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Post-vaccination surveillance and molecular epidemiology of highly pathogenic H5N1 avian influenza in Vietnam

A thesis presented
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Nomenclature

AAHL	Australian Animal Health Laboratory, Geelong
AHW	Animal Health Worker
AI	Avian influenza
CAHW	Community Animal Health Worker
CDC	Centers for Disease Control and Prevention, Atlanta
CI	Confidence interval
DAH	Department of Animal Health, Vietnam
DVS	District Veterinary Station
DIVA	Differentiating Infected from Vaccinated Animals
FAO	Food and Agriculture Organization of the United Nations
HA	Haemagglutinin
HI	Haemagglutination Inhibition (test)
HPAI	Highly pathogenic avian influenza
LabNet	National laboratory network (Vietnam)
LPAI	Low pathogenic avian influenza
M	Matrix protein

MARD	Ministry of Agriculture and Rural Development, Vietnam
NA	Neuraminidase
NCVD	National Centre for Veterinary Diagnostics, Vietnam
NP	Nucleoprotein
NS	Non-structural protein
NZAID	New Zealand Agency for International Development
OIE	World Organization for Animal Health
OR	Odds ratio
PA	Polymerase protein
PB	Transcriptase protein
PCR	Polymerase chain reaction
RAHO	Regional Animal Health Office
RDE	Receptor-Destroying Enzyme
ROC	Receiver Operating Characteristic (curve)
RRT-PCR	Real time reverse transcriptase polymerase chain reaction
SDAH	Sub-Department of Animal Health
SE	Standard error
SIVR	Sub-Institute of Veterinary Research
TADinfo	Transboundary Animal Disease Information System
USD	United States dollar
VND	Vietnamese Dong
USAID	United States Agency for International Development
UNICEF	United Nations International Children's Emergency Fund

WHO

World Health Organization

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Introduction

The long history and serious consequences of influenza has been the cause of concern to the global public for many decades. This was particularly the case when the novel avian influenza H5N1 virus first emerged in Hong Kong in 1997, causing the loss of many millions of poultry and 18 human lives (Claas et al., 1998; Subbarao et al., 1998). In the following years, highly pathogenic avian influenza H5N1 (HPAI H5N1) has been the cause of outbreaks of disease in poultry in several parts of mainland China (Broor, 2005). The disease was subsequently introduced into Vietnam, where it caused the first serious epidemic in poultry in late 2003 and early 2004. The government responded with massive culling of more than 45 million birds. This response was considered to be necessary, given concerns that the virus could cross the species barrier and cause an influenza pandemic in humans.

Measures to control the 2003 epidemic of HPAI H5N1 in Vietnam included stamping-out of infected flocks and those flocks within a 5 kilometre buffer area and restriction of trade. Consequently, the epidemic was brought under control in February 2004. A second wave of outbreaks started in late 2004. This second wave was the cause of much concern to local authorities, primarily because control measures were seen to be inefficient and the stamping-out policy caused numerous ethical and environmental concerns. Since September 2005 chickens and ducks in high risk areas have been vaccinated for HPAI H5N1 twice yearly and culling limited to affected flocks only. The Vietnamese government has spent many billions of US dollars to procure and administer vaccine for each vaccination round, and approximately 2,142 million doses were used up to December 2009 (MARD, 2009). At a cost of USD 0.02 per dose the drug cost alone of each vaccination round is estimated to be around USD 10 million (MARD, 2009). Both central and local governments have

also spent much time and effort to organise and implement these campaigns. Due to these high costs, it is necessary that efforts are made to evaluate the efficacy of vaccination as a tool for disease management.

Furthermore, although vaccination of poultry in high risk areas is thought to be one of the key reasons behind the reduced incidence of disease that has been observed in both poultry and humans since 2005, continued mass vaccination may produce faster antigenic drift of circulating influenza viruses due to ongoing selection pressure (Lee et al., 2004; Suarez et al., 2006; Escorcía et al., 2008). Hence, there is a strong need to revise the current vaccination campaign and better understand the epidemiology of influenza viruses in Vietnam.

This thesis is presented as a series of three papers prepared for publication. Each chapter represents the stage of preparation each paper has reached at the date of thesis submission. Chapter 2 provides a review of key topics in this subject area including its epidemiology, vaccination as a tool for control, and the molecular epidemiology of HPAI H5N1 in Vietnam. Since 2007 the Food and Agriculture Organization of the United Nations (FAO) has funded a post-vaccination surveillance project. Chapter 3 represents a descriptive epidemiological analysis of data collected during the course of this project for the years 2007, 2008 and 2009. A description of the spatial, temporal and individual bird-level factors associated with vaccination success provide a starting point for the development of targeted vaccination strategies that should enhance the efficacy of the programme as a whole. Chapter 4 quantifies determinants of HPAI vaccination success at the flock level. The data for these analyses comprised details from the post-vaccination surveillance programme for seven provinces in the Mekong River Delta in 2009. The aim of this chapter was to identify flock-level and province, district and commune-level influences on vaccination success.

