature **436:**191-192.

15. **Chen, J., F. Fang, Z. Yang, X. Liu, H. Zhang, Z. Zhang, X. Zhang, and Z. Chen.** 2009. Characterization of highly pathogenic H5N1 avian influenza viruses isolated from poultry markets in central China. Virus research **146:**19-28.

16. **Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster.** 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet **351:**472-477.

17. **Daoust, P. Y., F. S. Kibenge, R. A. Fouchier, M. W. van de Bildt, D. van Riel, and T. Kuiken.** 2011. Replication of low pathogenic avian influenza virus in naturally infected Mallard ducks (Anas platyrhynchos) causes no morphologic lesions. Journal of wildlife diseases **47:**401-409.

18. **Davis, C. T., A. L. Balish, E. O'Neill, C. V. Nguyen, N. J. Cox, X. Xiyan, A. Klimov, T. Nguyen, and R. O. Donis.** 2010. Detection and characterization of clade 7 high pathogenicity avian influenza H5N1 viruses in chickens seized at ports of entry and live poultry markets in Vietnam. Avian diseases **54:**307-312.

19. **Desvaux, S., V. Grosbois, T. T. Pham, S. Fenwick, S. Tollis, N. H. Pham, A. Tran, and F. Roger.** 2011. Risk factors of highly pathogenic avian influenza H5N1 occurrence at the village and farm levels in the Red River Delta Region in Vietnam. Transboundary and emerging diseases **58:**492-502.

20. **Eggert, D., and D. E. Swayne.** 2010. Single vaccination provides limited protection to ducks and geese against H5N1 high pathogenicity avian influenza virus. Avian diseases **54:**1224-1229.

21. **Ellis, T. M., R. B. Bousfield, L. A. Bissett, K. C. Dyrting, G. S. Luk, S. T. Tsim, K. Sturm-Ramirez, R. G. Webster, Y. Guan, and J. S. Malik Peiris.** 2004. Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian pathology **33:**492-505.

22. **Ellstrom, P., N. Latorre-Margalef, P. Griekspoor, J. Waldenstrom, J. Olofsson, J. Wahlgren, and B. Olsen.** 2008. Sampling for low-pathogenic avian influenza A virus in wild Mallard ducks: oropharyngeal versus cloacal swabbing. Vaccine **26:**4414-4416.

23. **Food and Agriculture Organization.** 2010. Fourth Report: Global Programme for the Prevention and Control of Highly Pathogenic Avian Influenza. Food and Agriculture Organization.

24. **Fielding, R., G. M. Leung, W. W. Lam, C. Q. Jiang, C. Sitthi-Amorn, L. V. Ahn, Y. M. Lu, and W. S. Zhang.** 2009. A pan-Asian survey of risk perception, attitudes and practices associated with live animal markets. Hong Kong medical journal **15 Suppl 6:**17-20.

25. **Gilbert, M., P. Chaitaweesub, T. Parakamawongsa, S. Premashthira, T. Tiensin, W. Kalpravidh, H. Wagner, and J. Slingenbergh.** 2006. Free-grazing ducks and highly pathogenic avian influenza, Thailand. Emerging infectious diseases **12:**227-234.

26. **Globig, A., C. Staubach, M. Beer, U. Koppen, W. Fiedler, M. Nieburg, H. Wilking, E. Starick, J. P. Teifke, O. Werner, F. Unger, C. Grund, C. Wolf, H. Roost, F. Feldhusen, F. J. Conraths, T. C. Mettenleiter, and T. C. Harder.** 2009. Epidemiological and ornithological aspects of outbreaks of highly pathogenic avian influenza virus H5N1 of Asian lineage in wild birds in Germany, 2006 and 2007. Transboundary and emerging diseases **56:**57-72.

27. **WHO/OIE/FAO H5N1 Evolution Working Group.** 2012. Continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature. Influenza and other respiratory viruses **6:**1-5.

28. **WHO/OIE/FAO H5N1 Evolution Working Group.** 2009. Continuing progress towards a unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: divergence of clade 2.2 viruses. Influenza and other respiratory viruses **3:**59-62.

29. **Guan, Y., J. S. Peiris, A. S. Lipatov, T. M. Ellis, K. C. Dyrting, S. Krauss, L. J. Zhang, R. G. Webster, and K. F. Shortridge.** 2002. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proceedings of the National Academy of Sciences of the United States of America **99:**8950-8955.

30. **Guan, Y., R. Webby, I. Capua, and J. Waldenstrom.** 2012. H5N1: How to track a flu virus. Nature **483:**535-536.

31. **Halvorson, D. A.** 2009. Prevention and management of avian influenza outbreaks: experiences from the United States of America. Revue scientifique et technique **28:**359-369.

32. **Henning, J., K. A. Henning, J. M. Morton, N. T. Long, N. T. Ha, T. Vu le, P. P. Vu, D. M. Hoa, and J. Meers.** 2011. Highly pathogenic avian influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Viet Nam. Preventive veterinary medicine **101:**229-240.

33. **Henning, J., H. Wibawa, J. Morton, T. B. Usman, A. Junaidi, and J. Meers.** 2010. Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. Emerging infectious diseases **16:**1244-1250.

34. **Hinshaw, V. S., W. J. Bean, R. G. Webster, and G. Sriram.** 1980. Genetic reassortment of influenza A viruses in the intestinal tract of ducks. Virology **102:**412-419.

35. **Hulse-Post, D. J., K. M. Sturm-Ramirez, J. Humberd, P. Seiler, E. A. Govorkova, S. Krauss, C. Scholtissek, P. Puthavathana, C. Buranathai, T. D. Nguyen, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster.** 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proceedings of the National Academy of Sciences of the United States of America **102:**10682-10687.

36. **Huo, X., R. Zu, X. Qi, Y. Qin, L. Li, F. Tang, Z. Hu, and F. Zhu.** 2012. Seroprevalence of avian influenza A (H5N1) virus among poultry workers in Jiangsu Province, China: an observational study. BMC infectious diseases **12:**93.

37. **Indriani, R., G. Samaan, A. Gultom, L. Loth, S. Indryani, R. Adjid, N. L. Dharmayanti, J. Weaver, E. Mumford, K. Lokuge, P. M. Kelly, and Darminto.** 2010. Environmental sampling for avian influenza virus A (H5N1) in live-bird markets, Indonesia. Emerging infectious diseases **16:**1889-1895.

38. **Ip, H. S., R. J. Dusek, and D. M. Heisey.** 2012. The effect of swab sample choice on the detection of avian influenza in apparently healthy wild ducks. Avian diseases **56:**114-119.

39. **Jindal, N., M. de Abin, A. E. Primus, S. Raju, Y. Chander, P. T. Redig, and S. M. Goyal.** 2010. Comparison of cloacal and oropharyngeal samples for the detection of avian influenza virus in wild birds. Avian diseases **54:**115-119.

40. **Jourdain, E., G. Gunnarsson, J. Wahlgren, N. Latorre-Margalef, C. Brojer, S. Sahlin, L. Svensson, J. Waldenstrom, A. Lundkvist, and B. Olsen.** 2010. Influenza virus in a natural host, the mallard: experimental infection data. PloS one **5:**e8935.

41. **Kalthoff, D., A. Globig, and M. Beer.** 2010. (Highly pathogenic) avian influenza as a zoonotic agent. Veterinary microbiology **140:**237-245.

42. **Keawcharoen, J., K. Oraveerakul, T. Kuiken, R. A. Fouchier, A. Amonsin, S. Payungporn, S. Noppornpanth, S. Wattanodorn, A. Theambooniers, R. Tantilertcharoen, R. Pattanarangsan, N. Arya, P. Ratanakorn, D. M. Osterhaus, and Y. Poovorawan.** 2004. Avian influenza H5N1 in tigers and leopards. Emerging infectious diseases **10:**2189-2191.

43. **Khuntirat, B. P., I. K. Yoon, P. J. Blair, W. S. Krueger, M. Chittaganpitch, S. D. Putnam, K. Supawat, R. V. Gibbons, S. Pattamadilok, P. Sawanpanyalert, G. L. Heil, J. A. Friary, A. W. Capuano, and G. C. Gray.** 2011. Evidence for subclinical avian influenza virus infections among rural Thai villagers. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **53:**107-116.

44. **Killian, M. L.** 2008. Avian influenza virus sample types, collection, and handling. Methods in molecular biology **436:**7-12.

45. **Killian, M. L.** 2008. Hemagglutination assay for the avian influenza virus. Methods in molecular biology **436:**47-52.

46. **Kim, J. K., P. Seiler, H. L. Forrest, A. M. Khalenkov, J. Franks, M. Kumar, W. B. Karesh, M. Gilbert, R. Sodnomdarjaa, B. Douangngeun, E. A. Govorkova, and R. G. Webster.** 2008. Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. Journal of virology **82:**11374-11382.

47. **Knipe, D. M., Howley, Peter M. (ed.).** 2007. Fields Virology. Lippincott Williams & Wilkins, Philadelphia, PA.

48. **Krauss, S., D. Walker, and R. G. Webster.** 2012. Influenza virus isolation. Methods in molecular biology **865:**11-24.

49. **Kuiken, T., J. van den Brand, D. van Riel, M. Pantin-Jackwood, and D. E. Swayne.** 2010. Comparative pathology of select agent influenza a virus infections. Veterinary pathology **47:**893-914.

50. **Kwon, Y. K., and D. E. Swayne.** 2010. Different routes of inoculation impact infectivity and pathogenesis of H5N1 high pathogenicity avian influenza virus infection in chickens and domestic ducks. Avian diseases **54:**1260-1269.

51. **Ladman, B. S., E. Spackman, and J. Gelb, Jr.** 2012. Comparison of pooling 11 or 5 oropharyngeal swabbings for detecting avian influenza virus by real-time reverse transcription-PCR in broiler chickens. Avian diseases **56:**227-229.

52. **Lam, T. T., C. C. Hon, P. Lemey, O. G. Pybus, M. Shi, H. M. Tun, J. Li, J. Jiang, E. C. Holmes, and F. C. Leung.** 2012. Phylodynamics of H5N1 avian influenza virus in Indonesia. Molecular ecology [Epub ahead of print].

53. **Lee, C. W., D. A. Senne, and D. L. Suarez.** 2004. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. Journal of virology **78:**8372-8381.

54. **Li, K. S., Y. Guan, J. Wang, G. J. Smith, K. M. Xu, L. Duan, A. P. Rahardjo, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. T. Estoepangestie, A. Chaisingh, P. Auewarakul, H. T. Long, N. T. Hanh, R. J. Webby, L. L. Poon, H. Chen, K. F. Shortridge, K. Y. Yuen, R. G. Webster, and J. S. Peiris.** 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature **430:**209-213.

55. **Li, Y., Z. Lin, J. Shi, Q. Qi, G. Deng, Z. Li, X. Wang, G. Tian, and H. Chen.** 2006. Detection of Hong Kong 97-like H5N1 influenza viruses from eggs of Vietnamese waterfowl. Archives of virology **151:**1615-1624.

56. **Li, Y., J. Shi, G. Zhong, G. Deng, G. Tian, J. Ge, X. Zeng, J. Song, D. Zhao, L. Liu, Y. Jiang, Y. Guan, Z. Bu, and H. Chen.** 2010. Continued evolution of H5N1 influenza viruses in wild birds, domestic poultry, and humans in China from 2004 to 2009. Journal of virology **84:**8389-8397.

57. **Lorusso, A., A. L. Vincent, M. R. Gramer, K. M. Lager, and J. R. Ciacci-Zanella.** 2012. Contemporary Epidemiology of North American Lineage Triple Reassortant Influenza A Viruses in Pigs. Current topics in microbiology and immunology.

58. **Loth, L., M. Gilbert, J. Wu, C. Czarnecki, M. Hidayat, and X. Xiao.** 2011. Identifying risk factors of highly pathogenic avian influenza (H5N1 subtype) in Indonesia. Preventive veterinary medicine **102:**50-58.

59. **Lubroth, J.** 2007. Control strategies for highly pathogenic avian influenza: a global perspective. Developments in biologicals **130:**13-21.

60. **Malik Peiris, J. S.** 2009. Avian influenza viruses in humans. Revue scientifique et technique **28:**161-173.

61. **Matrosovich, M., N. Zhou, Y. Kawaoka, and R. Webster.** 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. Journal of virology **73:**1146-1155.

62. **Matrosovich, M. N., T. Y. Matrosovich, T. Gray, N. A. Roberts, and H. D. Klenk.** 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proceedings of the National Academy of Sciences of the United States of America **101:**4620-4624.

63. **Middleton, D., J. Bingham, P. Selleck, S. Lowther, L. Gleeson, P. Lehrbach, S. Robinson, J. Rodenberg, M. Kumar, and M. Andrew.** 2007. Efficacy of inactivated vaccines against H5N1 avian influenza infection in ducks. Virology **359:**66-71.

64. **Moresco, K. A., D. E. Stallknecht, and D. E. Swayne.** 2012. Evaluation of different embryonating bird eggs and cell cultures for isolation efficiency of avian Influenza A Virus and Avian paramyxovirus serotype 1 from real-time reverse transcription polymerase chain reaction-positive wild bird surveillance samples. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc **24:**563-567.

65. **Myers, K. P., S. F. Setterquist, A. W. Capuano, and G. C. Gray.** 2007. Infection due to 3 avian influenza subtypes in United States veterinarians. Clinical infectious diseases **45:**4-9.

66. **Nidom, C. A., S. Yamada, R. V. Nidom, K. Rahmawati, M. Y. Alamudi, Kholik, S. Indrasari, R. S. Hayati, K. Iwatsuki Horimoto, and Y. Kawaoka.** 2012. Genetic characterization of H5N1 influenza viruses isolated from chickens in Indonesia in 2010. Virus genes **44:**459-465.

67. **OIE.** 2011. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Sixth ed, vol. I.

68. **Osterhaus, A. D., J. C. de Jong, G. F. Rimmelzwaan, and E. C. Claas.** 2002. H5N1 influenza in Hong Kong: virus characterizations. Vaccine **20 Suppl 2:**S82-83.

69. **Pantin-Jackwood, M. J., D. M. Smith, J. L. Wasilenko, C. Cagle, E. Shepherd, L. Sarmento, D. R. Kapczynski, and C. L. Afonso.** 2012. Effect of age on the pathogenesis and innate immune responses in Pekin ducks infected with different H5N1 highly pathogenic avian influenza viruses. Virus research [Epub ahead of print].

70. **Pantin-Jackwood, M. J., D. L. Suarez, E. Spackman, and D. E. Swayne.** 2007. Age at infection affects the pathogenicity of Asian highly pathogenic avian influenza H5N1 viruses in ducks. Virus research **130:**151-161.

71. **Pantin-Jackwood, M. J., and D. E. Swayne.** 2007. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. Avian diseases **51:**250-259.

72. **Pedersen, J. C.** 2008. Hemagglutination-inhibition test for avian influenza virus subtype identification and the detection and quantitation of serum antibodies to the avian influenza virus. Methods in molecular biology **436:**53-66.

73. **Pelzel, A. M., B. J. McCluskey, and A. E. Scott.** 2006. Review of the highly pathogenic avian influenza outbreak in Texas, 2004. Journal of the American Veterinary Medical Association **228:**1869-1875.

74. **Perdue, M. L., and D. L. Suarez.** 2000. Structural features of the avian influenza virus hemagglutinin that influence virulence. Veterinary microbiology **74:**77-86.

75. **Perkins, L. E., and D. E. Swayne.** 2003. Comparative susceptibility of selected avian and mammalian species to a Hong Kong-origin H5N1 high-pathogenicity avian influenza virus. Avian diseases **47:**956-967.

76. **Pfeiffer, J., D. L. Suarez, L. Sarmento, T. L. To, T. Nguyen, and M. J. Pantin-Jackwood.** 2010. Efficacy of commercial vaccines in protecting chickens and ducks against H5N1 highly pathogenic avian influenza viruses from Vietnam. Avian diseases **54:**262-271.

77. **Phuong do, Q., N. T. Dung, P. H. Jorgensen, K. J. Handberg, N. T. Vinh, and J. P. Christensen.** 2011. Susceptibility of Muscovy (Cairina Moschata) and mallard ducks (Anas Platyrhynchos) to experimental infections by different genotypes of H5N1 avian influenza viruses. Veterinary microbiology **148:**168-174.

78. **Pillai, S. P., M. Pantin-Jackwood, D. L. Suarez, Y. M. Saif, and C. W. Lee.** 2010. Pathobiological characterization of low-pathogenicity H5 avian influenza viruses of diverse origins in chickens, ducks and turkeys. Archives of virology **155:**1439-1451.

79. **Rudolf, M., M. Poppel, A. Frohlich, T. Mettenleiter, M. Beer, and T. Harder.** 2009. Efficacy of a commercial inactivated H5 influenza vaccine against highly pathogenic avian influenza H5N1 in waterfowl evaluated under field conditions. Revue scientifique et technique **28:**275-291.

80. **Saif, Y. M. (ed.).** 2008. Diseases of Poultry, Twelfth ed. Blackwell Publishing Professional, Ames, Iowa.

81. **Sakoda, Y., S. Sugar, D. Batchluun, T. O. Erdene-Ochir, M. Okamatsu, N. Isoda, K. Soda, H. Takakuwa, Y. Tsuda, N. Yamamoto, N. Kishida, K. Matsuno, E. Nakayama, M. Kajihara, A. Yokoyama, A. Takada, R. Sodnomdarjaa, and H. Kida.** 2010. Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory. Virology **406:**88-94.

82. **Salzberg, S. L., C. Kingsford, G. Cattoli, D. J. Spiro, D. A. Janies, M. M. Aly, I. H. Brown, E. Couacy-Hymann, G. M. De Mia, H. Dung do, A. Guercio, T. Joannis, A. S. Maken Ali, A. Osmani, I. Padalino, M. D. Saad, V. Savic, N. A. Sengamalay, S. Yingst, J. Zaborsky, O. Zorman-Rojs, E. Ghedin, and I. Capua.** 2007. Genome analysis linking recent European and African influenza (H5N1) viruses. Emerging infectious diseases **13:**713-718.

83. **Schrauwen, E. J., S. Herfst, L. M. Leijten, P. van Run, T. M. Bestebroer, M. Linster, R. Bodewes, J. H. Kreijtz, G. F. Rimmelzwaan, A. D. Osterhaus, R. A. Fouchier, T. Kuiken, and D. van Riel.** 2012. The multibasic cleavage site in H5N1 virus is critical for systemic spread along the olfactory and hematogenous routes in ferrets. Journal of virology **86:**3975-3984.

84. **Senne, D. A., B. Panigrahy, Y. Kawaoka, J. E. Pearson, J. Suss, M. Lipkind, H. Kida, and R. G. Webster.** 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian diseases **40:**425-437.

85. **Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster.** 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology **252:**331-342.

86. **Sims, L. D., J. Domenech, C. Benigno, S. Kahn, A. Kamata, J. Lubroth, V. Martin, and P. Roeder.** 2005. Origin and evolution of highly pathogenic H5N1 avian influenza in Asia. The Veterinary record **157:**159-164.

87. **Sims, L. D., T. M. Ellis, K. K. Liu, K. Dyrting, H. Wong, M. Peiris, Y. Guan, and K. F. Shortridge.** 2003. Avian influenza in Hong Kong 1997-2002. Avian diseases **47:**832-838.

88. **Slemons, R. D., and B. C. Easterday.** 1978. Virus replication in the digestive tract of ducks exposed by aerosol to type-A influenza. Avian diseases **22:**367-377.

89. **Songserm, T., R. Jam-on, N. Sae-Heng, N. Meemak, D. J. Hulse-Post, K. M. Sturm-Ramirez, and R. G. Webster.** 2006. Domestic ducks and H5N1 influenza epidemic, Thailand. Emerging infectious diseases **12:**575-581.

90. **Spackman, E.** 2009. The ecology of avian influenza virus in wild birds: what does this mean for poultry? Poultry science **88:**847-850.

91. **Spackman, E., J. Gelb, Jr., L. A. Preskenis, B. S. Ladman, C. R. Pope, M. J. Pantin-Jackwood, and E. T. McKinley.** 2010. The pathogenesis of low pathogenicity H7 avian influenza viruses in chickens, ducks and turkeys. Virology journal **7:**331.

92. **Spackman, E., M. J. Pantin-Jackwood, D. E. Swayne, and D. L. Suarez.** 2009. An evaluation of avian influenza diagnostic methods with domestic duck specimens. Avian diseases **53:**276-280.

93. **Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez.** 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of clinical microbiology **40:**3256-3260.

94. **Spackman, E., and D. L. Suarez.** 2008. Detection and identification of the H5 hemagglutinin subtype by real-time RT-PCR. Methods in molecular biology **436:**27-33.

95. **Spackman, E., and D. L. Suarez.** 2008. Type A influenza virus detection and quantitation by real-time RT-PCR. Methods in molecular biology **436:**19-26.

96. **Stallknecht, D. E., and S. M. Shane.** 1988. Host range of avian influenza virus in free-living birds. Veterinary research communications **12:**125-141.

97. **Starick, E., M. Beer, B. Hoffmann, C. Staubach, O. Werner, A. Globig, G. Strebelow, C. Grund, M. Durban, F. J. Conraths, T. Mettenleiter, and T. Harder.** 2008. Phylogenetic analyses of highly pathogenic avian influenza virus isolates from Germany in 2006 and 2007 suggest at least three separate introductions of H5N1 virus. Veterinary microbiology **128:**243-252.

98. **Sturm-Ramirez, K. M., T. Ellis, B. Bousfield, L. Bissett, K. Dyrting, J. E. Rehg, L. Poon, Y. Guan, M. Peiris, and R. G. Webster.** 2004. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. Journal of virology **78:**4892-4901.

99. **Sturm-Ramirez, K. M., D. J. Hulse-Post, E. A. Govorkova, J. Humberd, P. Seiler, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. Chaisingh, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster.** 2005. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? Journal of virology **79:**11269-11279.