Given the circulation of mixed genotypes and the difficulties in achieving optimum vaccination coverage under field conditions, there is a need to continuously monitor molecular change in circulating HPAI viruses to promptly detect changes in virus characteristics. Thus, Chapter 5 focuses on the molecular epidemiology of HPAI H5N1 isolates collected in Vietnam between 2008 and early 2010. The aim of this study was to investigate molecular characteristics of recent avian influenza virus isolates from clinically diseased and apparently healthy birds. The results of this study can be used to inform future surveil-

lance and control strategies in Vietnam.

Chapter 6 draws all of the concepts identified in Chapters 2 to 5 together, to develop some general conclusions.

Literature review

2.1 Introduction

Avian influenza (AI) is an infectious disease of poultry caused by influenza A viruses that are members of the family Orthomyxoviridae. Highly pathogenic avian influenza (HPAI) is characterised by high virulence and is caused by viruses of the H5 and H7 subtype (Alexander, 2000). The highly pathogenic H5N1 viruses responsible for avian influenza outbreaks in Asia, and more recently Europe and Africa, are considered mutants or reassortments of the first Asian H5N1 virus (Goose/GD/96) that was isolated from sick geese in southern China in 1996 (Guan et al., 2002; Chen et al., 2004; Li et al., 2004). HPAI is a disease of global concern because of the threat posed to food security in regions that depend on poultry as main source of protein and concerns that the H5N1 virus may mutate and cause a human influenza pandemic in which millions of human lives would be threatened (Li et al., 2004; Anonymous, 2005).

Although a substantial body of research relating to the epidemiology of AI viruses in both humans and animals has been undertaken, it is essential to update the disease situation, surveillance, and control strategies from time to time so that it can be used to direct future research into HPAI epidemiology and devise more effective programmes to control the disease. In affected countries such as Vietnam it is also important to understand the current HPAI surveillance system and vaccination strategies in place, and how these influence the course and development of disease outbreaks.

This chapter begins with an overview of avian influenza in terms of its aetiology, epidemiology and control. The following sections review the H5N1 vaccination and post-

vaccination surveillance strategies in Vietnam and discuss constraints faced by surveillance program currently in place. In developing countries like Vietnam where resources for disease surveillance are limited, an extensive surveillance system may not always be feasible. Therefore, it is important to identify the critical components of a surveillance system. Recommendations are given to show how to improve effectiveness of existing surveillance systems and how routine outbreak data can be analysed to orient future research into HPAI. This literature review is not intended to be exhaustive, rather to provide context and background for the research chapters that follow.

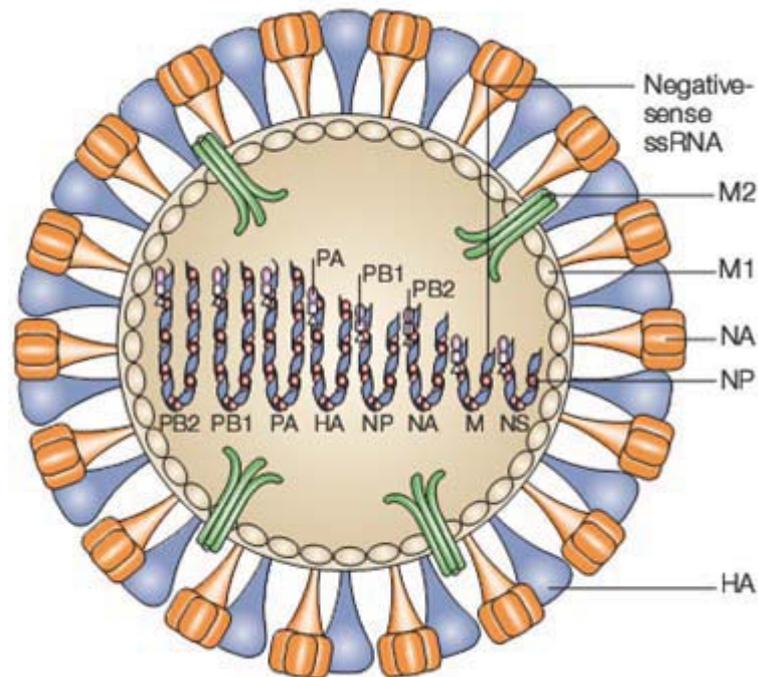
2.2 Avian influenza

2.2.1 Viral structure and characteristics

Avian influenza (AI) viruses belong to the Influenza Type A genus of the family Orthomyxoviridae. The virus is comprised of a negative single-stranded RNA with eight gene segments that encode at least ten different viral proteins. The structural proteins can be divided into:

- Three surface proteins, that is haemagglutinin (HA), neuraminidase (NA) and membrane ion channel (M2) proteins
- Internal proteins, including the nucleoprotein (NP) (Lo et al., 2008),
- The matrix protein (M1),
- Three transcriptases PB1, PB2, and PA proteins, and
- Two additional non-structural proteins NS1 and NS2 (Figure 2.1).

On the basis of antigenic differences, influenza viruses are divided into influenza virus A, B and C. Influenza A viruses are the only type known to infect birds and are classified on the basis of the antigenic properties of their haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins (Capua and Alexander, 2001; Swayne, 2008). Currently, 16 hemagglutinin subtypes (H1 – H16) and 9 neuraminidase subtypes (N1 – N9) are recognised. Each virus has one H and one N antigen which seem to be able to assort into any



Source: Horimoto T., 2005

Figure 2.1: Structure of the avian influenza virus.

combination. There are theoretically 144 possible combinations (16 H subtypes \times 9 N subtypes) that can be found in natural reservoir species, many of which have been isolated from avian species (Webster and Hulse, 2004; Alexander, 2007b).

AI viruses are classified as either highly pathogenic (HPAI) or low pathogenic (LPAI), based on the severity of clinical disease which they cause (OIE, 2009). AI viruses are defined as HPAI if they kill at least 75% of susceptible 4 to 6 week-old chickens within 10 days post-inoculation by the intravenous route (Swayne, 2008). Only H5 and H7 subtypes viruses can cause HPAI, but not all viruses of these subtypes are virulent (Capua and Alexander, 2001; Alexander, 2007b). AI viruses are defined as being LPAI if they kill less than 75% of 4 to 8 week-old birds (OIE, 2009). LPAI viruses are responsible for mild diseases, with few or no clinical symptoms in birds. Any AI subtype including H5 and H7 subtypes can be categorised as LPAI virus (Swayne, 2008). However, all confirmed LPAI H5 and H7 AI subtypes must be reported to the OIE because of their potential to mutate into highly pathogenic strains (Ausvetplan, 2007). Although there have been concerns about the change of LPAI to HPAI viruses, factors influencing the presence or absence of mutation and how quickly it occurs are not well understood (Capua and Alexander, 2009).

Table 2.1: Reservoir for HA and NA subtypes.

Host	HA subtypes	NA subtypes
Waterfowl	All 16 subtypes	All 9 subtypes
Pig	H1, H3, H4, H9	N1, N2
Horse	H3, H7	N7, N8
Cattle	H3	N2
Seal	H4, H7	N7
Whale	H3, H13	N2, N9
Cat, tiger	H5	N1
Human	H1, H2, H3, H5, H7	N1, N2, N3, N7

2.2.2 Epidemiology, ecology and evolution

Host range

Influenza A viruses have a wide range of hosts including birds, pigs, horses, cattle, seals, whales, cats, tigers and humans (Table 2.1). Wild aquatic birds, notably Anseriformes (ducks and geese) and Charadriiformes (gulls and shorebirds) are carriers of the full range of influenza virus A subtypes, and therefore constitute the natural reservoir of all influenza A viruses (Webster et al., 1992; Widjaja et al., 2004). These viruses are usually non-pathogenic in these species (Swayne, 2008) where they multiply predominantly in the gastrointestinal tract and are shed through faeces. Indeed, influenza viruses in wild aquatic birds have long been in a state of evolutionary equilibrium (stasis), and infected hosts usually show few signs of disease. In addition, some other species such as pheasants can serve as reservoirs and may also be carriers of influenza viruses (Humberd et al., 2006).

Transmission

When HPAI viruses are transmitted from the reservoir host to other species, including domestic birds, mammalian hosts and humans they may produce clinical disease. Influenza viruses spread from the infected host to others via secretions from the respiratory and intestinal tract. The most common means of transmission is direct contact (Capua and Alexander, 2001). Faeces from birds with asymptomatic infection can spread viruses via indirect contact such as contaminated feed, water, equipment and clothes. The mode of viral transmission from bird to bird is often complex and depends on the viral strain, af-

ected species and environmental factors (Kida et al., 1994; Webster et al., 1995; Monto, 2000; Alexander, 2007b).

Cross-species transmission can result in infection in mammals such as pigs, dogs, domestic cats, hamsters, mice, ferrets, stone martens, tigers, leopards, civets, macaques and humans (Choi et al., 2005; Gabriel et al., 2005; Govorkova et al., 2005; Lipatov et al., 2008; Qi et al., 2009). Within-host variation of AI viruses, the potential for contact, transmission, and mutability of AI viruses is likely to become more frequent as the number of species and their interactions increase.