100. **Suarez, D. L., M. L. Perdue, N. Cox, T. Rowe, C. Bender, J. Huang, and D. E. Swayne.** 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. Journal of virology **72:**6678-6688.

101. **Suguitan, A. L., Jr., Y. Matsuoka, Y. F. Lau, C. P. Santos, L. Vogel, L. I. Cheng, M. Orandle, and K. Subbarao.** 2012. The multibasic cleavage site of the hemagglutinin of highly pathogenic A/Vietnam/1203/2004 (H5N1) avian influenza virus acts as a virulence factor in a host-specific manner in mammals. Journal of virology **86:**2706-2714.

102. **Swayne, D. E. (ed.).** 2008. Avian Influenza. Blackwell Publishing, Ames, Iowa.

103. **Trock, S. C., and J. P. Huntley.** 2010. Surveillance and control of avian influenza in the New York live bird markets. Avian diseases **54:**340-344.

104. **Tumpey, T. M., D. L. Suarez, L. E. Perkins, D. A. Senne, J. G. Lee, Y. J. Lee, I. P. Mo, H. W. Sung, and D. E. Swayne.** 2002. Characterization of a highly pathogenic H5N1 avian influenza A virus isolated from duck meat. Journal of virology **76:**6344-6355.

105. **van der Goot, J. A., M. van Boven, M. C. de Jong, and G. Koch.** 2007. Effect of vaccination on transmission of HPAI H5N1: the effect of a single vaccination dose on transmission of highly pathogenic avian influenza H5N1 in Peking ducks. Avian diseases **51:**323-324.

106. **van der Goot, J. A., M. van Boven, A. Stegeman, S. G. van de Water, M. C. de Jong, and G. Koch.** 2008. Transmission of highly pathogenic avian influenza H5N1 virus in Pekin ducks is significantly reduced by a genetically distant H5N2 vaccine. Virology **382:**91-97.

107. **Villarreal, C.** 2007. Experience in control of avian influenza in the Americas. Developments in biologicals **130:**53-60.

108. **Villarreal, C. L.** 2006. Control and eradication strategies of avian influenza in Mexico. Developments in biologicals **124:**125-126.

109. **Vong, S., B. Coghlan, S. Mardy, D. Holl, H. Seng, S. Ly, M. J. Miller, P. Buchy, Y. Froehlich, J. B. Dufourcq, T. M. Uyeki, W. Lim, and T. Sok.** 2006. Low frequency of poultry-to-human H5NI virus transmission, southern Cambodia, 2005. Emerging infectious diseases **12:**1542-1547.

110. **Vong, S., S. Ly, M. D. Van Kerkhove, J. Achenbach, D. Holl, P. Buchy, S. Sorn, H. Seng, T. M. Uyeki, T. Sok, and J. M. Katz.** 2009. Risk factors associated with subclinical human infection with avian influenza A (H5N1) virus--Cambodia, 2006. The Journal of infectious diseases **199:**1744-1752.

111. **Wan, X. F., L. Dong, Y. Lan, L. P. Long, C. Xu, S. Zou, Z. Li, L. Wen, Z. Cai, W. Wang, X. Li, F. Yuan, H. Sui, Y. Zhang, J. Dong, S. Sun, Y. Gao, M. Wang, T. Bai, L. Yang, D. Li, W. Yang, H. Yu, S. Wang, Z. Feng, Y. Wang, Y. Guo, R. J. Webby, and Y. Shu.** 2011. Indications that live poultry markets are a major source of human H5N1 influenza virus infection in China. Journal of virology **85:**13432-13438.

112. **Wasilenko, J. L., A. M. Arafa, A. A. Selim, M. K. Hassan, M. M. Aly, A. Ali, S. Nassif, E. Elebiary, A. Balish, A. Klimov, D. L. Suarez, D. E. Swayne, and M. J. Pantin-Jackwood.** 2011. Pathogenicity of two Egyptian H5N1 highly pathogenic avian influenza viruses in domestic ducks. Archives of virology **156:**37-51.

113. **Webster, R. G.** 2004. Wet markets--a continuing source of severe acute respiratory syndrome and influenza? Lancet **363:**234-236.

114. **Webster, R. G., D. J. Hulse-Post, K. M. Sturm-Ramirez, Y. Guan, M. Peiris, G. Smith, and H. Chen.** 2007. Changing epidemiology and ecology of highly pathogenic avian H5N1 influenza viruses. Avian diseases **51:**269-272.

115. **Webster, R. G., M. Yakhno, V. S. Hinshaw, W. J. Bean, and K. G. Murti.** 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology **84:**268-278.

116. **World Health Organization**. Cumulative number of confirmed human cases of avian influenza A (H5N1) reported to WHO. World Health Organization. [Online, Accessed July 5, 2012].

117. **Xu, X., Subbarao, N. J. Cox, and Y. Guo.** 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology **261:**15-19.

118. **Yamamoto, Y., K. Nakamura, M. Okamatsu, M. Yamada, and M. Mase.** 2008. Avian influenza virus (H5N1) replication in feathers of domestic waterfowl. Emerging infectious diseases **14:**149-151.

119. **Yamamoto, Y., K. Nakamura, M. Yamada, and M. Mase.** 2010. Persistence of avian influenza virus (H5N1) in feathers detached from bodies of infected domestic ducks. Applied and environmental microbiology **76:**5496-5499.

120. **Zhang, Y., Y. Sun, H. Sun, J. Pu, Y. Bi, Y. Shi, X. Lu, J. Li, Q. Zhu, G. F. Gao, H. Yang, and J. Liu.** 2012. A Single Amino Acid at the Hemagglutinin Cleavage Site Contributes to the Pathogenicity and Neurovirulence of H5N1 Influenza Virus in Mice. Journal of virology **86:** 6924-6931.

Chapter 2

Prevalence and Seroprevalence of Avian Influenza Viruses in Domestic Duck Flocks in Indonesia

INTRODUCTION

Domestic ducks may play an important role in the spread and maintenance of avian influenza viruses, including HPAI H5N1 viruses. A number of studies have demonstrated that domestic duck flocks may be asymptomtically infected with HPAI H5N1 virus, allowing for dissemination of the virus without detection by flock owners or government livestock officials (5, 6, 15). An analysis of spacial distribution of HPAI H5N1 outbreaks in Thailand demonstrated that domestic duck flocks were the most important risk factor for the detection of HPAI H5N1 virus (1). In many countries in Asia, HPAI H5N1 virus has been detected in domestic duck flocks, with prevalence varying from 0.7% of domestic duck flocks in Vietnam during a period when no HPAI H5N1 outbreaks were reported (2) to 49.5% of domestic duck flocks in Thailand during an HPAI H5N1 virus outbreak (10).

Indonesia has a large domestic duck population, estimated at 34.3 million ducks (14). The vast majority of Indonesian duck flocks are raised on smallholder farms and are used for meat and egg production. Most domestic duck flocks are raised primarily as layer flocks but serve a number of purposes in addition to egg production. Some of the flocks are raised in an integrated production system on rice fields where they scavenge left over rice grains and eat worms, snails and other insects in the field, serving as a means of insect control. They also swim or walk around in the field, thereby aerating the field.

Domestic duck production and product distribution systems in Indonesia are very complex and involve an intricate network of producers (including villagers with small flocks),

middlemen (traders) and purchasers who all contribute to moving the ducks and duck products over a widespread geographical area. General production practices vary greatly and are poorly understood by the global community.

There were two primary objectives of this cross-sectional study. The first objective was to characterize domestic duck populations in Indonesia, including evaluation of husbandry and production practices, movement patterns, contact with wild birds and other animal species, contact with humans and usage of avian influenza virus vaccinations. The second objective was to determine avian influenza virus, particularly H5 avian influenza virus, prevalence and seroprevalence in these domestic duck flocks.

MATERIALS AND METHODS

Study Location

Three districts in the West Java province, Indramayu, Subang and Tangerang, were selected for this study. Indramayu was selected due to a high density of domestic ducks farmed in the district. Subang was selected due to a high density of commercial chicken production farms in the district. Tangerang was selected due to high production of live bird market birds, including domestic ducks and chickens, and a high density of live bird markets in the district. The districts are distributed throughout the West Java region, with a span of approximately 200 kilometers between the two farthest apart districts of Tangerang and Indramayu.

Flock Categorization and Selection

Prior to the start of the study, three categories were created to differentiate production systems of domestic duck flocks. These categories were created based on previous studies conducted to evaluate domestic duck flock production practices in Indonesia (14). Type I

flocks, or fully free-ranging flocks, typically reside on rice fields. These flocks do not return to a home premises and instead, move from rice field to rice field. Type II flocks, or partially free-ranged flocks, return to a home premises periodically and are semi-intensively reared. Type III flocks, or confined flocks, are intensively reared and are fully confined to one premises. They are typically housed in fenced-in areas open to the environment, with a barn or shed that provides shelter for the ducks.

Animal health officials in each district were informed of the study and the head of the district livestock office was asked for consent to work within the district. When consent was granted, the district animal health officials provided locations of and contact information for domestic duck flock owners that would qualify for participation in the study. Flocks were selected from flock information maintained in the district animal health office. Within each district, ten flocks of each flock type were selected. This resulted in participation of thirty flocks per district, for a total of ninety flocks. The number of flocks selected was based on an approximation of the number of flocks that could be contacted and sampled within the study timeframe.

The use of human and animal subjects was approved by the Research Integrity and Compliance Review Office at Colorado State University. All flock owners gave informed consent and all researchers followed approved animal handling and sampling protocols.

Owner Survey

The owner of each flock was contacted and asked to participate in the study. Researchers traveled to each participating flock where they administered a survey to the flock owner prior to collecting diagnostic samples. The survey consisted of 76 open-ended and close-ended questions

addressing topics on flock husbandry, production practices, bird movement, flock housing and environment, biosecurity practices and flock health.

Sample Collection and Testing

Samples were collected from 30 ducks in each flock. This number was based on a sample size calculation (Epi Z Epidemiologic Calculator, Fort Collins, CO) to detect presence or absence of disease. An assumption of 100 ducks per flock, test sensitivity of 90% and disease prevalence of 10%, with a confidence level of 95%, yielded a sample size of 28 ducks per flock. The sample size was increased to 30 ducks per flock to account for potential loss of sample in the field or laboratory. A total of 2700 ducks were sampled for this study.

The ducks in each flock were selected using a random sampling strategy and included both healthy and ill appearing ducks. An oropharyngeal swab, a cloacal swab and a blood sample were collected from each sampled duck. Swab samples were collected using polyester-tipped, plastic shafted swabs and placed immediately into 1ml of brain heart infusion broth. All samples were stored on ice or in a refrigerator during transport to the lab. All samples arrived in the lab within 72 hours of sample collection. Upon arrival in the lab, the blood samples were centrifuged and serum was separated into a new collection tube and stored in a -20°C freezer. Swab samples were stored in a -70°C freezer. All samples were assigned a unique identifying number with designations for the district location of the flock, flock production type (I, II or III), flock number, duck number and sample type.

Swab samples were tested for avian influenza virus by real-time reverse transcription polymerase chain reaction (rRT-PCR). Swab sample broths were pooled with five samples of the same swab type in each pool, yielding six pools of oropharyngeal samples and six pools of

cloacal samples per flock. RNA was extracted from the sample pools using the Ambion MagMAX-96 AI/NDV Viral Isolation Kit (Applied Biosystems, Foster City, CA), according to manufacturer's directions. Previously reported rRT-PCR protocols (11-13), utilizing the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems 7500 96-well plate thermocycler were used to test the RNA extractions for the presence of avian influenza virus. The sample pools were first tested using an avian influenza matrix gene assay, which is designed to detect all influenza A viruses. The matrix gene rRT-PCR assay was conducted on all individual samples from any pool with a cycle threshold value of 43 or less. All matrix positive individual samples were then tested for H5 avian influenza virus using a protocol that detects the H5 subtype hemagglutinin gene.

Serum samples were tested for anti-avian influenza virus antibodies using a commercial competitive ELISA (Flu Detect® BE, Synbiotics Corporation, Kansas City, MO) which detected antibodies to a recombinant avian influenza nucleoprotein antigen. Samples were tested and analyzed following manufacturer's directions.

Results Interpretation

Individual samples were considered positive if they had an rRT-PCR cycle threshold value of 40 or less. Sample pools were considered positive if they had an rRT-PCR cycle threshold value of 43 or less and contained an individual sample with a cycle threshold value of 40 or less. For negative sample pools, all individual duck samples were assumed to be negative. Individual ducks were considered positive for avian influenza virus if the oropharyngeal sample, cloacal sample or both samples were positive. Additionally, individual ducks were considered

positive for H5 virus if either sample or both samples were positive on the H5-specific rRT-PCR assay.

In order to determine overall flock-level prevalence, flocks were considered positive for avian influenza virus if at least one duck sample was positive on the matrix gene rRT-PCR. Flocks were considered positive for H5 avian influenza virus if at least one duck sample was positive on the H5-specific hemagglutinin gene rRT-PCR.

Individual ducks were considered seropositive on the cELISA if the sample to negative (S/N) ratio was less than the 0.60 cutoff established by the manufacturer. Flocks were considered seropositive for avian influenza virus if at least one duck serum sample was positive. For each flock, the percentage of seropositive ducks in the flock was calculated by dividing the number of positive ducks by the total number of ducks sampled in the flock.

Data Analysis

Flocks were stratified by flock type based on production practices (Type I, II or III). Data were statistically analyzed using SAS software version 9.2 (SAS Institute Inc., Cary, NC). The chi-square test for association was used to identify differences among flock types for the categorical variables. Probability values (p) less than or equal to 0.05 were considered statistically significant.

RESULTS

Survey Results

The majority of flock owners were male (83/90; 92.2%), with a median age of 45 years (age range 15-75). The overall education level of the flock owners was poor, as 22/90 (24.4%)

received no formal education, 16/90 (17.8%) did not finish elementary school, 35/90 (38.9%) completed elementary school but not junior high school, 10/90 (11.1%) completed junior high school but not high school and only 7/90 (7.8%) graduated from high school.

Fifty percent of the flock owners had significant experience as duck farmers, as 37/88 (42%) had more than 10 years of experience and 7/88 (8%) had 6 to 10 years of experience. Nine percent (8/88) were new to farming, with less than one year of experience, forty-one percent (36/88) had between 1 and 6 years of experience and two flock owners did not answer the question. Duck farming was the primary occupation for 44/90 (48.9%) of the farmers. For farmers citing a different primary occupation, rice farming was the most common and was cited by 30/90 (33.3%) flock owners.

More than one-half (48/90; 53.3%) of flock owners worked alone to care for their flocks. Thirty-two of ninety (35.6%) were cared for by the owner and one additional worker and 10/90 (11.1%) were cared for by the owner and two to five additional workers. For all flocks, the additional workers were family members or friends of the owner.

Table 2.1 – Flock Owner Characteristics

Flock Owner Characteristics	Percentage (%)
Owner Information	
Male	92.2
Education Level	
No formal Education	24.4
Some elementary school education	17.8
Some junior high School education	38.9
Some high school education	11.1
Graduated high school	7.8
Flock Owner Experience	
More than 10 years experience	42.0
6 to 10 years experience	8.0
1 to 6 years experience	41.0
Less than 1 year experience	9.1
Flock Owner Occupation	
Duck farming is primary occupation	48.9
Rice Farming is primary occupation	33.3
Other primary occupation	17.8

The flocks represented two primary production types, with 84/90 (93.3%) functioning as layer flocks and 6/90 (6.7%) functioning as meat flocks. In addition, two layer flocks also reported functioning as breeder/hatchery flocks. Most of the duck flocks that are primarily used as layers are sold as meat birds at the end of production, when meat prices are good, or to earn money. Of the layer flock owners that answered this question, 65/80 (81.3%) sold their layer ducks for meat.

The majority of flocks were composed of birds that were similar in age (76/90; 84.4%) as compared to multi-age flocks (14/90; 15.6%). Many of the flock owners did not know the exact age of the ducks in their flock. Of the duck flocks of known ages, owners reported ages from

less than one month old up to 18 months of age. Although not all flock owners reported the time span they planned to keep their flock, 69/86 (77.9%) planned to keep their flock between 1 and 3 years and 19/86 (22.1%) planned to keep their flock less than one year. Of the 17 flocks with a production cycle of less than one year, 6/17 (35.3%) were classified as meat production flocks.

Table 2.2 – Flock Characteristics

Flock Characteristics	Percentage (%)
Flock Purpose	
Layer flocks	93.3
(layers sold for meat at end of production)	81.3
Meat flocks	6.7
Flock Age	
Similar in age flock	84.4
Multi-age flock	15.6
Production Cycle	
Less than 1 year	22.1
1 to 3 years	77.9

The average flock size was 219 ducks per flock (range 31 – 1500 ducks). Of the three flock types, Type I flocks had the largest flocks, with an average flock size of 345 ducks per flock. Type II and Type III flocks were similar in size, both with an average of 155 ducks per flock. Of the 90 flock owners, 35 (38.9%) reported owning chickens, 32 (35.6%) reported owning Muscovy ducks and 8 (8.9%) reported owning pigeons. The average number of chickens kept was 12 (range 1 – 50), the average number of Muscovy ducks kept was 12 (range 1 – 34) and the average number of pigeons kept was 9 (range 3 – 15).

Table 2.3 – Average Flock Size

Average Flock Size	Type I	Type II	Type III	Average
Number of ducks	345	155	155	219

Ducks were purchased from a number of different sources. Of the flock owners that specified their purchase system, 43/89 (48.3%) purchased ducks from breeder flocks, 17/89 (19.1%), from retailers or street vendors (sales straight to purchaser), 15/89 (16.9%) from brokers or acquaintances (middlemen) and 8/89 (9.0%) from live bird markets. Six flock owners (6.7%) reported purchasing or raising hatching eggs and hatching their own chicks. Of the flock owners that reported the age of purchase for their current duck flock, 74/89 (83.1%) reported purchasing ducklings aged one month or younger and 15/89 (16.9%) reported purchasing older ducks, with a age range from 2 months to 15 months at the time of purchase.

Table 2.4 – Purchasing Systems

Purchasing System	Percentage (%)
Source of Ducks	
Breeder flocks	48.3
Retailer / street vendor	19.1
Broker / middleman (trader)	16.9
Live bird markets	9.0
Hatch own ducks	6.7
Age of Ducks Purchased	
Ducklings	83.1
Older ducks (older than 1 month)	16.9

Flock movement parameters were collected for Type I (fully free-ranging) and Type II (partially free-ranging) flocks. For Type I flocks, 4/30 (13.33%) ranged within a single village area, 12/30 (40%) ranged within a subdistrict area, 10/30 (33.33%) ranged within a district and

4/30 (13.33%) ranged within a province. For Type II flocks, 16/30 (53.3%) ranged within a single village area, 6/30 (20%) ranged within a subdistrict area, 5/30 (16.7%) ranged within a district, 2/30 (6.7%) ranged within a province and 1/30 (3.3%) ranged in more than one province. Type I flocks typically stayed in one area between one and two months. Twenty-five of thirty (83.3%) stayed one month and 5/30 (16.7%) stayed between 1 and 2 months. Type II flocks typically stayed between 1 day and 3 months. Twelve of thirty flocks (40%) stayed only one day and returned to their home premises each night, 7/30 (23.3%) stayed between 2 days and 1 month, 9/30 (30%) stayed between 1 and 2 months and 2/30 (6.7%) stayed 3 months. All Type I flocks were ranged in post-harvest rice fields. Additionally, one owner reported sometimes ranging his flock in irrigation waterways. For Type II flocks, 27/29 (93.1%) were ranged in post-harvest rice fields. Of these flocks, 7/27 (25.9%) were additionally ranged in rivers, ponds and/or irrigation waterways. Of the 2/29 (6.9%) Type II flocks that were not ranged in post-harvest rice fields, one was ranged in ponds and the other was ranged in irrigation waterways. It was common for free-ranging flocks to be ranged with other farmers' flocks. Twenty-nine of thirty (96.7%) Type I flocks were typically ranged with other flocks and 17/30 (56.7%) Type II flocks were ranged with other flocks in the same area. All Type I ducks were moved from one location to the next in a car or truck. Type II ducks were moved either in a car or truck (11/30; 36.7%) or were herded by the owner or flock workers (19/30; 63.3%).

Table 2.5 – Flock Movement

Flock Movement Parameters	Type I Flocks (%)	Type II Flocks (%)
Flock Movement (area of free-ranging)		
Single village area	13.3	53.3
Subdistrict	40.0	20.0
District	33.3	16.7
Province	13.3	6.7
Outside of province	0.0	3.3
Length of Time in One Location		
1 day, return to home premises at night	0.0	40.0
1 month or less	83.3	23.3
1 to 2 months	16.7	30.0
3 months	0.0	6.7
Commingling with Other Flocks		
Ranged with other flocks	96.7	56.7
Free-Ranging Area		
Post-harvest rice field	100.0	93.1
River, pond or irrigation waterways (primary)	0.0	6.9
River, pond or irrigation waterways (secondary)	3.3	25.9
Movement System		
Car or truck	100.0	36.7
Herded by owner to next location	0.0	63.3

Other animal species were reported by owners to commingle with their duck flocks in 25/30 Type I flocks and 28/30 Type II flocks. For Type I flocks, wild birds were most commonly reported (21/30; 70.0%), followed by village chickens (7/30; 23.3%), sheep (5/30; 16.7%), Muscovy ducks (4/30; 13.3%), goats (3/30; 10.0%), dogs (2/30; 6.7%) and cats (2/30; 6.7%). For Type II flocks, Muscovy ducks were most commonly reported (15/30; 50%), followed by village chickens (14/30; 46.7%), wild birds (13/30; 43.3%), goats (6/30; 20%), dogs (4/30; 13.3%), cats (3/30; 10.0%) and sheep (2/30; 6.7%).