Prevention and control HPAI

The first epidemic of HPAI caused by the H5N1 virus began in China in 1996, from where it spread to 61 countries throughout Asia and the Middle East, Africa and Europe (FAO, 2008). However, the true crisis began in 2003 which resulted in the culling of hundreds of thousands of chickens and ducks in ten countries. Economic losses due to these epidemics in the Asian poultry sector were estimated to be in the order of 20 billion USD. In addition, HPAI H5N1 has emerged as a threat to the livelihood of hundreds of millions of livestock farmers, jeopardising smallholders and commercial poultry production, and seriously impeding regional and international trade and market opportunities.

Various international organisations (OIE, FAO, UNICEF, The World Bank and The Asian Development Bank) and many countries have collaborated in an effort to control the disease in poultry so as to reduce the risk of human infection (FAO-OIE-WHO, 2005). A combination of control strategies, including early detection based on surveillance, stamping-out, movement restrictions, and vaccination have been applied in infected areas throughout the world.

Enhanced biosecurity of poultry farms is one of the key preventive measures. The aim here is to reduce the risk of virus introduction posed by people, animals and vehicles and equipment. Biosecurity is comprised of two elements, bio-containment (the prevention of spread of virus from infected premises) and bio-exclusion (measures to exclude infectious agents from uninfected premises). Enhanced biosecurity can be achieved through restrictions of movement, appropriate disinfection and use of protective clothing. Changes to industry practices (marketing systems, segregation of species, farming systems and prac-

tices and transport) to decrease the likelihood of transmission and spread from backyard poultry flocks is considered a long-term strategy in reducing the risk of HPAI spread in Vietnam. Control of the movement of poultry and poultry products also plays an important role in limiting virus spread from infected to uninfected areas. Destruction of infected and at-risk poultry (stamping out) and decontamination of infected farms as well as removal or disinfection of potentially infectious material are critical to eradicate the virus from affected premises and to achieve effective disease control.

Vaccination is one of the key strategies in controlling the HPAI outbreaks in some countries. In Mexico (1994 – 1995) and Central America (2001 – 2005) vaccination was successfully applied to control HPAI H5N2 outbreaks (Senne, 2007). Vaccination has also been used in combination with other measures (stamping out and movement restrictions) to control and prevent HPAI H5N1 outbreaks in China, Indonesia, Vietnam (Capua and Marangon, 2004; FAO, 2007; Sims, 2007), Nigeria and Egypt (FAO, 2007).

Stamping-out is the preferred option for an outbreak of HPAI and should be used in all flocks exhibiting clinical disease. It has been highly effective in controlling confined outbreaks of HPAI where there is limited and low risk of reintroduction. However, where mass culling is either not desirable or not feasible, vaccination may be considered as an option to support other control measures. Vaccination can be used either as a tool to support eradication or as a tool to control the disease and reduce viral load in the environment.

In short, a combination of strategies including biosecurity, surveillance, and elimination of infected birds, reduction of host susceptibility to infection (e.g. vaccination) and education need to be used to effectively reduce the losses associated with avian influenza in poultry populations.

2.3 HPAI surveillance in Vietnam

Surveillance is one of the key measures in controlling HPAI. Both active and passive approaches have been applied in Vietnam since 2005 (Figure 2.2). Passive surveillance has relied mainly on reports from poultry owners and local people (community based surveillance) (Desvaux et al., 2007). Active activities include post-vaccination monitoring and