Table 2.6 – Comingling with Other Animals

Flock Commingling	Type I Flocks	Type II Flocks
	(%)	(%)
Commingling reported by flock owners		
Yes	83.3	93.3
No	16.7	6.7
Commingling animal species (all species reported)		
Wild birds	70.0	43.3
Village chickens	23.3	46.7
Village Muscovy ducks	13.3	50.0
Sheep	16.7	6.7
Goats	10.0	20.0
Dogs	6.7	13.3
Cats	6.7	10.0

Live ducks and duck products, including eggs and meat, were sold through a variety of routes. The majority of ducks and duck products (75/90; 83.3%) were sold to collectors or brokers (middlemen), retailers or vendors (sold directly to consumers) or slaughter facilities (ducks sold for meat). One owner (1.1%) sold eggs to a company (contract producer), ten (11.1%) sold products to markets, two (2.2%) sold to neighbors and two (2.2%) did not report where they sold products.

Table 2.7 – Sale of Ducks and Duck Products

Sale of Ducks and Duck Products	Percentage (%)
Sold to middlemen, retailers or slaughter facilities	83.3
Sold to live bird markets	11.1
Sold to neighbors	2.2
Sold to company	1.1
Not reported	2.2

Type I flocks had the least contact with veterinary professionals, with 0/30 (0%) reporting contact with veterinarians and 4/30 (13.3%) reporting contact with para-veterinarian professionals. Five of thirty (16.7%) Type II flocks reported contact with veterinarians and para-veterinarian professionals and 4/30 (13.3%) reported contact with para-veterinarian professionals. Two of thirty (6.7%) Type III flocks reported contact with veterinarians and para-veterinarian professionals and 12/30 (40%) reported contact with para-veterinarian professionals. Of the 12 Type III flocks reporting contact with para-veterinarian professionals, 9 came from one district where 9/10 (90%) flocks had contact with para-veterinarian professionals.

Vaccines and antibiotics were not widely used in the flocks. Not including reports of avian influenza virus vaccination, 6/90 (6.7%) flock owners reported that their flock had been vaccinated. Three of six flocks reported that their flock had been vaccinated with a Newcastle's disease virus vaccine. The other three of six owners did not know which vaccines were administered. Twelve of ninety owners (13.3%) reported administering antibiotics to their current flock.

Flock owners were asked if they commonly see clinical signs of illness in their flocks and 82/90 (91.1%) answered yes. The common clinical signs reported included paralysis (57/90; 63.3%), coughing and/or sneezing and/or respiratory illness (32/90; 35.6%), torticollis (7/90; 7.8%) and diarrhea (5/90; 5.6%). When asked to report the primary response to dealing with sick ducks, just over half (47/90; 52.2%) of flock owners medicate sick ducks, while 32/90 (35.6%) do not treat sick ducks, 7/90 (7.8%) sell sick ducks, 2/90 (2.2%) dispose of sick ducks and 1/90 (1.1%) slaughter and consume sick ducks. One owner did not report which actions were taken with sick ducks. When asked to report the secondary response taken if the sick ducks do not respond to medication or if their illness does not resolve, an additional 16/90 (17.8%)

flock owners reported selling sick ducks and 14/90 (15.6%) reported slaughtering sick ducks for consumption. Owners were also asked to report mortality rate per month. The reports varied widely, from 0% -20% per month.

Five flock owners stated that their flock was previously tested for avian influenza by government veterinary officials. Two of the five flock owners reported that their flock was positive for avian influenza when tested. The two flock owners did not know if their flock was positive for low pathogenic or highly pathogenic avian influenza virus. Both owners reported that their flocks were vaccinated against avian influenza virus. Additionally, two other flock owners reported that their birds were vaccinated against avian influenza virus by government veterinary officials.

Table 2.8 – Flock Health Information

Flock Health Information	Percentage (%)
Pharmaceuticals Administered	
Flock Vaccinated (non-AIV vaccines)	6.7
Antibiotics Administered	13.3
Owner Reported Illness	
Owner reports illness is common	91.1
Clinical Signs of Illness Reported	
Paralysis	63.3
Coughing/sneezing/respiratory disease	35.6
Torticollis	7.8
Diarrhea	5.6
Treatment of Sick Ducks (primary response)	
Medicated	52.2
No treatment	35.6
Sold	7.8
Euthanize and dispose of sick ducks	2.2
Slaughter	1.1
Treatment of Sick Ducks (secondary response)	
Sold	17.8
Slaughtered	15.6
Avian Influenza	
Tested for avian influenza	5.6
Tested positive for avian influenza	2.2
Vaccinated against avian influenza	4.4

Although not all flocks owners reported the methods used for disposing of dead ducks, those that did report reported a number of different methods. Flock owners were asked to report all methods used and many flock owners reported using more than one method of disposal. Fifty-nine of eighty-one (72.8%) buried dead ducks, 33/81 (40.7%) threw dead ducks in a river

or irrigation waterway, 24/81 (29.6%) burned dead ducks, 6/81 (7.4%) used or sold dead ducks as fish or poultry feed and 2/81 (2.5%) processed dead ducks for human consumption.

Table 2.9 – Disposal of Dead Ducks

Disposal of Dead Ducks (all methods used were reported)	Percentage (%)
Burial	72.8
Throw dead ducks in river or other waterway	40.7
Burning	29.6
Sold as fish or poultry feed	7.4
Processed for consumption	2.5

Avian Influenza Virus Prevalence

Based on rRT-PCR testing, 35/90 (38.9%) flocks were positive for avian influenza virus. When evaluated by flock type, 19/30 (63.3%) Type I flocks, 9/30 (30%) Type II flocks and 7/30 (23.3%) Type III flocks were positive for avian influenza virus. The prevalence of avian influenza virus in Type I flocks was significantly higher than Type II ($p = .01$) and Type III flocks ($p = .002$). Of the three districts, Subang had 9 (30%) positive flocks, Tangerang had 13 (43.3%) positive flocks and Indramayu also had 13 (43.3%) positive flocks.

The average number of positive ducks within a positive flock was 10 (30%), with a range of 1-30 positive ducks per flock. When evaluated by flock type, the average number of positive ducks per flock was 11 (36.7%; range 1 – 30) for Type I flocks, 12 (40%; range 1 – 28) for Type II flocks and 7 (23.3; range 1 – 26) for Type III flocks. In total, 361/2700 (13.4%) ducks tested positive for avian influenza virus. Of the positive ducks, 179/361 (49.6%) ducks had a positive oropharyngeal (OP) sample, 100/361 (27.7%) had a positive cloacal (CL) sample and 82/361 (22.7%) had both a positive oropharyngeal and cloacal sample.

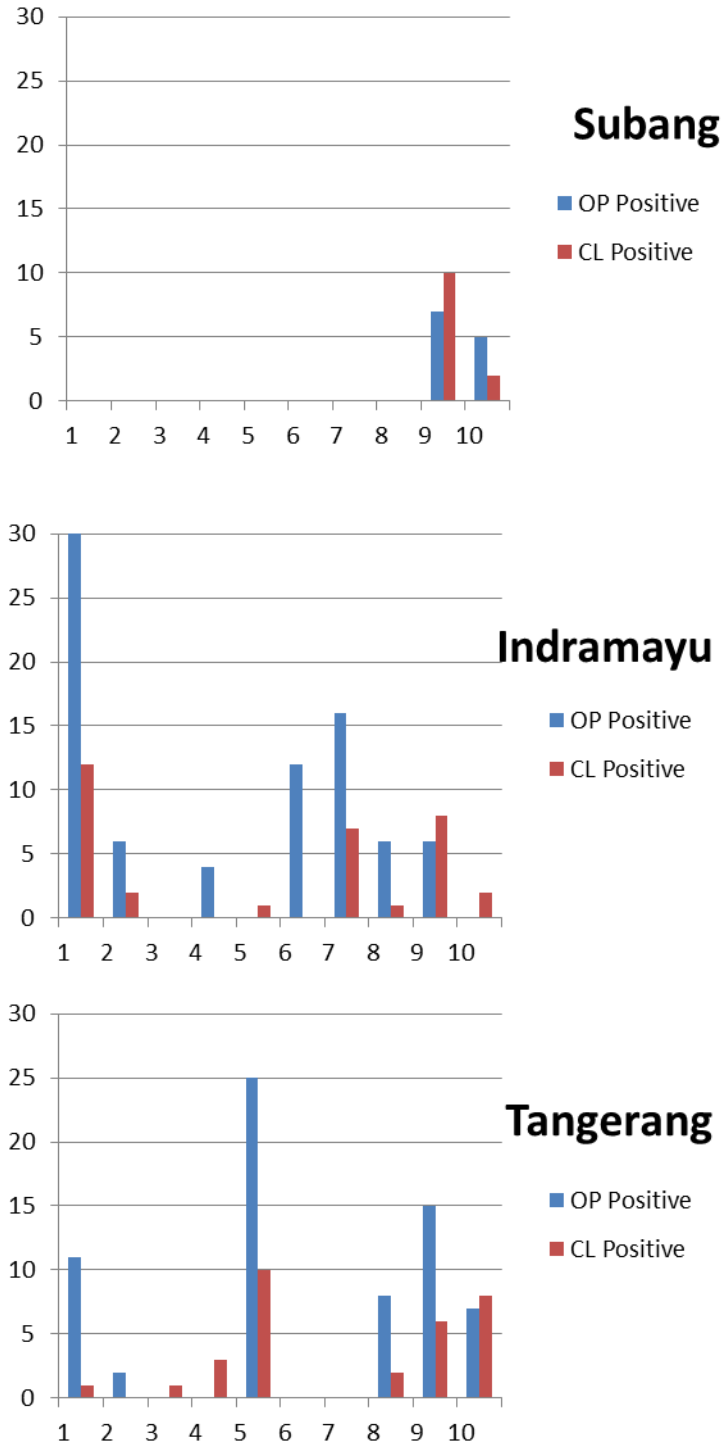

Figure 2.1 – Detection of avian influenza virus in Type I (fully free-ranging) domestic duck flocks in three districts in West Java, Indonesia. The X-axis represents the flock numbers in each district. The Y-axis represents the number of positive ducks. Opharyngeal (OP) and cloacal (CL) positive individual duck samples are depicted.

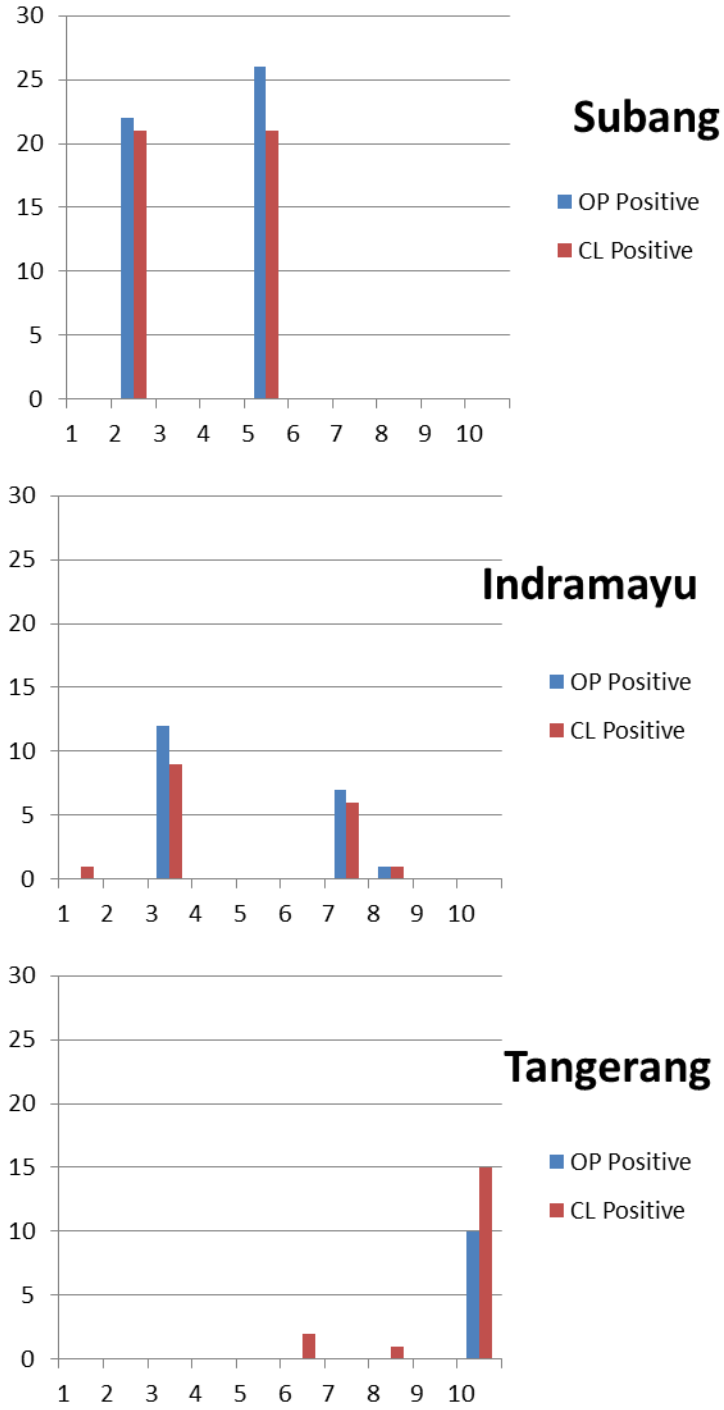

Figure 2.2 – Detection of avian influenza virus in Type II (partially free-ranging) domestic duck flocks in three districts in West Java, Indonesia. The X-axis represents the flock numbers in each district. The Y-axis represents the number of positive ducks. Opharyngeal (OP) and cloacal (CL) positive individual duck samples are depicted.

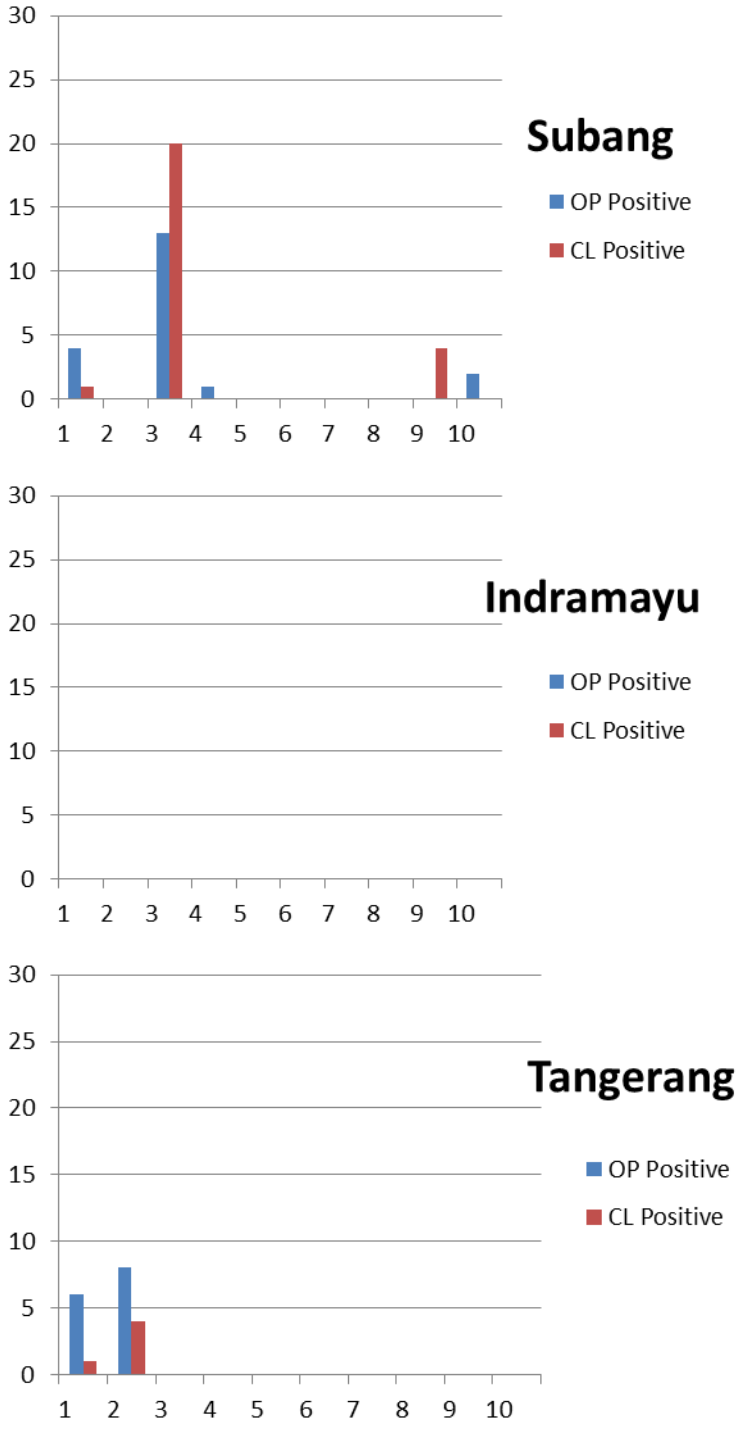

Figure 2.3 – Detection of avian influenza virus in Type III (confined) domestic duck flocks in three districts in West Java, Indonesia. The X-axis represents the flock numbers in each district. The Y-axis represents the number of positive ducks. Opharyngeal (OP) and cloacal (CL) positive individual duck samples are depicted.

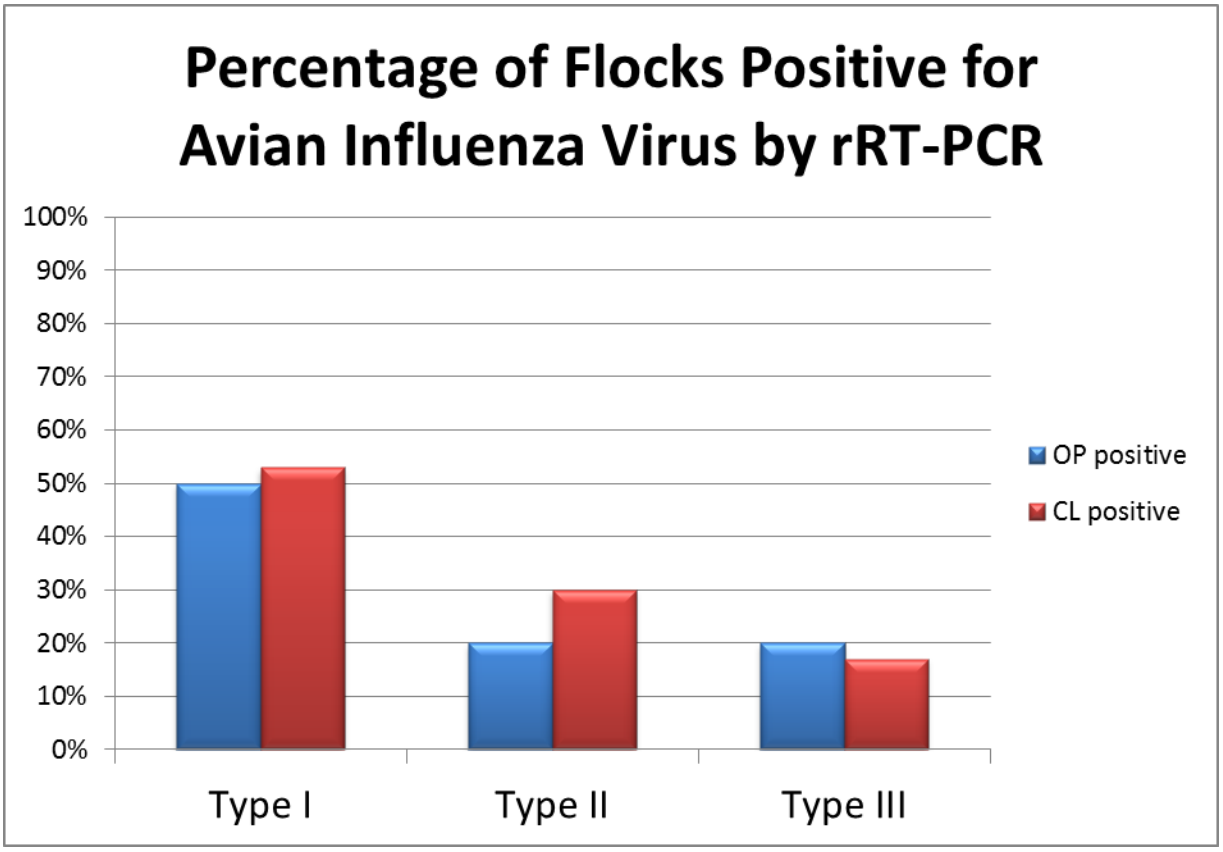

Figure 2.4 – Flock-based prevalence of avian influenza virus for all three districts combined. Prevalence is categorized by flock type (x-axis).

H5 Avian Influenza Virus Prevalence

Fourteen of ninety flocks (15.6%) had one or more ducks that tested positive for H5 subtype avian influenza virus. In total, 29 ducks tested positive for H5 virus, with a range of 1 to 6 positive ducks per flock. All of the flocks with H5 positive ducks also contained non-H5 avian influenza virus positive ducks. When evaluated by flock type, 8/30 (26.7%) Type I flocks, 3/30 (10%) Type II and 3/30 (10%) Type III flocks had one or more H5 positive duck flocks. There was no statistical difference between flock types and H5 virus prevalence ($p = 0.12$). Of the 29 ducks, 27 (93.1%) were positive on an oropharyngeal sample and 2 (6.9%) were positive on a cloacal sample.