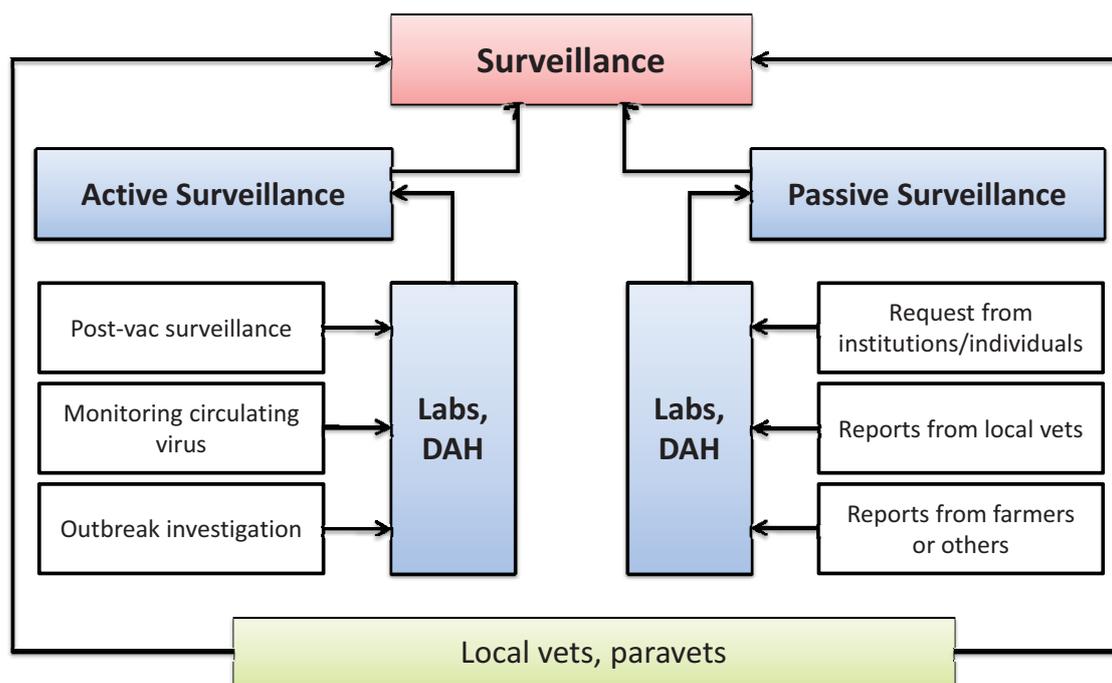


Figure 2.2: Structure of HPAI surveillance strategies in Vietnam.

surveillance for circulation of HPAI H5N1 virus, which have been implemented closely particularly during high risk periods of the year (Taylor and Dung, 2007; Gilbert et al., 2008; Long, 2008, 2009; Hoa et al., 2010; Long, 2010). However, active surveillance is associated with a high cost and requires substantial human and laboratory resources. Passive surveillance, therefore, has been used as the predominant system to detect outbreaks with a central role taken by local animal health workers.

In Vietnam, the Department of Animal Health (DAH) under the Ministry of Agriculture and Rural Development (MARD) takes a lead role in driving animal health surveillance. Subdivisions within the DAH include the National Centre for Veterinary Diagnosis (NCVD) and the Regional Animal Health Offices (RAHOs, $n = 7$) (Figure 2.4). The provincial Sub-Departments of Animal Health (SDAHs, $n = 64$) provide direct technical supervision to field veterinarians to carry out both active and passive surveillance activities such as sampling. The next level down includes the District Veterinary Stations (DVSSs, $n = 705$). Field staff at the lowest level are communal Animal Health Workers (AHWs, $n = 11,055$) who provide basic veterinary services and play a role in collecting and submitting of epidemiological records through a reporting system (Figure 2.3). A national hotline and many local phone numbers have been set up, free of charge for callers. These hotlines were established for reporting suspected cases of HPAI and other major