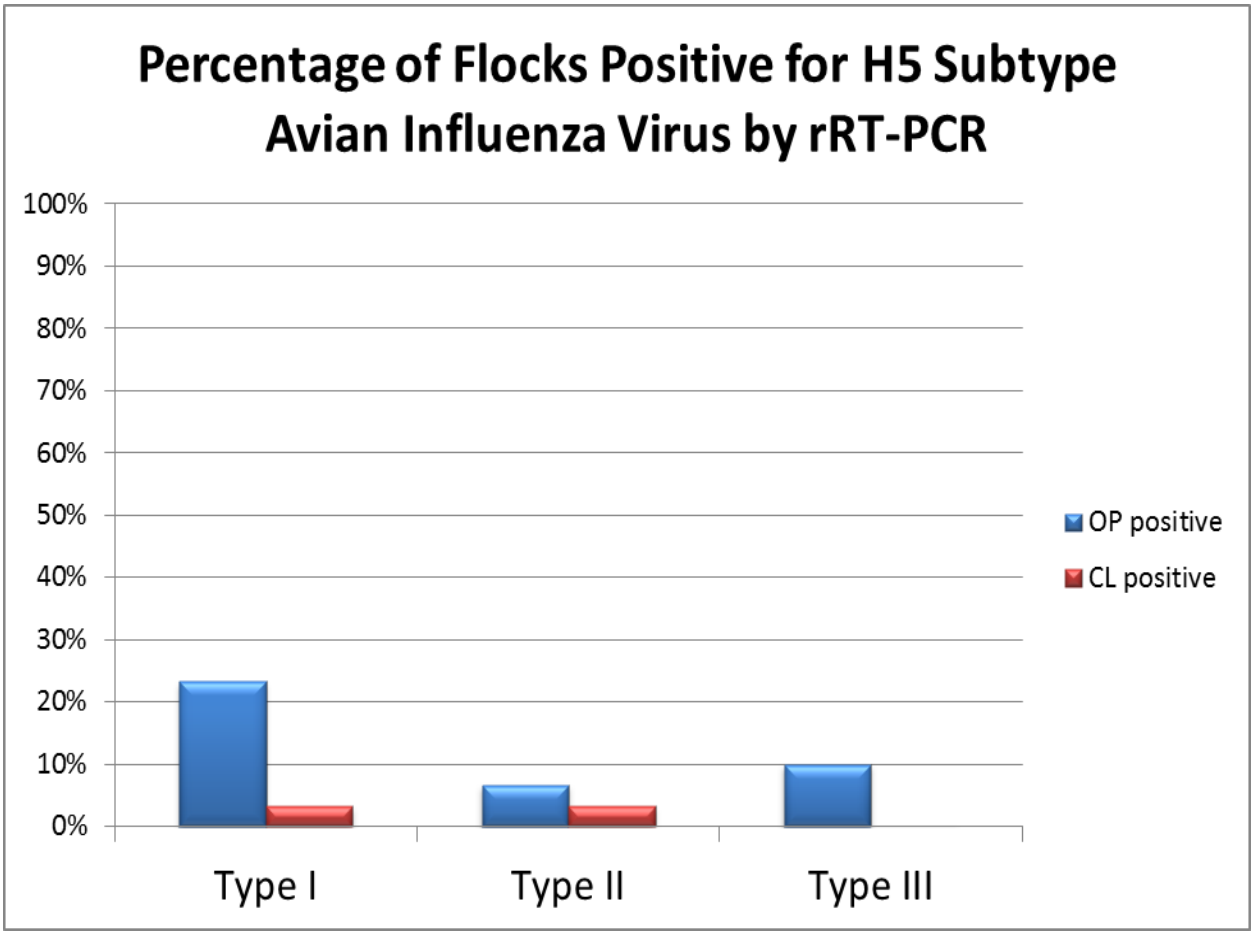

Figure 2.5 – Flock-based prevalence of H5 subtype avian influenza virus for all three districts combined. Prevalence is categorized by flock type (x-axis).

Avian Influenza Virus Seroprevalence

Blood samples were collected from all flocks; however, field veterinarians were not always able to collect 30 blood samples per flock due to difficulty in collecting samples from small ducks and/or owner unwillingness to have blood collected from some ducks. In addition, some of the blood samples collected were small in volume and after centrifugation and serum separation, did not yield the minimum amount of serum required for testing. The full 30 samples were collected from 48/90 (53.3%) flocks. For the remaining 42/90 (46.7%) flocks, an average of 27 (range 19-29) ducks were sampled.

Eighty-seven of ninety flocks (96.7%) were seropositive for avian influenza virus. The three seronegative flocks were Type III flocks in the Subang district. The individual duck seroprevalence was 59.1% for Type I ducks, 60.8% for Type II ducks and 55.1% for Type III ducks. There was a significant difference detected in the seropravelence levels between Type II and Type III flocks ($p = 0.016$). Overall, the Tangerang district had the highest percentage of seropositive ducks per flock, with 70.1%, followed by Indramayu with 57.6% and Subang with 45.6%. Statistically significant differences were detected for seroprevalence for all districts ($p < 0.0001$).

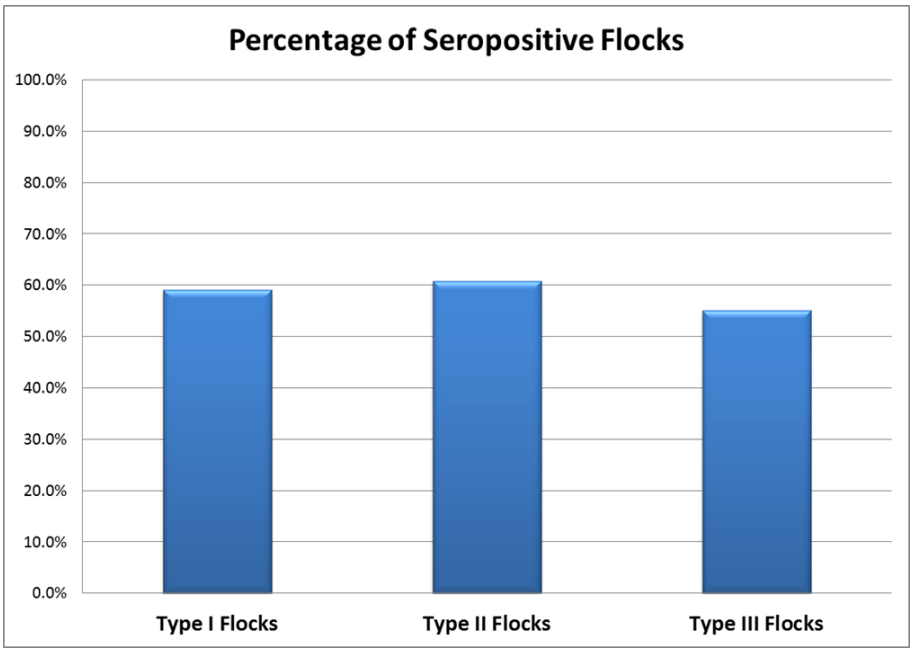

Figure 2.6 – Flock-based seroprevalence for avian influenza virus, categorized by flock type.

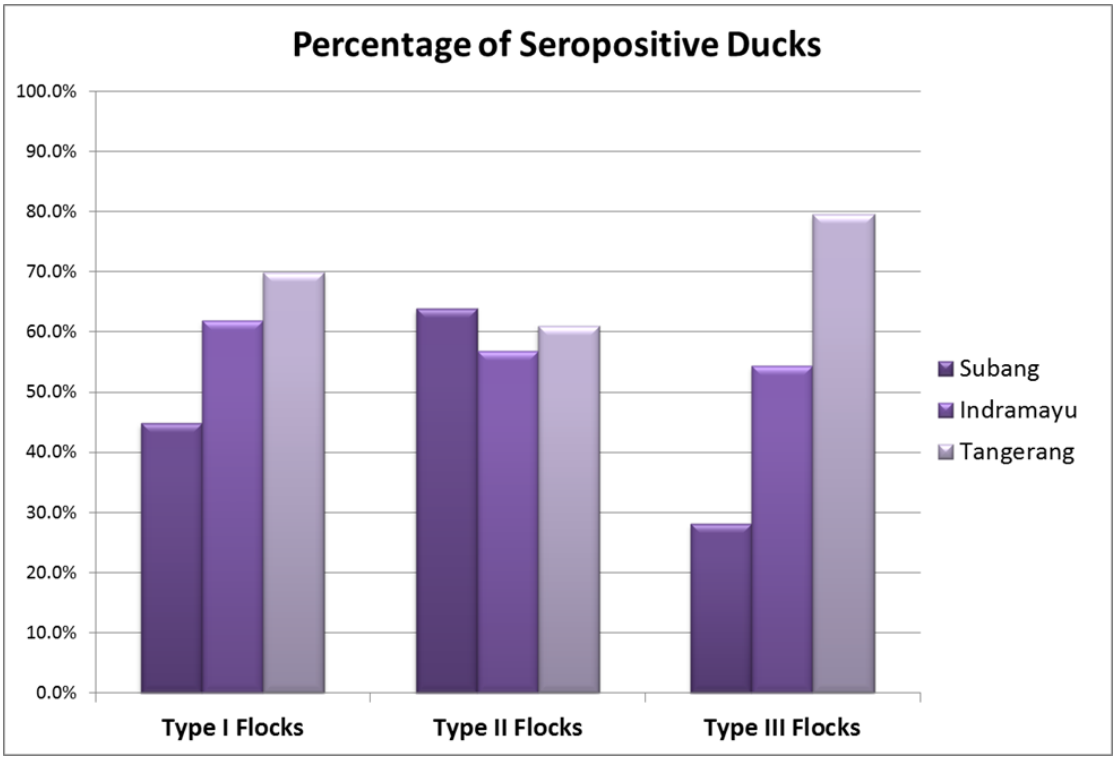

Figure 2.7 – Individual duck-level seroprevalence, categorized by flock type and district.

DISCUSSION

Domestic duck flocks are part of intricate animal production and movement systems in Indonesia. The results of this study demonstrate that domestic duck populations are raised in complex production systems, are highly mobile, have significant contact with wild and domestic birds and mammals, are frequently ill and are provided with little formal veterinary care.

For this study, domestic duck flocks were classified into a three-tiered system. All flocks evaluated in this study readily fell into one of the three flock types, Type I, II or III, based on their production system. This classification system allowed for a more in-depth evaluation of study results by comparing production and movement practices, giving insight into the effects of these practices on disease status. The categorization system also provides a framework for a method to estimate typical behaviors and management styles of domestic duck flocks.

The average flock size was 219 ducks, with reported flock sizes ranging from 31 to 1500 ducks. With an average of 345 ducks per flock, Type I flocks were larger than Type II and Type III flocks which averaged 155 ducks. Because Type I flocks are extensively ranged on post-harvest rice fields, owners may be able to keep larger flocks to increase egg production, as a structured confinement space is not utilized. Domestic ducks in Indonesia are raised as both layer and meat flocks. Most duck flocks are raised primarily as layer flocks, with a production cycle of 3 years or less. Most layer flocks were ranged on post-harvest rice fields, which allow the ducks to scavenge for food and provides service to the rice fields by way of insect control and aeration and fertilization of the field. Flocks raised strictly for meat production have a shorter production cycle when compared to layer flocks, as they are typically sold for slaughter prior to reaching one year of age.

Flock ducks were obtained from a variety of different sources, including breeder flocks, retailers, street vendors, middlemen, acquaintances and live bird markets. Few owners reported hatching their own ducklings. When starting a new flock, most owners purchased ducklings of one month or less in age. While most of the flock owners reported that their flock was composed of birds similar in age, they also reported that they often added or removed ducks from their flock. Much of the individual duck movements of adding ducks to or subtracting ducks from the flock were based on flock size, production or monetary needs. For example, most layer ducks were sold as meat ducks for slaughter at the end of their production cycle or if the owner needed a source of income. This type of movement of ducks provides frequent opportunities for disease introduction, as compared to all-in, all-out management practices.

Live ducks and duck food products, including eggs and meat, were sold through a variety of routes. The majority of ducks and duck products were sold to brokers or middlemen, retailers, street vendors, slaughter facilities and live bird markets. These complicated movement systems make movement of ducks and duck products extremely hard to trace. Of particular concern to extensive movement of ducks and duck products are the middlemen, who purchase ducks and duck products from many different flock owners and sell products through diverse distribution systems, including other middlemen, retailers, restaurants, slaughter facilities and live bird markets. Such an extensive and complex network of movement may allow for widespread disease dissemination in a short timeframe.

The flocks themselves were also moved extensively throughout their production cycle. Type III flocks were raised in confinement; however, the purchase and sales of ducks and duck products, as previously described, contribute significant levels of movement to their overall production system. The Type I fully free-ranging ranging flocks exhibit the most extensive

movement. Eighty-seven percent of the flocks were moved outside of their village area throughout their subdistrict or district area and thirteen percent ranged throughout their entire province. All Type I flocks were ranged on post-harvest rice fields and owners reported staying in one location for only one to two months at a time before moving the flock to a new location. Forty-seven percent of Type II partially free ranging flocks were moved outside of their village throughout their subdistrict or district area, seven percent ranged throughout their province and three percent ranged outside of their province. Many Type II flocks were only ranged during the day and returned to their home base at night, so were often ranged in the same location daily. However, many flocks were ranged on a variety of different locations, staying in one location between one day and three months. Type II flocks were ranged on post-harvest rice fields, rivers, ponds and irrigation waterways. This practice of moving and ranging ducks on rice fields, rivers, ponds and irrigation waterways may contribute to significant environmental contamination and disease dissemination. Ninety-seven percent of Type I flocks and fifty-seven percent of Type II flocks were ranged with other owners' flocks, providing opportunities for disease transmission between flocks.

Flock owners reported significant interaction of their flocks with other birds and mammals, creating the potential for disease transmission between both domestic and wild bird and mammal populations. In addition to their duck flock, many reported owning chickens, Muscovy ducks and pigeons. Type I and Type II flock owners also reported commingling of their flock with domestic and wild birds and mammals, including wild birds, village chickens, village Muscovy ducks, goats, sheep, dogs and cats, when the birds were ranging. Seventy percent of Type I flock owners reported interaction of their flock with wild birds, including wild

waterfowl, creating a connection for transmission of avian influenza viruses from the wild bird reservoir to domestic duck populations.

Flock owners utilized a number of different methods for disposal of dead duck carcasses. In order of reported use and starting with the most commonly reported method, owners disposed of dead ducks via burial (73%), throwing them in rivers or irrigation waterways (41%), burning (30%), feeding them to fish or poultry (7%) and processing for human consumption (3%). If properly conducted, burial and burning are suitable methods for disposal. Throwing carcasses in rivers and irrigation waterways may result in environmental contamination and may potentially expose wild and domestic birds and mammals to infectious organisms. Feeding dead ducks to fish may contaminate water sources and may be of concern in areas were poultry are raised in close proximity to aquaculture ponds. Processing and human consumption of dead ducks is risky considering the ducks may have died from a zoonotic disease. In Indonesia, many carcasses are disposed of improperly due to difficulties in finding a proper space for burial in a population dense island with a high ground water table and frequent flooding events. While burning is a good option, many owners are hesitant to burn large numbers of ducks in a village area because neighbors may object. The flock owners in our study did not report use of composting and likely lack knowledge on this subject.

The majority of flock owners were middle-aged men with a poor educational background. Most flock owners cite duck farming or rice farming as their primary job and most of them have significant experience in raising domestic duck flocks. While significant experience with duck farming gives flock owners knowledge in general production best-practices, the low education level of most of the flock owners is concerning, as general

knowledge of scientific and veterinary concepts such as disease transmission and prevention may be insufficient.

Just over half of flock owners cared for their flocks on their own. The other half of flock owners reported utilizing between one and five additional workers, who were family members or friends of the flock owner. Although few people are in daily contact with domestic duck flocks, the interaction between the flock owners or workers and the ducks is very high. Most Type I flock owners essentially lived with their flocks on the post-harvest rice fields. This type of human-duck interaction may allow for exposure of the owners and workers to zoonotic diseases, including HPAI H5N1 virus.

More than ninety percent of flock owners reported that they commonly see clinical signs of illness in their flocks, including paralysis, coughing, sneezing, torticollis and diarrhea. Owners also reported varying levels of mortality. The causes of the significant levels of illness and mortality reported by flock owners was not elucidated in this study, as only assays for influenza viruses were conducted. Further studies of infectious diseases in domestic ducks in Indonesia are needed to determine which infectious diseases are involved and evaluate production practices that could be addressed to decrease disease prevalence and minimize the impacts on domestic flock production.

Many owners attempt to medicate sick ducks. Ducks that were not treated or ducks that did not improve with treatment were sold, slaughtered for human consumption or euthanized and disposed of. The sale of sick ducks is particularly concerning due to the high potential for dissemination of infectious disease to other bird populations in previously described distribution networks.

Vaccines and antibiotics were not commonly used by flock owners. Few owners reported vaccinating their flocks and of those that did report vaccine use, most could not identify which vaccines were used. Five flock owners reported that their flocks were vaccinated for avian influenza virus, with two of the flock owners reporting that their flock was vaccinated because the ducks tested positive for avian influenza virus. These reports on avian influenza virus diagnosis and vaccination demonstrate some government interactions with domestic duck flocks; however, less than one-third of flock owners reported contact with veterinary professionals. Of the flock owners that reported contact, most were in contact with para-veterinary professionals and only a few were in contact with veterinarians. Type I flocks had the least amount of contact, as they had no contact with veterinarians and only 4 of the 30 flocks had contact with para-veterinary professionals. This lack of contact demonstrates that flock owners may not be properly educated on disease recognition and prevention, may not be reporting disease issues to veterinarians and may not be receiving disease diagnostic and response services from the government.

The flock-level prevalence of avian influenza virus was 39%. The results from the survey demonstrate that domestic duck flocks in Indonesia are at high risk for exposure to avian influenza viruses, which corresponds with the high prevalence detected. Domestic duck flocks have a long production cycle, with some flocks in production up to three years, as compared to poultry production cycles. The flocks do not practice all-in, all-out management and instead, frequently add and remove ducks from the flock. Ducks and duck food products are purchased from and sold to many different sources, including live bird markets and middlemen, and many of these systems are not end-points in the distribution chain but instead further distribute the ducks and duck products. The flocks move extensively and have significant contact with other

domestic duck flocks, domestic birds and mammals and wild birds and mammals. Dead birds are often disposed of improperly in water systems or are fed to fish or poultry or processed for human consumption. The flock owners report that their birds are frequently ill and that they provide little formal veterinary care for their flocks. All of these practices may result in avian influenza introduction to domestic duck flocks, dissemination of the virus between flocks and to other bird or mammal populations and environmental contamination with avian influenza viruses.

At 63%, the prevalence of avian influenza virus was significantly higher in Type I flocks than Type II and Type III flocks, which had prevalence levels of 30% and 23%, respectively. When evaluated in conjunction with survey data, Type I flocks may have the highest prevalence of avian influenza virus due to a number of factors, including longer production cycles of up to three years, extensive movement of the flock throughout the production cycle, commingling with other Type I flocks, high levels of contact with wild birds on the post-harvest rice fields and lack of formal veterinary care. Although not specifically evaluated, wild birds may be the source of introduction for many of the avian influenza viruses detected in Type I flocks, resulting in a higher prevalence in these flocks as compared to Type II and Type III flocks.

On average, 30% of the birds in an avian influenza virus positive flock were individually positive for the virus. As avian influenza viruses have high morbidity, the majority of the ducks in the flock would be expected to test positive at some point during the cycle of the outbreak. Because samples were collected on one day, the virus may have been detected at the beginning or end of the outbreak, when not all of the ducks are shedding virus. There were a number of the flocks in our study where all or almost all of the birds were positive for avian influenza virus, indicating that the samples were collected during the peak of the outbreak.

H5 subtype avian influenza virus was detected in 16% of flocks. In most of the H5 virus positive flocks, only a few birds, with a range from 1 to 6 birds, were positive and in most of the flocks, other ducks tested positive for avian influenza virus but did not test positive on the H5 subtype assay. A previous study by Henning, et al. conducted in Indonesia from 2007-08, demonstrated an H5 virus flock-level prevalence of 2.5% (3). The higher detected flock-level prevalence in our study may be indicative of increased circulation of H5 virus in domestic ducks. However, 10/14 of the H5 positive flocks in this study were given positive status based on detection of only 1 positive bird, indicating the potential detection of false positive cases, potentially due to cross-reaction of the assay resulting in detecting a non-H5 virus as an H5 virus, or a low level of within flock circulation that is difficult to detect due to inefficient viral transmission from duck to duck or mortality of infected ducks. The rRT-PCR assay used is targeted to detect Asian-origin HPAI H5N1 virus but may detect other H5 viruses, so sequencing is required to characterize the virus detected.

In total, 1% of the ducks tested in the study were positive for H5 subtype avian influenza virus. This demonstrated an overall low prevalence of H5 avian influenza virus. However, since the impact of H5 viruses, particularly HPAI H5N1 virus, and concern over spread of these viruses is so high, any detection should be considered significant. Although we did not specifically test for H7 subtype virus, it is likely that most of the avian influenza viruses detected in this study are low pathogenic viruses.

Half of the avian influenza virus positive ducks tested positive on the oropharyngeal sample but not on the cloacal sample. The other half of ducks tested positive on cloacal sample (28%) or on both oropharyngeal sample and cloacal sample (23%). These results are interesting considering that in many surveillance studies, only cloacal samples are collected for ducks, as it

is expected that ducks tend to shed avian influenza viruses primarily in cloacal secretions (4, 9, 16). However, more attention has been focused on collection of oropharyngeal samples, due to HPAI H5N1 virus tropism for the respiratory system (7, 8). The results of this study demonstrate the necessity to collect both oropharyngeal and cloacal samples for surveillance programs. For the H5 subtype assay, almost all of the ducks were positive on the oropharyngeal sample and only a few were positive on the cloacal sample, demonstrating the predilection for ducks to shed H5N1 virus in respiratory secretions and further indicating that collection of oropharyngeal samples during surveillance programs is essential.

The flock-level seroprevalence was very high, with 97% of flocks positive for avian influenza virus antibodies. Only three seronegative flocks were detected and all were located in the district with the lowest avian influenza virus prevalence, as compared to the other two districts. There were significant differences in individual duck seroprevalence, with the highest seroprevalence detected in Type II ducks, with 61% of ducks positive for avian influenza antibodies, followed by 59% of Type I ducks and 55% of Type III ducks. The high number of seropositive flocks corresponds with the high prevalence of avian influenza virus in domestic duck flocks and demonstrates that although Type I flocks have the highest avian influenza virus prevalence, all flock types are exposed to a wide variety of avian influenza viruses. Type I flocks may be more frequently exposed to different avian influenza viruses, resulting in a higher virus prevalence but similar seroprevalence to the other flocks.