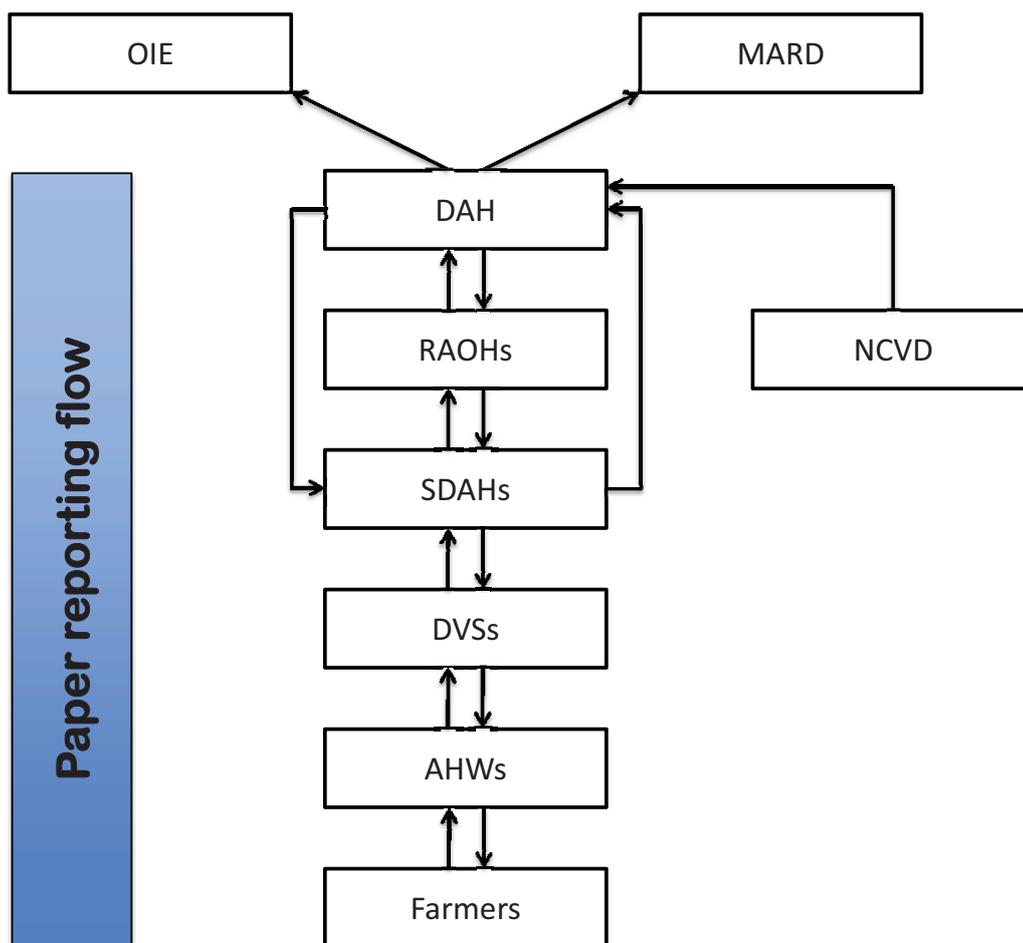


Figure 2.3: Diagram outlining reporting flow for endemic animal diseases in Vietnam.

diseases such as foot-and-mouth disease (FMD) and porcine reproductive and respiratory syndrome (PRRS).

Laboratory system

In Vietnam, the National Centre for Veterinary Diagnosis and Regional Animal Health Laboratories coordinate sampling and carry out laboratory testing (Figure 2.4). Before 2004, the national veterinary laboratory capacity for HPAI diagnosis was limited to only conventional techniques such as virus isolation and serological testing (HA, HI). After the first HPAI epidemic, molecular techniques such as Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) specific with the HPAI H5N1 subtype, was used to identify samples containing H5N1 viral sequences. Since 2006, real-time RT-PCR has been used in active surveillance programs and to confirm disease outbreaks. A national laboratory network (LabNet) was established in 2006 to enable sharing of information between central and regional laboratories. The country also has several provincial laboratories capable

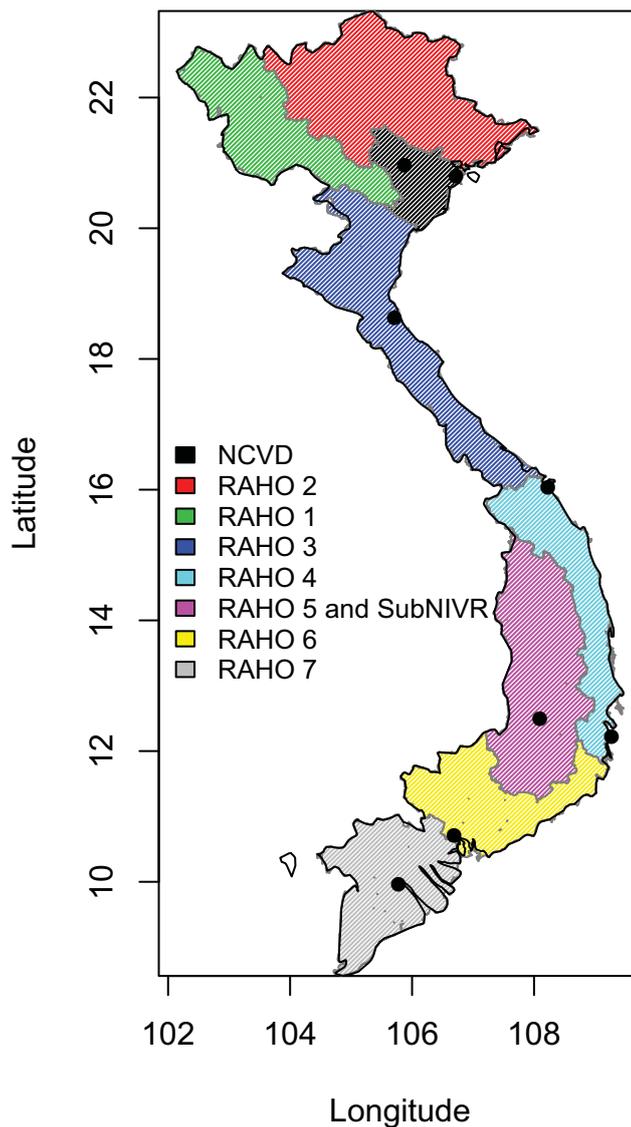


Figure 2.4: Map of Vietnam showing regional boundaries and the veterinary diagnostic laboratories servicing each region. Points (●) indicate the location of each lab.

of HPAI diagnosis using both serological and molecular tests.

Data management

At the time of writing this review, handwritten records are collected in the field and then collated and transcribed into spreadsheets in provincial offices. These are then forwarded to regional offices by email. In 2006, the Transboundary Animal Disease Information System (TADinfo) has been customised to facilitate data management for diseases such as HPAI, FMD and PRRS (Figure 2.5). TADinfo data can be viewed by the central office (DAH) and extracted for descriptive analyses. The information is then shared with

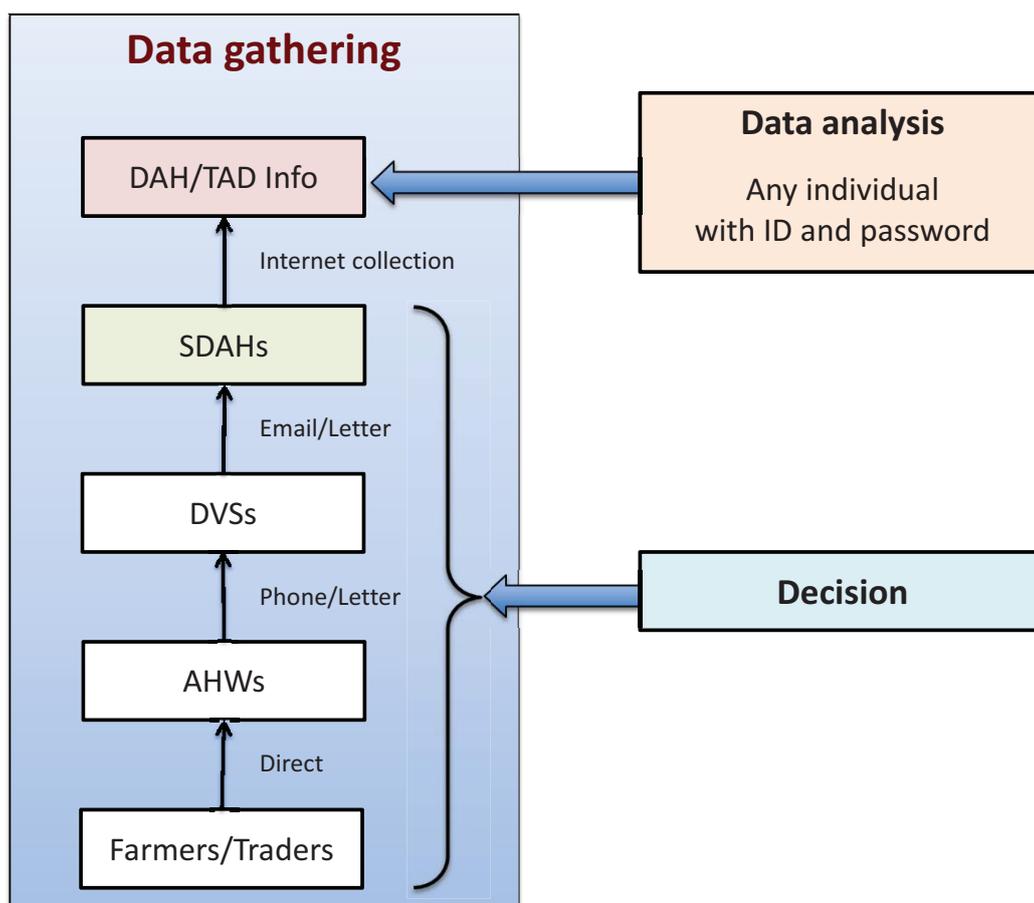


Figure 2.5: Diagram showing the structure of TADinfo as currently deployed in Vietnam.

regional and provincial partners and the public via the governmental webpage.¹ While this facility represents a major step forward in terms of allowing the DAH to collect animal health data in a consistent format a gap still exists in terms of getting the system to produce reports to facilitate decision making (Long, 2007b).

2.4 Vaccination

2.4.1 Rationale and benefits of vaccination

To control AI and then to eradicate it from a country, vaccination should be viewed as a single component of a comprehensive control strategy, which includes enhancement of biosecurity, surveillance, management of poultry movement and elimination of infected and at-risk poultry during the early stage of outbreaks. A major concern around the use

¹URL: (<http://www.dah.gov.vn>)

of vaccine is that suppression of clinical expression of disease might result in relaxing of the intensity of other components of control allowing disease to spread into previously uninfected areas. In addition, it has been suggested that widespread use of vaccination may facilitate viral mutation (Louis, 2006).

A major outcome from vaccination is that it increases the proportion of the poultry populations that is protected against AI infection. This is the result of the immune response elicited against the haemagglutinin protein (HA), and to a lesser extent, against the neuraminidase protein (NA) (Suarez and Schultz-Cherry, 2000). Immunological protection is not given by the immune response to internal or non-structural proteins, such as the nucleoprotein or matrix protein. Protection is provided against the individual HA or/and NA subtype, which is included in the vaccine.