This study had several limitations. Response bias may be a contributing source of error in the results. Flock owners may have under-reported or over-reported their practices and instead reported their interpretation of best-practices if they perceived that negative implications would result from their participation in this study. Participating flocks were selected based on a

convenience sample, which may result in selection bias. In addition, the sample size for this study was relatively small, which may limit the ability to detect differences among flock types.

The results of this study provide information on domestic duck flocks in Indonesia, including characterization of general production and movement practices and owner reported flock health parameters. Domestic duck flocks are at high risk for introduction of avian influenza virus and other infectious diseases. Of most concerning is the potential introduction of HPAI H5N1 virus. The flocks may also be involved in dissemination of avian influenza viruses to other duck flocks, poultry flocks and wild bird populations through the extensive movement networks created by flock owners for movement of ducks and duck products. Transmission of zoonotic avian influenza virus, particularly H5N1 virus, is of concern for flock owners and flock workers due to high levels of interaction between humans and ducks. The flocks have a high level of prevalence and seroprevalence for avian influenza virus, demonstrating the important role domestic ducks play in the maintenance of avian influenza viruses in Indonesia.

References

1. **Gilbert, M., P. Chaitaweesub, T. Parakamawongsa, S. Premashthira, T. Tiensin, W. Kalpravidh, H. Wagner, and J. Slingenbergh.** 2006. Free-grazing ducks and highly pathogenic avian influenza, Thailand. Emerging infectious diseases **12:**227-234.

2. **Henning, J., K. A. Henning, J. M. Morton, N. T. Long, N. T. Ha, T. Vu le, P. P. Vu, D. M. Hoa, and J. Meers.** 2011. Highly pathogenic avian influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Viet Nam. Preventive veterinary medicine **101:**229-240.

3. **Henning, J., H. Wibawa, J. Morton, T. B. Usman, A. Junaidi, and J. Meers.** 2010. Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. Emerging infectious diseases **16:**1244-1250.

4. **Hinshaw, V. S., W. J. Bean, R. G. Webster, and G. Sriram.** 1980. Genetic reassortment of influenza A viruses in the intestinal tract of ducks. Virology **102:**412-419.

5. **Hulse-Post, D. J., K. M. Sturm-Ramirez, J. Humberd, P. Seiler, E. A. Govorkova, S. Krauss, C. Scholtissek, P. Puthavathana, C. Buranathai, T. D. Nguyen, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster.** 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proceedings of the National Academy of Sciences of the United States of America **102:**10682-10687.

6. **Kim, J. K., N. J. Negovetich, H. L. Forrest, and R. G. Webster.** 2009. Ducks: the "Trojan horses" of H5N1 influenza. Influenza and other respiratory viruses **3:**121-128.

7. **Kim, J. K., P. Seiler, H. L. Forrest, A. M. Khalenkov, J. Franks, M. Kumar, W. B. Karesh, M. Gilbert, R. Sodnomdarjaa, B. Douangngeun, E. A. Govorkova, and R. G. Webster.** 2008. Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. Journal of virology **82:**11374-11382.

8. **Pantin-Jackwood, M. J., and D. E. Swayne.** 2007. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. Avian diseases **51:**250-259.

9. **Slemons, R. D., and B. C. Easterday.** 1978. Virus replication in the digestive tract of ducks exposed by aerosol to type-A influenza. Avian diseases **22:**367-377.

10. **Songserm, T., R. Jam-on, N. Sae-Heng, N. Meemak, D. J. Hulse-Post, K. M. Sturm-Ramirez, and R. G. Webster.** 2006. Domestic ducks and H5N1 influenza epidemic, Thailand. Emerging infectious diseases **12:**575-581.

11. **Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez.** 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of clinical microbiology **40:**3256-3260.

12. **Spackman, E., and D. L. Suarez.** 2008. Detection and identification of the H5 hemagglutinin subtype by real-time RT-PCR. Methods in molecular biology **436:**27-33.

13. **Spackman, E., and D. L. Suarez.** 2008. Type A influenza virus detection and quantitation by real-time RT-PCR. Methods in molecular biology **436:**19-26.

14. **Center for Indonesian Veterinary Analytical Studies,** 2006. A Review of Free Range Duck Farming Systems in Indonesia and Assessment of their Implication in the Spreading of the Highly Pathogenic (H5N1) Strain of Avian Influenza (HPAI). A Report from the Center for Indonesian Veterinary Analytical Studies (CIVAS) for the Food and Agricultural Organization (FAO) of the United Nations. [Online, Accessed July 5, 2012].

15. **Sturm-Ramirez, K. M., D. J. Hulse-Post, E. A. Govorkova, J. Humberd, P. Seiler, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. Chaisingh, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster.** 2005. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? Journal of virology **79:**11269-11279.

16. **Webster, R. G., M. Yakhno, V. S. Hinshaw, W. J. Bean, and K. G. Murti.** 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology **84:**268-278.

Chapter 3

Maintenance of Avian Influenza Viruses in Domestic Duck Flocks in Indonesia

INTRODUCTION

Previous studies have demonstrated that ducks can shed HPAI H5N1 virus for a period of up to 17 days post infection (1, 5, 7). This extended period of shedding, combined with the knowledge that domestic ducks may be asymptomatically infected by HPAI H5N1 virus (3, 4, 8), has raised global concern that domestic ducks may be a source of maintenance of the virus (2, 5, 12).

In the previously described cross-sectional study, the flock-level point prevalence for avian influenza virus in domestic duck flocks in Indonesia was almost 40%. In addition, the flock-level point prevalence for H5 subtype avian influenza virus was 15%. These results demonstrate the high level of prevalence for avian influenza viruses in domestic duck flocks and support the concern that domestic ducks may play an important role in the ecology of HPAI H5N1 virus.

The knowledge gained in the first study was used to develop a longitudinal study, in order to monitor avian influenza viruses in domestic duck flocks over time. The objectives of this study were to estimate the incidence of avian influenza virus, including H5 subtype viruses, in domestic duck populations and to evaluate the persistence of avian influenza viruses and duration of shedding. For this study, ducks were placed in domestic duck flocks for a seven month period which included the two meteorological seasons in Indonesia. Although these study ducks were not all avian influenza virus naïve ducks at the time of placement, they are referred to as “sentinel” ducks for ease of reference in this dissertation. The sentinel ducks were sampled

monthly. The repeated sampling of the sentinel ducks provided an evaluation of viral persistence and duration of viral shedding.

MATERIALS AND METHODS

Flock Categorization and Selection

All study flocks were categorized into one of three flock types, based on production styles and movement practices, just as they were for the cross-sectional study. Fully free-ranging flocks with no home premises were classified as Type I flocks. Partially free-ranging flocks that periodically returned to a home premises were classified as Type II flocks. Confined or intensively reared flocks that did not free range were classified as Type III flocks. In addition, the same three districts in West Java used in the cross-sectional study, Subang, Tangerang and Indramayu, were selected for this study. Two flocks of each flock type were selected per district, for a total of 18 flocks.

The study was approved by district government livestock officials. The livestock officials assisted in identifying qualifying flocks for participation in this study and provided contact information for the flock owners. The Research Integrity and Compliance Review Office at Colorado State University approved the use of human and animal subjects. All flock owners gave informed consent prior to participation in the study and all researchers followed approved animal handling and sampling protocols.

Study Timeframe

The study was conducted over a seven month period from September to March. These months correspond with the end of the dry season (September – October) and most of the rainy

season (November – March) in Indonesia and were selected to allow for seasonal variation and to determine if there is enhanced avian influenza transmission during the rainy months. Each flock was visited monthly for each of the seven months of the study.

Flock Survey

A survey was administered to participating flock owners during every visit, with the exception of the first month when the sentinels were sampled and placed in the flocks. The survey included open-ended and close-ended questions addressing flock size, health history and flock mortality over the past month. Current GPS coordinates were collected during each visit to capture movement parameters of Type I and II flocks. Based on the previous study, it was expected that Type I fully free-ranging flocks would move every month or every other month and Type II partially free-ranging flocks would move to new locations daily, weekly or monthly, depending on the flock.

Sentinel Selection and Identification

Based on information from the previous study, a sample size estimate was calculated using a population of 200 ducks (previous study showed an average of 219 ducks per flock) and an expected prevalence of 20% (the lowest prevalence in the previous study was in Type III flocks, with a prevalence of 23.3%). With a confidence level of 95% and test sensitivity of 90%, the estimated sample size was 15 ducks, based on a calculation to detect presence or absence of disease. The number was increased to 30 ducks per flock to account for the expected attrition of sentinel ducks.

In an effort to allow for testing of the same ducks throughout the study, each flock owner was provided with funding to purchase thirty sentinel ducks. The flock owners were asked to purchase ducks through their normal procurement system. The flock owner was instructed to keep the sentinels in a pen separated from the rest of his flock until the first set of samples was collected from the sentinel ducks during month 1. If the owner refused to introduce new ducks into his flock, he was permitted to select 30 ducks from his current flock to serve as sentinel ducks. The 30 sentinels were numbered and tagged by research personnel for identification purposes, so individual birds could be followed throughout the study. Ducks were given a numbered metal wing tag and a leg band and were additionally marked by the owner with cloth around the wing or via another marking system. The owners were asked to maintain the sentinels throughout the period of the study. If the sentinels lost their identification tags during the study, they were retagged with a new identification number. In specific instances, if the number of sentinel ducks was diminishing due to death or loss, research personnel selected non-sentinel ducks from the owner's flock to replace the lost sentinel ducks. These ducks were identified and tracked as replacement sentinels.

Sample Collection and Testing

One the first and last months of the study (months 1 and 7), 30 sentinel ducks and 30 non-sentinel flock ducks, or as many as were alive and available, were sampled. On months 2 through 6, only the sentinel ducks were sampled. During month 1, sentinel duck samples were collected within 7 days of purchase of the sentinel ducks and all ducks were kept in a pen separated from the rest of the flock but on the same premises until the time of sampling. After the first sampling, all sentinel ducks were released to live with the rest of the flock. If one or

more of the 30 individually identified sentinel ducks were not available on the sampling day due to loss or death of the duck, all available sentinel ducks were sampled.

During each monthly flock visit, research personnel collected both an oropharyngeal and cloacal swab from each sampled duck. Polyester-tipped, plastic shafted swabs were used to collect the sample and swabs were immediately placed in 1ml of brain heart infusion broth. All samples were labeled with flock and individual duck identifying accession numbers. All samples were kept refrigerated immediately after collection and during transport to the laboratory. All samples were received by the laboratory within 72 hours of collection and were stored in a -70°C freezer.

Pools composed of five oropharyngeal or five cloacal samples per pool were made, for 6 pools per flock. When the total number of samples collected for one flock was less than 30, the samples were evenly divided into 6 pools. Individual samples were also maintained for further testing. RNA was extracted from each sample using the Ambion MagMAX-96 AI/NDV Viral Isolation Kit (Applied Biosystems, Foster City, CA), according to manufacturer's directions. Two previously reported avian influenza virus real-time reverse transcription polymerase chain reaction (rRT-PCR) assays were used to test the samples for avian influenza virus (9-11) using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems 7500 96-well plate thermocycler. The first assay was designed to detect the matrix gene of avian influenza virus, identifying all avian influenza viruses. Individual samples that were part of matrix positive pools were tested for H5 subtype avian influenza virus using a second assay designed to detect the H5 subtype hemagglutinin gene. Individual samples were considered positive for H5 avian influenza virus if the cycle threshold value was 40 or less.

Results Interpretation

All sample pools were considered positive for avian influenza virus if the rRT-PCR cycle threshold value was 40 or less. Flocks were considered positive for avian influenza virus if one or more sample pools was positive by rRT-PCR. Individual samples were considered positive for H5 avian influenza virus if the rRT-PCR cycle threshold value was 40 or less.

Data Analysis

Data were statistically analyzed using SAS software version 9.2 (SAS Institute Inc., Cary, NC). Incidence rates for avian influenza virus in Type I, II and III flocks were evaluated and compared using Poisson regression with generalized estimating equation to account for clustering. Probability values (p) less than or equal to 0.05 were considered statistically significant.

RESULTS

Maintenance and Sampling of Sentinel Ducks

Thirty sentinel ducks were obtained for each flock and individually identified at the onset of the study. Most of the flock owners (11/18; 61.1%) agreed to obtain new ducks to serve as sentinel ducks and did so through their typical procurement system. Some of the flock owners declined to introduce new ducks into their flock (7/18; 38.9%) and instead were permitted to select 30 ducks from their current flock to serve as sentinel ducks.

Every month, research personnel sampled the 30 sentinel ducks or as many ducks were alive and locatable at the time of the visit. Overall, there was a general attrition of the sentinel ducks every month due to loss or death. During the first month of the study, six flocks lost 2

(two flocks), 3 (two flocks), 6 (one flock) or 10 (one flock) sentinel ducks due to death or loss of the duck. Because this was the beginning of the study, the research personnel selected additional ducks from the owner's flock to serve as sentinels. Replacement sentinel ducks were tagged in three flocks during the remainder of the study, in order to increase sentinel numbers. Tangerang flock 3 replaced 1 duck during month 4, Subang flock 4 replaced 5 ducks during month 3 and Indramayu flock 2 replaced 2 ducks during month 5. The majority of lost or dead ducks were not replaced, so the total number of ducks sampled per flock per month was variable and typically less than 30 ducks per flock. On average for all flocks, there were 30 sentinel ducks sampled per flock on the first month, 29 on the second month, 28 on the third month, 26 on the fourth month, 25 on the fifth month, 22 on the sixth month and 21 on the seventh month. When categorized by flock type, Type III flocks maintained the highest number of sentinel ducks, with 25 sentinel ducks on the seventh month, while Type I and Type II flocks had 20 and 19 sentinel ducks, respectively. One Type I flock and flock owner, could not be located in month 6 so was not sampled. The following month, the flock and owner were located, but only 7 sentinel ducks remained in the flock due to mortality of 12 sentinel ducks in the preceding month.

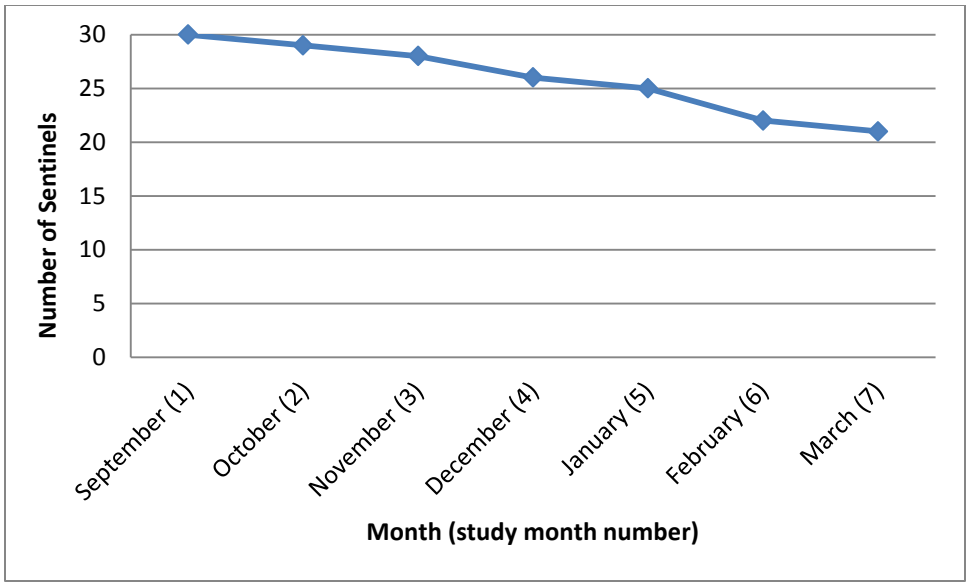

Figure 3.1 – Average number of sentinels sampled per month

Flock Characteristics

The average duck age at the beginning of the study was 9.7 months. The average flock size on the last month of the study was 281 ducks per flock and the median flock size was 128 ducks per flock (range 20-2000). One large Type II flock of 2000 ducks was an outlier and when removed, the average and median number of ducks per flock on the last month of the study were 179 and 105, respectively (range 20-500). During the study, individual flock sizes were highly variable due to addition, sale, loss or death of ducks. Flock sizes were not uniformly collected during the first 2 months of the study but were collected for the last 5 months of the study. The large 2000 duck flock added, removed or lost at least 2750 ducks over the last 5 months of the study. The other 17 duck flocks added, removed or lost an average of at least 211 ducks per flock (range 4 – 556) over the last 5 months of the study.

Six of the duck farmers (6/11; 54.5%) purchased ducks or ducklings from brokers or middlemen. The other 5 farmers (5/11; 45.5%) purchased fertile eggs and hatched their own

ducklings. Two of the flocks (Indramayu flocks 2 and 4) replaced their flock with new birds on the last month of the study.

Flock Health

Ten of eighteen (55.6%) flocks reported significantly increased mortality in their flock in one or more months during the study. Seven of eighteen flocks (38.9%) reported increased mortality during two or more months during the study. An additional 3 flocks (16.7%) reported clinical illness without increased mortality during at least one month during the study. Flock owners reported a variety of clinical signs of illness in their duck flocks and some reported more than one clinical sign of illness in one or more months. Paralysis and ataxia were most commonly reported and were reported by 8/18 (44.4%) flocks in one or more months, followed by respiratory illness, which was reported by 5/18 (27.8%) flocks in one or more months, and diarrhea, which was reported by 2/18 (11.1%) flocks in one or more months. Four of eighteen flock owners (22.2%) reported unexpected mortality with no preceding clinical signs of illness.

As previously discussed, there was a general attrition of the sentinel ducks over the course of the study. On average, 11 sentinel ducks were lost for each flock. In most cases, the flock owner reported that the sentinel duck was known to have died. Some of the sentinel ducks could not be accounted for so were presumed dead; however, some of the ducks may have actually been alive but lost due to movement of the flock or commingling with other flocks or loss of all sentinel identification tags.

Detection of Avian Influenza Virus Positive Flocks

Flocks were considered positive for avian influenza virus during a specific month of the study if at least one sentinel duck sample pool was positive during the month in question. Throughout the study, 17/18 (94.4%) flocks were positive for avian influenza virus at least one month during the study. This equates to an incidence of 94 avian influenza positive flocks per 100 domestic duck flocks over a 7 month period. A number of flocks were frequently positive throughout the study, with 12/18 (66.7%) flocks positive in two or more months, 7/18 (38.9%) flocks positive in three or more months, 5/18 (27.8%) flocks positive in four or more months and 1/18 (5.6%) flocks positive all seven months of the study. Overall, 13/18 (72.2%) flocks were positive during the dry season and 12/18 (66.7%) flocks were positive during the rainy season.

Of the newly procured groups of sentinel ducks, 7/11 (63.6%) tested positive for avian influenza virus upon or shortly after introduction to the new flock. However, in all but 1 of these flocks (6/11; 54.5%) the non-sentinel flock ducks also tested positive for avian influenza virus.

In order to determine the level of dissemination of avian influenza viruses from month to month, numbers of positive pools per month were evaluated for each flock. In half of the flocks (9/18; 50%), avian influenza virus was detected in the sentinel ducks in two or more consecutive months at some point during the study. There were 5 (27.8%) flocks that had high levels of detection of avian influenza virus (50% or more of the sample pools were positive) in two or more non-consecutive months, with either a lower level of detection of or no detection of avian influenza virus in the intermediate months. There was 1 flock (5.6%) where in one month at least 50% of the sample pools were positive and no detection of avian influenza virus in the preceding or following month. In the first month of the study, 5 (27.8%) flocks were positive for

avian influenza virus in at least 50% of the sample pools, with no positive pools in the month immediately following.

When categorized by flock type, Type I flocks were most frequently positive for avian influenza virus. On average, Type I flock sentinels were positive for 4 out of 7 months (range 1 – 7 months) of the study, Type II flock sentinels were positive for 2.3 months (range 1 – 5 months) and Type III flock sentinels were positive for 1.5 months (range 0 – 2) out of 7 months of the study.

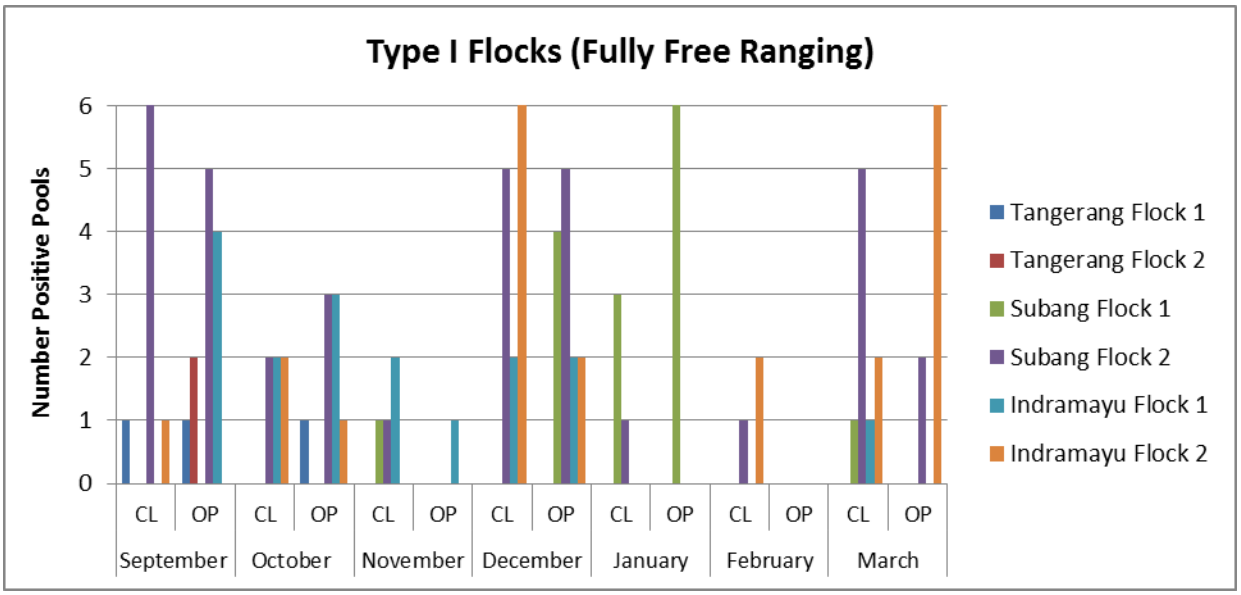

Figure 3.2 – Frequency of detection of avian influenza virus and number of avian influenza virus positive pools for each flock over a seven month period for Type I flocks. CL = cloacal sample, OP = oropharyngeal sample.

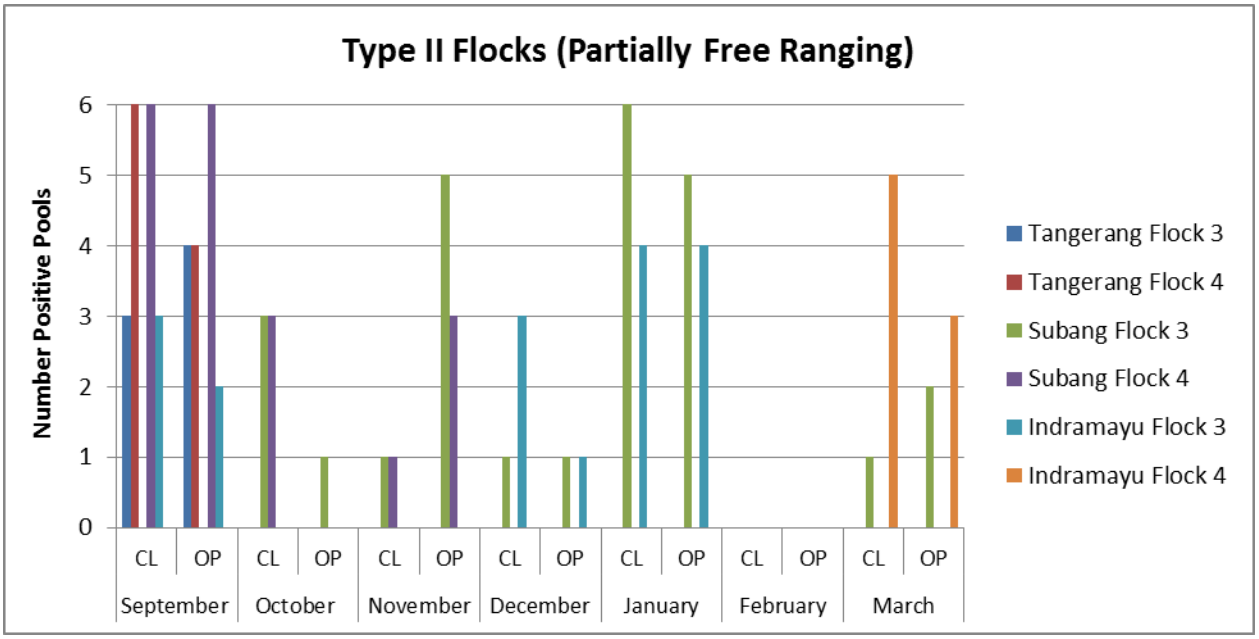

Figure 3.3 – Frequency of detection of avian influenza virus and number of avian influenza virus positive pools for each flock over a seven month period for Type II flocks. CL = cloacal sample, OP = oropharyngeal sample.

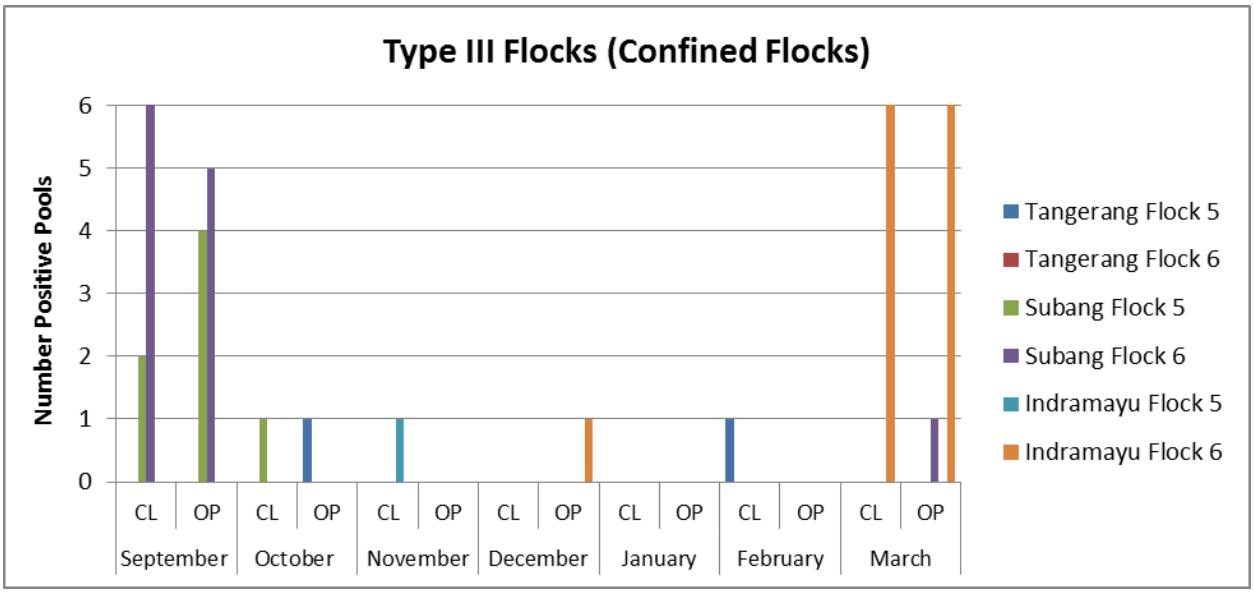

Figure 3.4 – Frequency of detection of avian influenza virus and number of avian influenza virus positive pools for each flock over a seven month period for Type III flocks. CL = cloacal sample, OP = oropharyngeal sample.

A flock was considered positive for avian influenza virus in one month if one or more of either of the oropharyngeal or cloacal sample pools, or both, were positive. In testing of both the sentinels (months 1 – 7) and non-sentinels (months 1 and 7) in the 18 study flocks, there were 65 positive detections, where at least one sample pool is positive in one month. In most (43/65; 66.2%) instances, one or more oropharyngeal and one or more cloacal sample pools were positive, with an average of 6.9 avian influenza virus positive pools. In 7/65 (10.8%) instances, one or more oropharyngeal sample pools were positive but no cloacal pools were positive, with an average of 2.0 positive oropharyngeal pools. In 15/65 (23.1%) instances, one or more cloacal pools were positive but no oropharyngeal pools were positive, with an average of 1.3 positive cloacal pools.

The presence or absence of avian influenza virus in the sentinels was compared to sentinel mortality for months 2 – 7, with the exception of the flock that was not sampled in

month 6. For avian influenza virus positive flocks, in 20/107 (18.7%) months, the owner reported mortality of one or more sentinel ducks and in 15/107 (14.0%) months the flock was positive and the owner did not report sentinel duck mortality. For avian influenza virus negative flocks, in 48/107 (44.9%) months, the owner reported mortality of one or more sentinel ducks and in 24/107 (22.4%) months the owner did not report sentinel duck mortality.

Detection of H5 Avian Influenza Virus Positive Flocks

Individual samples that were part of avian influenza virus positive sample pools were tested for H5 subtype avian influenza virus. Overall, 9/18 (50%) flocks were positive during the study for H5 subtype virus based on testing of both sentinel and non-sentinel ducks. For all flocks, the detection of H5 subtype virus was singular, with no H5 virus detected in the months before or after the month of detection. The detection of H5 subtype virus in individual ducks is shown in Figures 3.5 – 3.7. For these figures, non-sentinel ducks sampled on month 1 and month 7 were also included because Indramayu flock number 4 had a high number of positive non-sentinel flock ducks, as compared to the number of positive sentinel ducks in month 7. Additionally, Indramayu flock number 3 and Tangerang flock number 5 had positive non-sentinel ducks and no positive sentinel ducks in months 1 and 7, respectively.

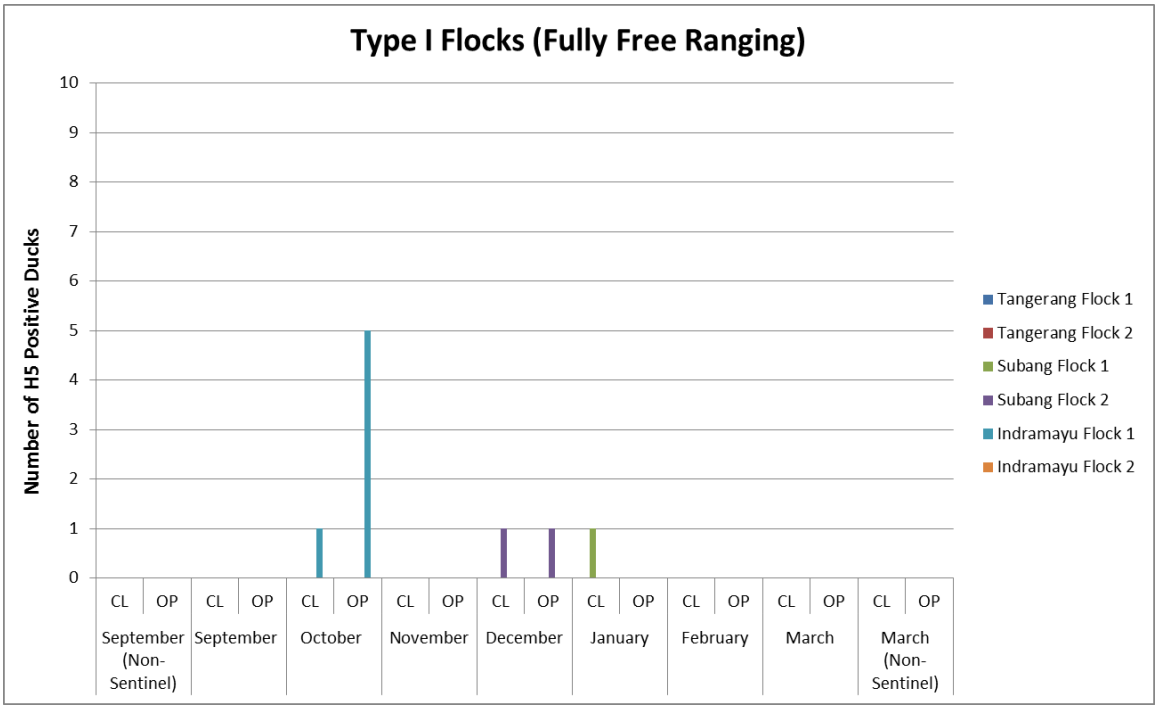

Figure 3.5 – Frequency of detection of H5 subtype avian influenza virus and number of H5 virus positive pools for each flock over a seven month period for Type I flocks. CL = cloacal sample, OP = oropharyngeal sample.

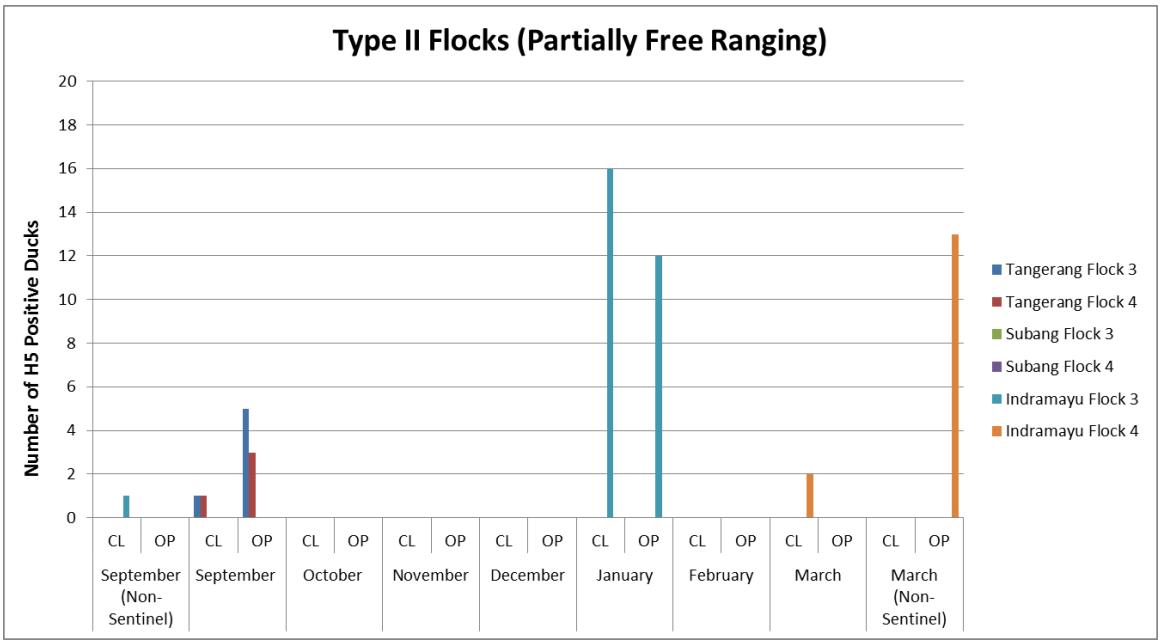

Figure 3.6 – Frequency of detection of H5 subtype avian influenza virus and number of H5 virus positive pools for each flock over a seven month period for Type II flocks. CL = cloacal sample, OP = oropharyngeal sample.

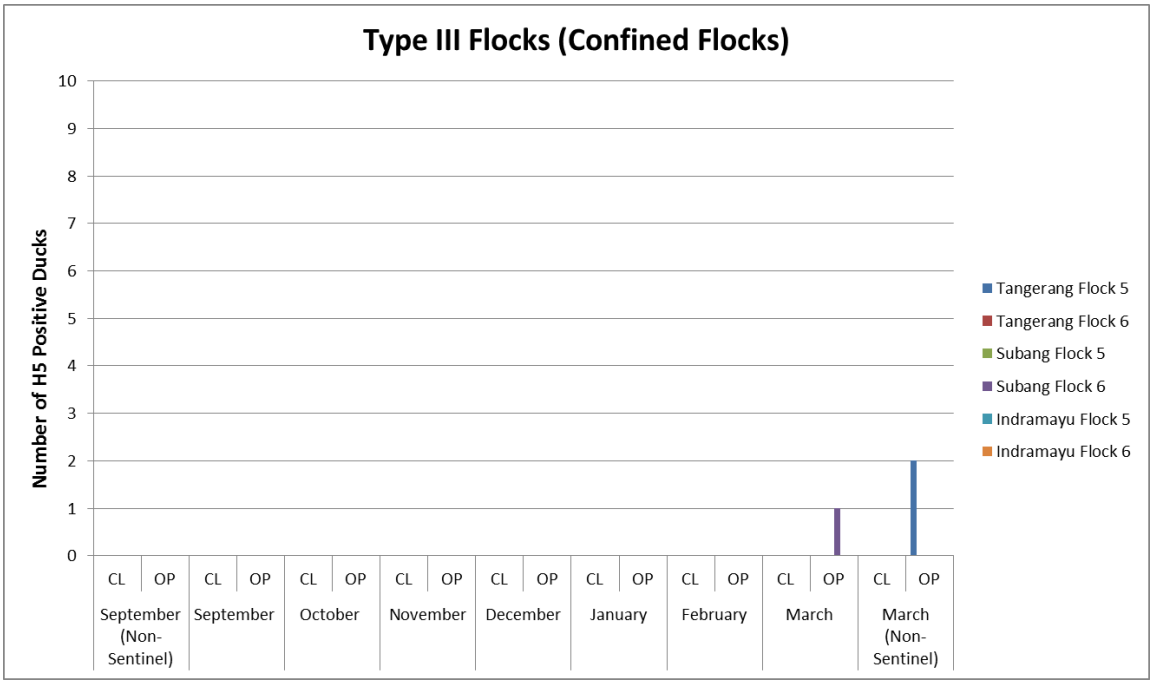

Figure 3.7 – Frequency of detection of H5 subtype avian influenza virus and number of H5 virus positive pools for each flock over a seven month period for Type III flocks. CL = cloacal sample, OP = oropharyngeal sample.

On average, 17.7% of ducks in a flock classified as an H5 virus positive flock were individually positive for H5 virus. Of the H5 positive ducks, 13/53 (24.5%) were positive on both the oropharyngeal and cloacal sample, 29/53 (54.7%) were positive on oropharyngeal sample only and 11/53 (20.8%) were positive on cloacal sample only.

Three of the flocks were positive for H5 virus during month 1, when sentinels were newly introduced to the flock so no mortality data is available. Of the remaining H5 positive flocks, 5/7 (71.4%) reported no sentinel mortality during the month the H5 virus was detected. Of the 2/7 (28.6%) flocks that did report sentinel mortality, the flocks reported 10 and 2 dead sentinels.

DISCUSSION

The purpose of this study was to follow domestic duck flocks over time, to evaluate the incidence of avian influenza virus in this population, as well as evaluate virus persistence and shedding. Flock owners were asked to maintain a group of sentinel ducks for this study, which would provide a consistent population to test. According to the original study design, the intent was to raise a population of avian influenza virus negative ducks to use as the naïve sentinel ducks. Based on the results from the previous study, it was determined that avian influenza virus seronegative ducks would be difficult to obtain so the next option was to raise a flock of naïve ducks to use as sentinels. Unfortunately, a closed facility in which to raise naïve ducks was available. In addition, the flock owners were very hesitant to allow ducks to be introduced into their flock that did not originate from their typical procurement system. They were very concerned that our study ducks would be inferior as production ducks in comparison to their current ducks and would hinder the overall flock production. They also had some concerns that

our ducks could introduce disease into their flocks. Because the use of naïve ducks was impractical for this study, flock owners were asked to procure ducks through their regular route. Most of the flock owners procured new ducks, although some of them declined to add new birds to the flock. In these flocks, the owners were permitted to select 30 of their current flock birds to serve as sentinels.

Maintaining the sentinel ducks within each flock proved challenging. Based on the study design, each of the sentinel ducks was to be sampled monthly for the seven months of the study. However, many flock owners had difficulties maintaining the sentinel ducks and research personnel were not able to sample every sentinel duck every month due to death of the duck or inability to locate the duck due to loss when commingling with other flocks or loss of the sentinel identification tags. Locating individual ducks every month also proved challenging, as some of the ducks could not be located certain months, despite significant efforts, due to difficulties in identifying the duck in large, open areas or inability to catch the duck. In addition, some of the flock owners inadvertently sold or removed sentinel ducks from the flock. Although a considerable number of the sentinel ducks were lost or died during the period of the study, on average, 21 original sentinel ducks remained as part of the flock on the last month, which exceeded the sample size of 15 ducks required to determine the presence or absence of disease. All flocks maintained at least 15 sentinels throughout the study, with the exception Indramayu flock 1, in which only 7 sentinel ducks remained on the last month of the study. Type III flock owners were able to maintain the most sentinels throughout the study, likely because the ducks are in confinement so easier to maintain, do not experience mortality associated with free-ranging and are harder to lose, as compared to Type I fully free-ranging and Type II partially free-ranging flocks.

Over the course of the study, individual flock sizes were highly variable, as ducks were frequently added and removed from their flock. On average, individual flock sizes changed by 211 ducks over the last five months of the study. Exact numbers of added/removed/lost ducks were difficult to determine since flock size was reported monthly. The difference in numbers could be determined, but this did not exactly account for what actually occurred in the flocks. A decrease in flock size of 200 ducks did not necessarily reflect an exact decrease in 200 ducks but instead, for example, could be the removal or loss of 300 ducks and addition of 100 new ducks. Even though exact numbers could not be ascertained, the results demonstrate the frequent addition and subtraction of ducks in domestic duck flocks and the potential for introduction of infectious disease into the flock.

Owners were asked monthly if overall mortality was higher than expected during the previous month. Just over half of the flock owners reported a significant increase in duck mortality in one or more months and 39% reported increased mortality in two or more months during the study. An additional 17% of flocks reported clinical illness in the absence of increased mortality. These results are especially concerning as they demonstrate that domestic duck flocks frequently experience significant illness, which often results in an increase in duck mortality. Since the sentinel ducks were only tested for influenza viruses, the causes of flock illness and/or increased mortality were not determined; however, many of the clinical signs of illness reported, particularly paralysis and other neurologic clinical signs and unexplained sudden death, are consistent with severe infectious diseases, including HPAI H5N1 virus and other duck transboundary animal diseases such as duck virus hepatitis and duck parvovirus. While not all of the reports of clinical illness and mortality may be due to an outbreak of an infectious disease, it

remains possible that many of them are, demonstrating the potential frequent introduction of infectious diseases into domestic duck flocks.

Interestingly, the owner reports of increased flock morality did not always correlate with sentinel duck mortalities, as owners reported increases over their "expected" level of mortality. Many of the flock owners reported the expected loss of a few ducks per month, so the loss of a few sentinels every month was not out of the ordinary for the flock owners. Over the course of the study, an average of one-third of the sentinels were reported dead or lost from the flock. A significant majority of the sentinels were reported as dead, with death confirmed by the owner, but some were never found, so it is not known if the duck was lost, sold or died in the field. Regardless, significant mortality of sentinels was detected, further demonstrating the frequent mortality experienced by domestic duck flocks.

Although the sentinel ducks were sampled within 7 days of purchase and were separated from the owner's flock until the time of sampling, the results cannot be interpreted to determine if the sentinel ducks transmitted avian influenza to the flock ducks or vice versa. Most of the introduced sentinels tested positive for avian influenza virus at the outset of the study, demonstrating that the addition of new ducks into a flock can serve as a source of introduction of influenza virus into the flock. The flock owners reported many production practices that may serve as a source of introduction of avian influenza viruses, including frequent addition of new ducks, significant contact between their duck flocks with other duck flocks and other wild and domestic birds, and frequent sale of duck products to middlemen and live bird markets. Additional risk factor analyses may elucidate specific practices that increase the likelihood of introduction of avian influenza virus.

As determined by this study, the incidence of avian influenza virus in domestic duck flocks in Indonesia over a 7 month timeframe is 94 out of every 100 duck flocks (94% of the flocks). This incidence was not unexpected, since the previous study estimated the overall point prevalence for avian influenza virus to be 38.9% of flocks. Interestingly, many of the flocks were frequently positive for avian influenza virus throughout the study, with 67% positive in two or more months, 39% positive in three or more months and 28% positive in four or more months. One flock was positive every month during the study.

Based on the rRT-PCR results alone, it cannot be determined if multiple positive detections for one flock within the 7 month period are due to a single avian influenza virus that is moving through the flock over 2 or more months in a smoldering fashion or if the positive results are due to the detection of two or more different avian influenza virus, due to separate introductions of different viruses. In half of the flocks, avian influenza virus was detected in two or more consecutive months, indicating that the same virus may be slowly moving through the flock with continual flock shedding over a number of months. In almost 35% of the flocks, there was a high level of detection of avian influenza virus in two or more non-consecutive months, with either a low level of detection or no detection of avian influenza virus in the intermediate months. This may indicate flock infection with two different avian influenza viruses during the study. Further testing of samples, including virus isolation and sequencing, could provide insight on frequency of infection with two or more different viruses in this short timeframe.

Incidence of avian influenza viruses typically peak during the rainy season and decline during the dry season, likely due to environmental persistence of the virus. In this study, 72% of the flocks were positive in the first two months of the study, which occurred during the dry season in Indonesia. Sixty-seven percent of the flocks were positive in the last five months of

the study, which occurred in the rainy season in Indonesia. These results suggest that while the historical incidence of avian influenza virus is higher in the rainy season, the importance of disease detection in the dry season should not be overlooked.

Similar to the results of the previous study, Type I fully free-ranging flocks had the highest incidence of avian influenza virus, as compared to Type II and Type III flocks (stats to be inserted). The higher incidence may be due to a number of production practices, including frequent movement of the flocks, commingling with other flocks, longer production life of the ducks and significant interaction with wild birds while free ranging.

Overall, 50% of the flocks were positive for H5 subtype avian influenza virus at some point during the study. In a positive flock, an average of 18% of the ducks were positive. In all cases, H5 virus was detected in a singular month, with no H5 virus detected in the months before or after. These results suggest that H5 viruses may move quickly though the flock and the period of viral shedding by individual ducks may be less than one month, which is consistent with other studies (5, 7). On the last month of the study, Indramayu flock 4 had a high number of positive non-sentinel ducks (13) and a lower positive number of sentinel ducks (2). Studying this flock proved interesting because a few days prior to sampling, the owner had replenished his flock with new birds (non-sentinels) and, based on test results, likely purchased H5 positive birds that then transmitted virus to the sentinel ducks.

Both oropharyngeal and cloacal samples were collected for testing to provide information on viral shedding by domestic ducks. For most avian influenza virus positive flocks, both oropharyngeal and cloacal samples were positive. When only one positive sample type was detected in a flock, cloacal samples were positive more often than oropharyngeal samples, as expected. For the H5 positive flocks, more than half of the ducks were positive on

oropharyngeal sample alone, as expected, since ducks primarily shed H5N1 virus in respiratory secretions (7). The significant detection of avian influenza virus in oropharyngeal samples highlights the importance of collecting this sample, in addition to cloacal samples, when testing domestic ducks.

The avian influenza testing results were evaluated against mortality or loss of sentinel ducks but no correlation was made, as expected, since avian influenza viruses, with the exception of HPAI H5N1 viruses, typically do not cause clinical illness or mortality in duck flocks. Sentinel mortality was reported in specific months for both avian influenza virus positive and negative flocks and was detected in a higher level in negative flocks. Likewise, no mortality was reported in specific months for both positive and negative flocks. Similarly, there was no correlation between increased mortality for an H5 virus positive flock, as the owners reported no sentinel mortality for most of the H5 positive flocks. Even for the flocks that did report mortality, because other causes of mortality were not ruled out, H5 avian influenza virus could not be attributed as the cause of the increased mortality. While mild to moderate mortality has been associated with H5N1 virus in ducks (6, 7), from the results of this study, it appears that mortality may be limited.

A population of sentinel ducks proved difficult to maintain and the attrition of sentinel ducks decreased the sample size every month of the study. However, the impact of the decreased samples size was minimized, because the study started with double the number of ducks needed as determined by the sample size calculation. Maintenance of the minimum sample size, with the exception of one flock, allowed for detection of the presence or absence of avian influenza viruses.

Due to the inability to introduce avian influenza virus naïve sentinel ducks into the study flocks, there was no way to assure that the sentinel ducks had no prior exposure to avian influenza virus. Ducks with prior exposure may be seropositive for avian influenza virus antibodies, which could result in protection from infection and thus, may impact the results if a duck flock was exposed to an avian influenza virus but did not shed detectable levels of virus. However, because antibodies are only somewhat cross-protective due to specificity to a certain virus, even if the ducks had antibodies to specific viruses, they were likely exposed to different viruses where antibody protection was low or insignificant due to the extremely high genetic variation within avian influenza viruses.

An rRT-PCR cycle threshold cutoff value of 40 cycles was used to determine positive status of an rRT-PCR assay. This value was selected to allow for high sensitivity for the detection of avian influenza virus positive flocks. In addition, the previous study showed that pooling of samples will results in a higher cycle threshold value for the pooled sample, as compared to the individual sample. Using a cycle threshold cutoff value that is lower may result in misclassifying positive samples as negative. With the higher sensitivity cutoff value, the potential for false positives increases, and some of the flocks classified as positive in this study may not have been truly positive. The determination of the cutoff value was based on results from the previous study.

Also, the individual samples from matrix positive pools were not tested, due to funding limitations. Therefore, we can determine overall flock-based virus status, but cannot determine the numbers of individual ducks within a flock that are positive or negative. This issue does not significantly limit study findings, as poultry flocks are typically classified with a positive or negative disease status based on the concept that if even one bird is positive, the flock is

considered positive. In this study, if one sample pool was positive, at least one duck was considered positive, and the entire flock was classified as positive.

Characterization of the avian influenza viruses detected in this study should be conducted. Characterizing the viruses would provide essential information on avian influenza virus infections in domestic duck flocks. Of most concern is the detection of H5 avian influenza viruses in this study, as some of these detected viruses may be HPAI H5N1 viruses. Current knowledge on HPAI H5N1 virus outbreaks in domestic duck flocks, particularly in Indonesia, is limited. In addition, there are few HPAI H5N1 virus sequences in GenBank, making tracking of virus mutation over time a challenge. Because of the nature of HPAI H5N1 virus, including ability to mutate, zoonotic potential and impact on poultry production, characterization of this virus is of the utmost importance.

References

1. **Bingham, J., D. J. Green, S. Lowther, J. Klippel, S. Burggraaf, D. E. Anderson, H. Wibawa, D. M. Hoa, N. T. Long, P. P. Vu, D. J. Middleton, and P. W. Daniels.** 2009. Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. Avian pathology **38:**267-278.

2. **Guan, Y., R. Webby, I. Capua, and J. Waldenstrom.** 2012. H5N1: How to track a flu virus. Nature **483:**535-536.

3. **Henning, J., K. A. Henning, J. M. Morton, N. T. Long, N. T. Ha, T. Vu le, P. P. Vu, D. M. Hoa, and J. Meers.** 2011. Highly pathogenic avian influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Viet Nam. Preventive veterinary medicine **101:**229-240.

4. **Henning, J., H. Wibawa, J. Morton, T. B. Usman, A. Junaidi, and J. Meers.** 2010. Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. Emerging infectious diseases **16:**1244-1250.

5. **Hulse-Post, D. J., K. M. Sturm-Ramirez, J. Humberd, P. Seiler, E. A. Govorkova, S. Krauss, C. Scholtissek, P. Puthavathana, C. Buranathai, T. D. Nguyen, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster.** 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proceedings of the National Academy of Sciences of the United States of America **102:**10682-10687.

6. **Pantin-Jackwood, M. J., D. L. Suarez, E. Spackman, and D. E. Swayne.** 2007. Age at infection affects the pathogenicity of Asian highly pathogenic avian influenza H5N1 viruses in ducks. Virus research **130:**151-161.

7. **Pantin-Jackwood, M. J., and D. E. Swayne.** 2007. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. Avian diseases **51:**250-259.

8. **Songserm, T., R. Jam-on, N. Sae-Heng, N. Meemak, D. J. Hulse-Post, K. M. Sturm-Ramirez, and R. G. Webster.** 2006. Domestic ducks and H5N1 influenza epidemic, Thailand. Emerging infectious diseases **12:**575-581.

9. **Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez.** 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of clinical microbiology **40:**3256-3260.

10. **Spackman, E., and D. L. Suarez.** 2008. Detection and identification of the H5 hemagglutinin subtype by real-time RT-PCR. Methods in molecular biology **436:**27-33.

11. **Spackman, E., and D. L. Suarez.** 2008. Type A influenza virus detection and quantitation by real-time RT-PCR. Methods in molecular biology **436:**19-26.

12. **Sturm-Ramirez, K. M., D. J. Hulse-Post, E. A. Govorkova, J. Humberd, P. Seiler, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. Chaisingh, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster.** 2005. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? Journal of virology **79:**11269-11279.

Chapter 4

Characterization of Indonesian Avian Influenza Viruses

INTRODUCTION

Influenza viruses continually undergo antigenic shift and drift, resulting in the frequent emergence of new viruses. The HPAI H5N1 virus is of particular concern to the global health community, as this virus is highly pathogenic in poultry (14, 15) and can be transmitted directly from poultry to humans, causing severe and often lethal infection in humans (1, 11). Monitoring of HPAI H5N1 viruses is essential for the detection and evaluation of divergent strains.

The Asian-origin HPAI H5N1 virus lineage has been divided into multiple clades based on the amino acid sequences of the hemagglutinin surface glycoprotein. From the original clade 0 progenitor virus detected in geese in Guangdong Province, China in 1996, 20 distinct virus clades have been identified. Due to increasing divergence, second-, third- and fourth-order clades have been assigned (2, 3).

Indonesian HPAI H5N1 viruses are designated as clade 2.1 viruses, with 2.1.3 viruses currently circulating (8, 9, 13). Indonesian lineage viruses have remained relatively stable as compared to other HPAI H5N1 virus lineages; however, very little virus sequence information has emerged from Indonesia, due to government restrictions on removal of HPAI H5N1 positive samples from the country, as well as limited in-country molecular capabilities. Continued monitoring of the virus is crucial to understanding circulating viruses and detecting emergence of viruses with pandemic potential.

MATERIALS AND METHODS

Diagnostic Samples

One hundred and fifty domestic duck oropharyngeal and cloacal swab samples collected in Indonesia were shipped to Colorado State University for further analysis. The Indonesian Ministry of Agriculture and Bogor Agricultural University granted approval for analysis of the 150 samples, prior to removal of the samples from Indonesia. The samples were collected as part of the two previously discussed cross sectional and longitudinal avian influenza studies. Samples were selected based on results of rRT-PCR performed in our Indonesian research laboratory, with H5 positive samples receiving priority.

Virus Isolation in Embryonated Eggs and RNA Extraction

Samples were vortexed and centrifuged, then a 0.5ml portion of the supernatant was aliquoted for inoculation. The inoculum was treated with 0.5ml of an antibiotic and antifungal solution, containing 20,000 units/ml penicillin G, 0.004 gm/ml streptomycin sulfate, 0.65mg/ml kanamycin sulfate, 2.0 gm/ml gentamicin sulfate and 0.02 mg/ml amphotericin B, for 1 hour to kill bacteria and fungi present in the sample. Each sample was inoculated into two 9- to 11-day old specific-pathogen-free (SPF) embyronated chicken eggs (0.2ml sample per egg) via the allantoic route. Eggs were incubated for 72 hours at 99^{o}F and candled twice daily. Eggs with dead or dying embyros were refrigerated for 4 hours and harvested. Eggs with live embyros were refrigerated overnight, prior to harvest. Approximately 3-5ml of amniotic allantoic fluid (AAF) was harvested from each egg. Aliquots of the harvested AAF were inoculated into another two SPF embyronated chicken eggs for a second passage, following the same procedure (7, 10, 19).

A hemagglutination assay was used to test the amniotic allantoic fluid from both the passage 1 and passage 2 eggs for the presence of an infectious agent with hemagglutinating activity. Using a microtiter plate, 50µl of phosphate buffered saline was added to every well in a row on the plate, followed by 50 µl of AAF in the first well. The contents of the first well were mixed, then two-fold dilutions were made down the row of the plate. 50 µl of 0.5% chicken red blood cells (Colorado Serum Company, Denver, CO) were added to every well and the plate was left at room temperature for 30 minutes. The assay plate was evaluated for the presence of hemagglutination (6, 10).

For each passage, the amniotic allantoic fluid harvested from two eggs inoculated with the same sample were combined, yielding one passage 1 AAF sample and one passage 2 AAF sample per original swab sample. RNA was extracted from all AAF samples, including hemagglutination negative AAF, using the Ambion MagMAX-96 AI/NDV Viral Isolation Kit (Applied Biosystems, Foster City, CA), according to manufacturer's directions.

Virus isolation, hemagglutination assays and RNA extraction were performed in a biosafety level 3 laboratory, following established biosafety protocols.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

All RNA samples were screened for avian influenza virus using the previously described avian influenza virus matrix gene and H5 hemagglutinin subtype gene rRT-PCR protocols. All rRT-PCR matrix positive samples were selected for sequencing.

Two protocols were used to amplify the hemagglutinin gene for sequencing. The first protocol, designed by Hoffmann, et al. (5) requires the generation of cDNA. RNA was transcribed into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR

(Invitrogen, Life Technologies, Grand Island, NY) and the Hoffman primer Uni12 (AGCAAAAGCAGG). This was followed by amplification of the cDNA using the Qiagen One-Step RT-PCR Kit (Qiagen, Valencia, CA) and the hemagglutinin gene primers designed by Hoffmann, et al.

The second protocol, designed by the USDA, allows for direct RT-PCR amplication, using the Qiagen One-Step RT-PCR Kit (Qiagen, Valencia, CA) and RNase Inhibitor (Promega, Madison, WI). The PCR reaction followed USDA protocols(18) and utilized USDA designed hemagglutinin gene primers(17).

Table 4.1 – Primer sets used for RT-PCR amplification of the hemagglutinin gene

	Forward Primer	**Reverse Primer**
E. Hoffmann, et al	Bm-HA-1: TATTCGTCTCAGGGAGCAAAAGCAGGGG	Bm-NS-890R: ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT
USDA	H-T7: TAATACGACTCACTATAAGTAGAAACAAGGGTG	HggA or Hggt: CTCTTCGAGCAAAAGCAGGGGA CTCTTCGAGCAAAAGCAGGGGT

The PCR products were purified from agarose gel using the Qiagen QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), following manufacturer's instructions.

Sequencing

The RT-PCR products were sequenced using the same amplification primers used for RT-PCR. The sequence reaction was prepared using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and processed using an ABI 3130xL Genetic Analyzer (Applied Biosystems, Foster City, CA) by the Colorado State University Proteomics and Metabolomics Facility.

Sequence Analysis

A GenBank nucleotide BLAST search was performed to identify the sample sequences and compare to similar sequences. Sequences were aligned and further analyzed using the Influenza Research Database (16). The Influenza Research Database was also used to generate a phylogenetic tree for the Indonesian H5N1 viruses sequenced.

RESULTS

Diagnostic Samples

All of the samples evaluated were tested by rRT-PCR in an Indonesian research laboratory at Bogor Agricultural University run by collaborators on this study. The samples were shipped on dry ice to Colorado State University. All of the samples evaluated were positive by rRT-PCR for avian influenza virus. In addition, most of the samples were also positive for H5 virus by rRT-PCR. The majority of the samples (121/150; 80.7%) were from the 7 month long longitudinal study, although 29/150 (19.3%) were from the cross-sectional point prevalence study. All of the cross-sectional study samples were individual oropharyngeal samples. From the longitudinal study, 42/121 (34.7%) were individual oropharyngeal samples, 22/121 (18.2%) were individual duck cloacal samples, 37/121 (30.6%) were pooled oropharyngeal samples and 20/121 (16.5%) were pooled cloacal samples.

Virus Isolation

Avian influenza virus was isolated from 38/150 (25.3%) samples. Of the 38 isolated viruses, 2 were from the cross-sectional study and 36 from the longitudinal study. All of the

isolated viruses were positive for hemagglutinating activity and were positive for avian influenza virus by rRT-PCR.

Sequencing

Thirty-three sequences were generated from the thirty-eight avian influenza viruses isolated. Viruses sequenced include Asian-origin HPAI H5N1 virus and H7, H3 and H2 viruses. These viruses were identified in samples originating from 8 different flocks. Identification of viruses is described in the following table.

Table 4.2 – Avian Influenza Viruses Identified

Virus Subtype	Number of Samples	Number of Flocks
H5N1 virus	21	3
H5N1 virus + H7 virus	2	1
H5N1 virus + H3 virus	1	same flock as H5N1 + H7 virus positive
H7 virus	6	3
H2 virus	1	1
H3 virus	2	same flock as H5N1 + H7 virus positive

HPAI H5N1 virus was isolated from three flocks – one from the Tangerang district during the first month of the study (Tangerang flock 3), one from the Indramayu district during the fifth month of the study (Indramayu flock 3) and the last from the Indramayu district during the seventh month of the study (Indramayu flock 4). The Indramayu flock that was positive during month 7 was also positive for H7 and H3 viruses. All HPAI H5N1 flocks were Type II flocks.

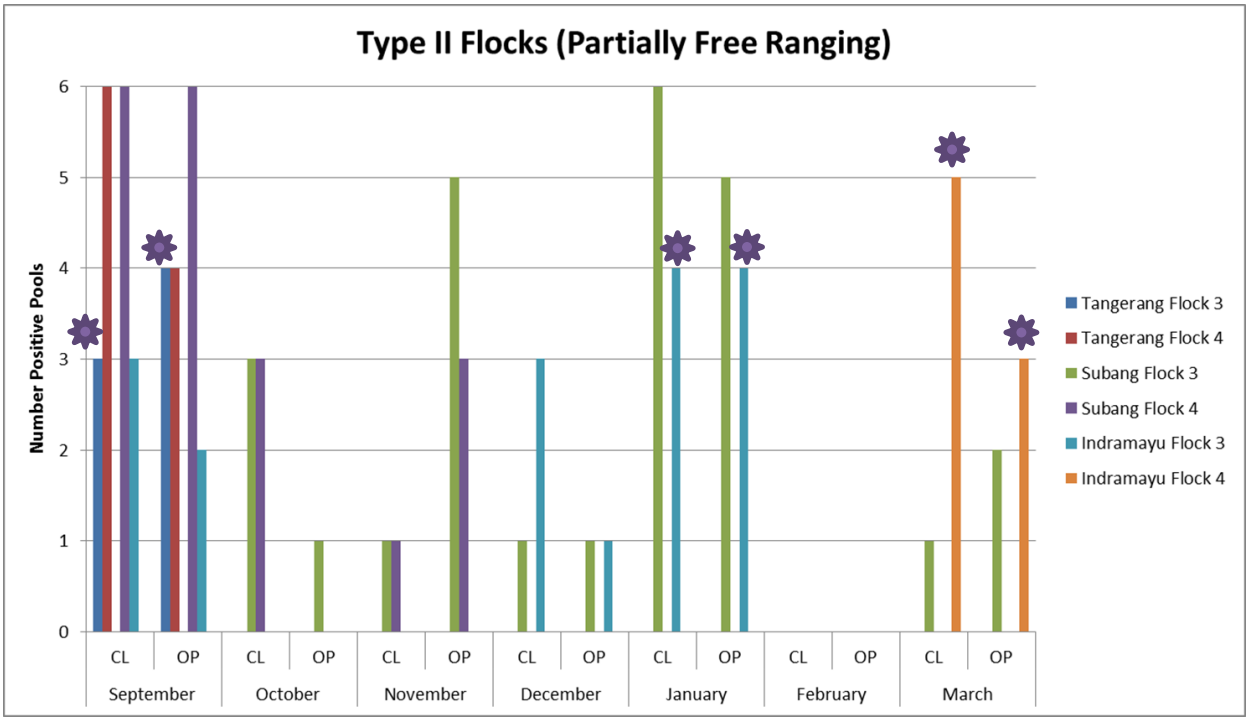

Figure 4.1 - Detection of avian influenza virus and number of avian influenza virus positive pools for each flock over a seven month period for Type II flocks. H5N1 positive flocks are denoted using a purple star symbol.

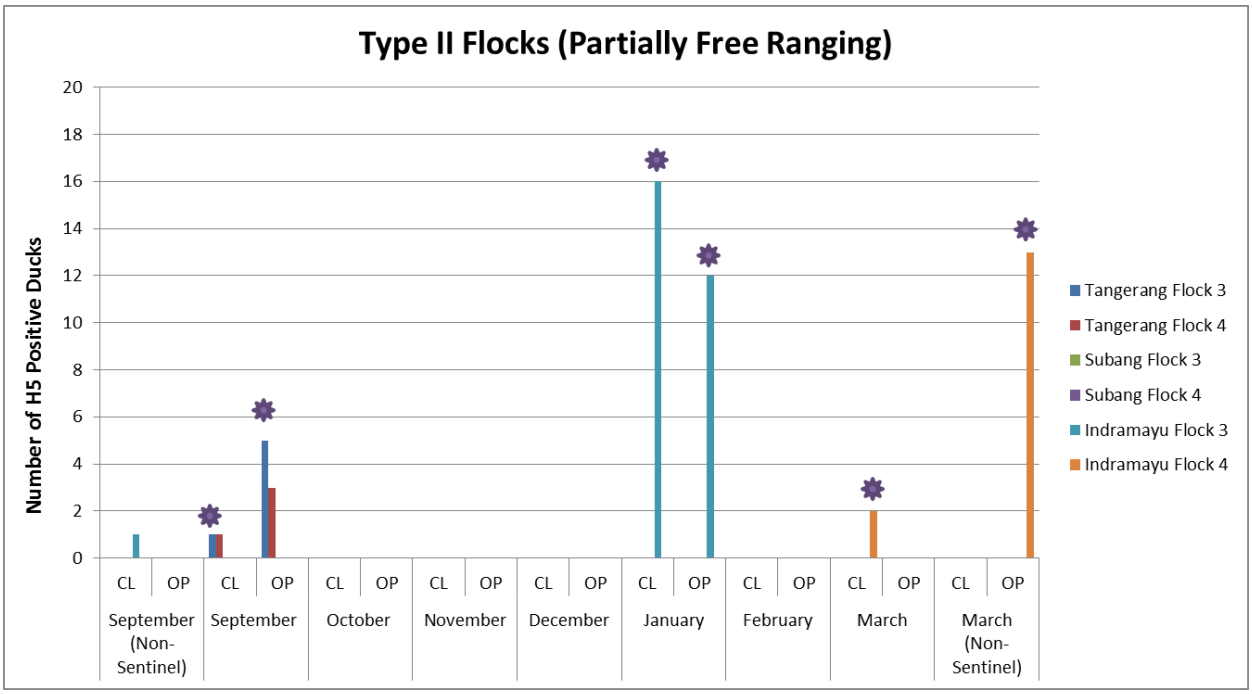

Figure 4.2 - Detection of H5 subtype avian influenza virus and number of positive pools for each flock over a seven month period for Type II flocks. H5N1 positive flocks are denoted using a purple star symbol.

All HPAI H5N1 viruses were compared to sequences in the GenBank genetic sequence database and were characterized as 2.1.3 Indonesian lineage viruses. The phylogenetic tree in Figure 4.1 describes the relationship of the characterized study H5N1 viruses to other Indonesian HPAI H5N1 viruses. Viruses sequenced for this study are named using the nomenclature A/domestic duck/West Java/(sample number)/2010. For the purposes of creating an easy to read phylogenetic tree, not all sequenced HPAI H5N1 viruses characterized for this study were included; however, all of the viruses were similar in nature.

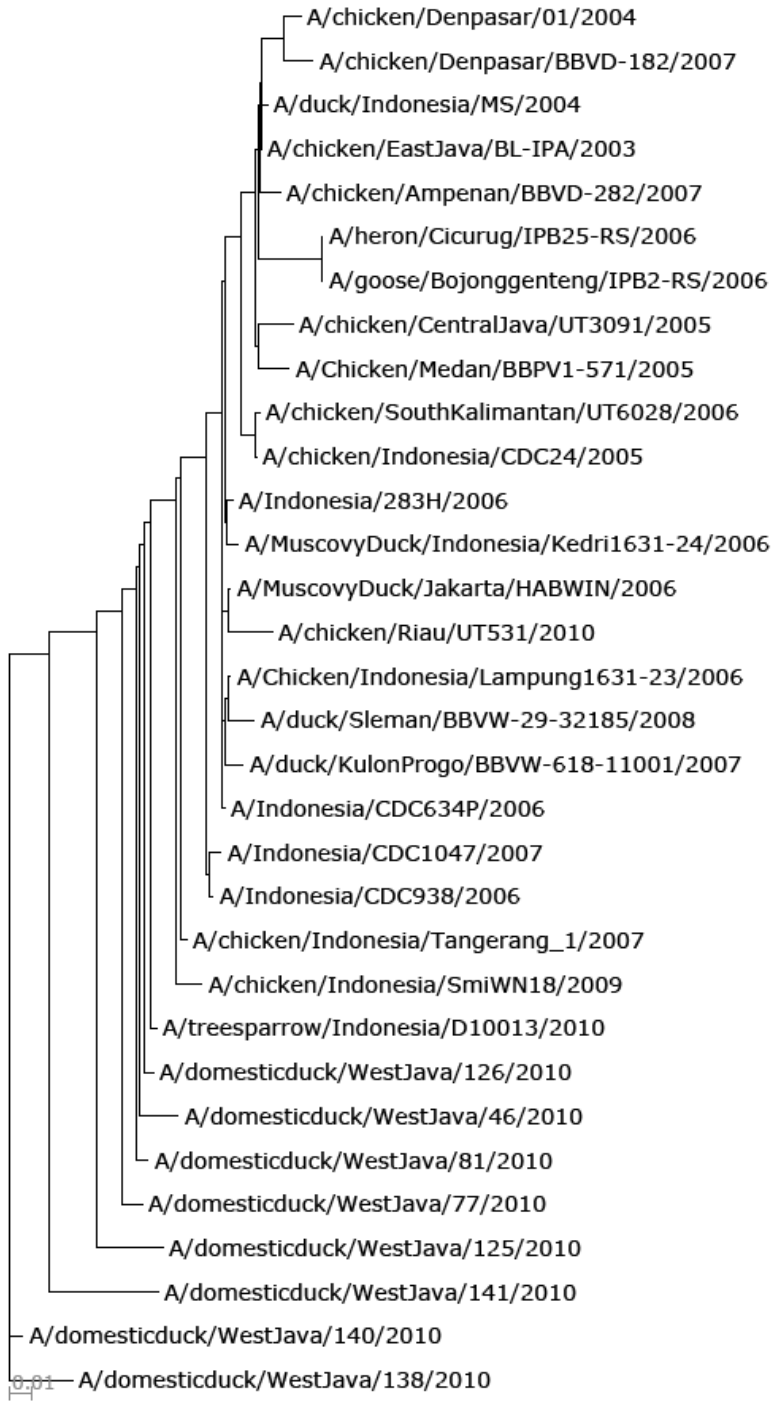

Figure 4.3 – Phylogenetic relationships of domestic duck H5N1 virus isolates and Indonesian H5N1 viruses

DISCUSSION

Obtaining samples for sequencing from Indonesia was difficult, due to government restrictions protecting Indonesian interests in influenza vaccine production. Additionally, Indonesians have legitimate concerns over inadequate international recognition for their work in the global HPAI H5N1 epizootic. Due to these barriers, up to three years elapsed between the time the samples were collected and the time the samples were shipped to the United States for sequencing. During this period, samples were maintained in a -70^{o}C freezer; however, complications in maintaining stable electricity to power the freezer resulted in a number of incidences where the power was lost and the freezer temperature increased for short time, leading to potential inactivation of the virus due to thawing and refreezing of samples. Additionally, there was a multi-day delay in delivery of the airmailed sample package from Indonesia, which may have resulted in thawing of the samples and inactivation of the virus. Virus was isolated from 7% of the cross sectional study samples, indicating that significant freezer temperature instability had occurred. The longitudinal study samples were collected more recently and viruses were more readily isolated from these samples, demonstrated by isolation of avian influenza virus from 30% of the samples. Overall, live virus was isolated from 25% of the samples, which should be considered successful, considering the difficulties in maintaining an appropriate freezing temperature for the samples and the challenging conditions surrounding field collection of samples.

Sequences were generated from 87% of the isolated avian influenza viruses. The failure in sequencing five of the virus isolates may be due to incorrect identification as avian influenza viruses, PCR primer mismatch or low titer of virus present in the sample making amplification of the virus difficult. Attempts to sequence these viruses are ongoing.

All of three of the HPAI H5N1 positive flocks were Type II partially free-ranging flocks. The reason for this finding cannot be ascertained due to the small sample size. Type II flocks are moved frequently to free range, so they have significant contact with village poultry and wild birds that may serve as a source for virus introduction. Additionally, ducks are added and removed frequently from the flocks, which also provides opportunities for virus introduction. However, these characteristics are common with Type I and Type III flocks, so the further studies are needed to elucidate specific risk factors for HPAI H5N1 introduction in domestic duck flocks. The owner of the Tangerang flock that was positive during the first month had just purchased sentinels for the purposes of this study. Five of the thirty newly purchased sentinel ducks but no owner flock ducks were positive for HPAI H5N1 virus, demonstrating that the owner introduced the virus with the introduction of the sentinel ducks.

All of the HPAI H5N1 virus detections were limited to one month during the study for each flock. The Indramayu flock positive during month 5 was not positive for H5 virus in the preceding or following month. The Tangerang flock positive during month 1 was not positive for H5 virus during month 2, which is interesting as the virus was introduced with the new sentinel ducks and expected to spread to the flock owners' ducks, indicating that the duration of the outbreak lasted less than 1 month. The Indramayu flock positive during month 7 was not positive in the preceding month but was not followed after the seventh month, so the duration of the outbreak cannot be ascertained. Regardless, from this limited data, it appears that the duration of an HPAI H5N1 virus outbreak in a domestic duck flock likely lasts less than 1 month. However, because the flocks are frequently moved and commingled with other flocks and because ducks are frequently added and removed from the flocks, significant opportunities to spread HPAI H5N1 virus exist during the outbreak period.

When HPAI H5N1 virus positive samples were inoculated into embyronated chicken eggs, embryo death typically occurred within 24 hours, demonstrating the high virulence of this virus. However, none of the HPAI H5N1 virus positive flocks reported significant clinical illness or increases in mortality in their flock. These findings are consistent with studies that have revealed that Indonesian lineage HPAI H5N1 viruses induce mild disease in domestic duck flocks and older ducks are less likely to show clinical signs of illness than younger ducks (4, 12).

All of the HPAI H5N1 viruses isolated originated from three flocks sampled during the longitudinal study. All of the viruses aligned with clade 2.1.3 Indonesian lineage viruses. With 98% similarity, the closest relative of the viruses was an H5N1 virus isolated from a tree sparrow in East Java in 2010. As depicted by the phylogram, the viruses were similar to many H5N1 viruses detected in various avian species from Indonesia since 2003. While all of the viruses are genetically similar, a considerable amount of genetic diversity exists within the Indonesian H5N1 lineage. The H5N1 viruses detected in this study were genetically different than other characterized Indonesian H5N1 viruses, demonstrating that circulating HPAI H5N1 viruses have complex biological characteristics.

Continual monitoring of virus evolution is also important for influencing vaccine development. Knowledge of the genetic composition of currently circulating viruses can be used to create an efficacious vaccine that matches field virus and is an instrumental part of implementing an effective vaccination program. The use of highly efficacious vaccines is essential for reducing viral replication and shedding and lowering the potential for selection of antigenic variants.

One of the HPAI H5N1 positive flocks was also positive for H3 and H7 subtype viruses. This finding is highly concerning due to the predilection of avian influenza viruses for

reassortment. The presence of the H7 virus is particularly concerning since this virus has the potential to become a highly pathogenic virus. Two ducks in this flock were co-infected with both H5N1 virus and H7 virus and one duck was co-infected with H5N1 virus and H3 virus, creating the prime opportunity for reassortment of the viruses within an individual duck.

The viruses detected in this study were 97% similar to H5N1 virus human isolates from 2006 and 2007. Continual mutation of H5N1 virus provides opportunities for the virus to acquire the ability to transmit more efficiently from human to human and may result in the emergence of a pandemic level virus. The need for monitoring of H5N1 evolution is critical to the early detection of novel viruses.

References

1. **Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster.** 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet **351:**472-477.

2. **WHO/OIE/FAO H5N1 Evolution Working Group.** 2012. Continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature. Influenza and other respiratory viruses **6:**1-5.

3. **WHO/OIE/FAO H5N1 Evolution Working Group.** 2009. Continuing progress towards a unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: divergence of clade 2.2 viruses. Influenza and other respiratory viruses **3:**59-62.

4. **Henning, J., H. Wibawa, J. Morton, T. B. Usman, A. Junaidi, and J. Meers.** 2010. Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. Emerging infectious diseases **16:**1244-1250.

5. **Hoffmann, E., J. Stech, Y. Guan, R. G. Webster, and D. R. Perez.** 2001. Universal primer set for the full-length amplification of all influenza A viruses. Archives of virology **146:**2275-2289.

6. **Killian, M. L.** 2008. Hemagglutination assay for the avian influenza virus. Methods in molecular biology **436:**47-52.

7. **Krauss, S., D. Walker, and R. G. Webster.** 2012. Influenza virus isolation. Methods in molecular biology **865:**11-24.

8. **Lam, T. T., C. C. Hon, P. Lemey, O. G. Pybus, M. Shi, H. M. Tun, J. Li, J. Jiang, E. C. Holmes, and F. C. Leung.** 2012. Phylodynamics of H5N1 avian influenza virus in Indonesia. Molecular ecology **21:** 3062-3077.

9. **Nidom, C. A., S. Yamada, R. V. Nidom, K. Rahmawati, M. Y. Alamudi, Kholik, S. Indrasari, R. S. Hayati, K. Iwatsuki Horimoto, and Y. Kawaoka.** 2012. Genetic characterization of H5N1 influenza viruses isolated from chickens in Indonesia in 2010. Virus genes **44:**459-465.

10. **OIE.** 2011. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Sixth ed, vol. I.

11. **Osterhaus, A. D., J. C. de Jong, G. F. Rimmelzwaan, and E. C. Claas.** 2002. H5N1 influenza in Hong Kong: virus characterizations. Vaccine **20 Suppl 2:**S82-83.

12. **Pantin-Jackwood, M. J., D. L. Suarez, E. Spackman, and D. E. Swayne.** 2007. Age at infection affects the pathogenicity of Asian highly pathogenic avian influenza H5N1 viruses in ducks. Virus research **130:**151-161.

13. **Poetranto, E. D., M. Yamaoka, A. M. Nastri, L. A. Krisna, M. H. Rahman, L. Wulandari, R. Yudhawati, T. E. Ginting, A. Makino, K. Shinya, and Y. Kawaoka.** 2011. An H5N1 highly pathogenic avian influenza virus isolated from a local tree sparrow in Indonesia. Microbiology and immunology **55:**666-672.

14. **Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster.** 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology **252:**331-342.

15. **Sims, L. D., J. Domenech, C. Benigno, S. Kahn, A. Kamata, J. Lubroth, V. Martin, and P. Roeder.** 2005. Origin and evolution of highly pathogenic H5N1 avian influenza in Asia. The Veterinary record **157:**159-164.

16. **Squires, R. B., J. Noronha, V. Hunt, A. Garcia-Sastre, C. Macken, N. Baumgarth, D. Suarez, B. E. Pickett, Y. Zhang, C. N. Larsen, A. Ramsey, L. Zhou, S. Zaremba, S. Kumar, J. Deitrich, E. Klem, and R. H. Scheuermann.** 2012. Influenza Research Database: an integrated bioinformatics resource for influenza research and surveillance. Influenza and other respiratory viruses [Epub ahead of print].

17. **Suarez, D. L., M. Garcia, J. Latimer, D. Senne, and M. Perdue.** 1999. Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. Journal of virology **73:**3567-3573.

18. **USDA/APHIS/VS, N. V. S. L.** 2009. Amplification and Purification of PCR Products for Direct Sequencing of Hemagglutinin, Neuraminidase, and Matrix Genes of Swine Influenza Virus, p. 1-17, vol. Standard Operating Procedure, Document SOP-BPS-9035.01.

19. **Woolcock, P. R.** 2008. Avian influenza virus isolation and propagation in chicken eggs. Methods in molecular biology **436:**35-46.

Chapter 5

Conclusions

With the emergence of HPAI H5N1 virus, the evolutionary stasis of influenza A virus in the wild waterfowl reservoir has been disrupted. Since late 2003, HPAI H5N1 virus has spread in an unprecedented manner across Asia, Africa and Europe and has been associated with significant economic and public health consequences. Unlike other influenza A viruses, HPAI H5N1 virus can cause significant illness and mortality in waterfowl, although variation in disease severity in domestic duck flocks has been reported. The aims of the studies described in this dissertation were to assess the role of domestic ducks in maintenance and spread of avian influenza viruses, particularly HPAI H5N1 virus, in order to appropriately target disease surveillance and control programs.

The findings of these studies demonstrate that avian influenza virus, including HPAI H5N1 virus, prevalence and incidence are high in domestic duck flocks in Indonesia. This is likely due to a combination of risky practices involved in domestic duck production that may result in avian influenza virus introduction into a flock, spread from flock to flock, and environmental contamination. Domestic duck flocks in Indonesia are moved frequently to different free-range sites, including post-harvest rice fields and irrigation waterways. Ranging the flocks in an open environment allows for significant contact with wild birds, village poultry and wild and domestic mammals, and potential spread to these in-contact species. The flocks are often ranged in conjunction with other flocks, allowing for spread of the virus from flock to flock. Domestic duck purchasing and sales systems, involving breeders, middlemen, vendors

and live bird markets, are extremely complex and provide significant opportunities for commingling of ducks with other wild and domestic avian and mammalian species and dissemination of the virus throughout this complex network. Housing of ducks in free-ranging areas and in live bird markets and other bird distributions sites may result in significant environmental contamination. Improper disposal of dead ducks, including improper burial, feeding of dead ducks to fish or poultry and throwing ducks in village water systems, may also result in significant environmental contamination and potential spread of disease.

The investigations in this dissertation provided evidence that domestic duck flocks can be asymptomatically infected with HPAI H5N1 virus, allowing the virus to replicate silently and efficiently, as no correlation was made between clinical illness and mortality and H5 virus or HPAI H5N1 virus flock infection. Interestingly, in the longitudinal study, when H5 subtype virus was detected in a flock, the virus was only detected in one month during the study, demonstrating that field outbreaks are short-lived and virus may not persist in the flock for longer than one month. However, due to the significant movement of these flocks and contact with other wild and domestic birds and mammals, significant opportunities exist for dissemination of the virus within this timeframe.

Domestic duck flocks should be made a specific focus of all HPAI H5N1 surveillance and control programs. Effective enhanced surveillance and control programs could significantly reduce risks from HPAI H5N1 virus on human and animal health. Most disease surveillance programs rely on passive surveillance and easily detectable disease outbreaks. Because domestic ducks can serve as silent carriers of the virus, active surveillance must be conducted to detect outbreaks in these flocks. In addition, these studies confirmed that avian influenza viruses in general, and particularly H5 subtype viruses, are shed in respiratory secretions. Based on

these results, collection of both oropharyngeal and cloacal samples from ducks should be conducted. If funds are limited, interventions should be focused on the longer-lived and frequently moved Type I fully free-ranging and Type II partially free-ranging flocks. Type I flocks had the highest prevalence and incidence of avian influenza virus infection, potentially due to interaction with wild bird and continual commingling with other flocks. While only a small number of positive samples from this study were sequenced, all of the HPAI H5N1 virus isolations came from Type II samples, which may be associated with a higher prevalence in these flocks, potentially due to sometimes daily movement and significant interactions with village poultry.

These studies also determined that domestic duck flocks in Indonesia are frequently ill and are provided with little formal veterinary care. Significant levels of mortality were common in the flocks and disease issues were rarely reported to government livestock officials. These findings demonstrate that domestic duck flocks experience significant disease issues, potentially the result of infections with HPAI H5N1 virus and other transboundary duck infectious diseases, and further substantiate that domestic duck flocks should be the focus of significant government interventions. In addition, further studies should evaluate the prevalence of other infection diseases in domestic duck flocks in Indonesia in order to understand potential causes for the significant levels of illness and mortality in the flocks.

The potential for HPAI H5N1 virus to undergo antigenic drift and shift and emerge as a novel pandemic-level virus that could cause severe and lethal disease in humans is highly concerning. Continued exposure and circulation of HPAI H5N1 virus in domestic ducks may drive mutations to make the virus more adapted and more pathogenic in this species. In one flock in the longitudinal study, three different viruses, H3 and H7 viruses and HPAI H5N1 virus,

were isolated. This type of situation is alarming because waterfowl may serve as a mixing vessel through co-infection with one or more influenza viruses. For these reasons, the need for monitoring HPAI H5N1 virus, as well as other avian influenza viruses, evolution is critical. In addition, monitoring virus evolution is essential for the design and continual update of vaccinations that are highly efficacious against currently circulating virus strains, in order to effectively prevent mortality and viral shedding in an infected flock and prevent selection of antigenic variant viruses through selective pressure.

Additional studies are needed to further evaluate HPAI H5N1 outbreaks in domestic ducks. While experimental studies have demonstrated that ducks shed HPAI H5N1 virus for 3-17 days, depending on the virus strain and duck species, little work has been done to document the period of shedding in a natural field setting. Additionally, the experimental infection trials typically use Pekin or Muscovy ducks, which are different breeds than the typical runner duck breeds comprising most Asian domestic duck flocks. Since breed-related differences in viral pathogenicity and shedding have been detected, studies using true domestic duck flock breeds should be conducted. At this point, the use of HPAI H5N1 vaccinations in domestic duck flocks is questionable, as vaccine efficacy has not been verified in a field setting. Further field trials are needed to determine if currently available vaccines should be used in HPAI H5N1 control programs in endemic countries. Finally, while the findings of the studies described in this dissertation established the high level of prevalence and incidence of avian influenza viruses in domestic ducks and interaction of domestic duck flocks with village poultry flocks, further studies are needed to specifically demonstrate viral transmission between domestic ducks and poultry.

The results of these studies provide insight on the role of domestic ducks in the maintenance and spread of influenza viruses. Domestic duck flocks in Indonesia are frequently infected with avian influenza viruses, including HPAI H5N1 virus. HPAI H5N1 virus infections in domestic ducks are often asymptomatic, despite significant viral shedding by a high percentage of ducks in a flock. This creates a situation where the virus outbreak goes undetected, allowing for maintenance of the virus. In addition, many of the practices associated with domestic duck flock production provide opportunities for virus spread. Domestic duck flocks should be a focus for government interventions, particularly in HPAI H5N1 endemic countries.