Vaccination can be a valuable part of control program when administered correctly with following goals: (1) to protect vaccinated population against clinical disease and death (Swayne, 2003; Capua et al., 2004), (2) to increase the infectious dose needed to get vaccinated birds infected (Capua et al., 2004), (3) to reduce the amount and duration of virus shedding (Swayne, 2003; Tumpey et al., 2004; Tian et al., 2005; Swayne, 2006), (4) to reduce the transmission level to such an extent that major outbreaks can be prevented (Van der Goot et al., 2005), and (5) to provide at least 20 weeks protection following a single vaccination for chickens (with two or more injections in turkeys and ducks) (Swayne, 2006).

2.4.2 Factors affecting vaccination efficacy

The efficacy of vaccination is influenced by a range of factors related to the animal, composition of the vaccine itself and the way it is administered (maintenance of cool chain, vaccination technique). Indeed, vaccination failure can be defined using various criteria, such as disease prevention, disease mitigation or immune response. Primary failure (for example, lack of sero-conversion or sero-protection) needs to be distinguished from secondary failure (waning immunity). Factors affecting vaccination efficacy can be categorised into animal factors, vaccine factors and compliance and administrative issues. Each of these are discussed below.

Animal factors

Species and age are the main factors affecting vaccine efficacy (Barefoot et al., 2009). The use of vaccine for individual poultry species requires strict compliance with manufacturer's instructions. For instance, chickens can be vaccinated with the vaccine for H5N2 subtype as soon as they are one day old (Steensels et al., 2009) whereas it is recommended that ducks are vaccinated with the H5N1 vaccine once they have reached two weeks of age (Tian et al., 2005; DAH, 2006). Younger ducks may respond poorly when they are vaccinated earlier as their immunity system is still immature (Capua and Alexander, 2009; Barefoot et al., 2009). Other animal-level factors such as flock size, health status (e.g. parasitism), nutrition and environmental stress (temperature, relative humidity) may also reduce the ability to mount an adequate immune response (Butcher and Miles, 1994).

Vaccine factors

Currently, two types of vaccines are available for the control of AI: inactivated vaccines based on adjuvant whole virions and live recombinant vaccines. The former can be produced from master seed viruses isolated from the field, whilst the latter is generated by reverse genetic technology. The H5N1 vaccine that is currently used in Vietnam is a reassortant vaccine. This vaccine is comprised of the A/Harbin/Re-1/2003 (Re-1), which was generated by plasmid-based reverse genetics from the HA and NA genes of the GSGD/96 virus (Tian et al., 2005).

Recombinant vector-based vaccines are comprised of a vector virus expressing the haemagglutinin protein of avian influenza. For example, the Merial vaccine, Trovac AVI H5 (TROVAC-H5) is an avian influenza fowlpox vector vaccine, which contains a live recombinant fowlpox (recFP), expressing the hemagglutinin gene of an AI H5 subtype isolate. This vaccine has been used widely in countries such as the USA, Mexico and even for some years in Vietnam. In 2008 Vietnam stopped using this vaccine because it did not show good efficacy in laboratory challenge experiments (DAH, 2008*b*). The reasons for this could be that the vaccine strain was different from field circulating strains.

It has been pointed out that to be effective the strain of virus included in a vaccine needs to be the same as the strain of virus circulating in the environment (Lee et al., 2004). In

addition, factors related to vaccine manufacture (lot variation) may also lead to variability in vaccine efficacy. Quality standards released by international bodies must be respected by manufacturers and in addition, quality certification from regulatory and independent institutions should be obtained before widespread field use (Ka-Oudt et al., 2008). Vaccine production requires relatively long manufacturing times (48 months) and AI vaccines have a relatively short shelf life of about 2 years. This means that the number of doses needed to implement and sustain an AI vaccination programme should be evaluated carefully. Vaccine banks for emergency vaccination should be set up and maintained, and the vaccine need to be routinely evaluated based on continuous surveillance of the antigenic characteristics of the circulating viruses.

Vaccine delivery

Vaccine delivery encompasses a range of issues including preparation of sufficient amounts of vaccine for national campaigns and outbreak control and maintenance of a reliable cool chain from the point of manufacture to the point of administration. In areas where vaccination is to be carried out training of vaccinators and education of livestock owners is critical to ensure that campaigns can be implemented efficiently and effectively. Vaccination failure may also occur due to non-compliance with recommended schedules, for example failure to present animals for booster immunisations (Stevenson, 1990). Storage and delivery is an important determinant of the success of a vaccination program and for this reason manufacturer's recommendations concerning storage and delivery should be strictly adhered to (Ka-Oudt et al., 2008). Breaks in the cold chain are likely to reduce the potency of vaccine and contribute to primary vaccine failure.

2.4.3 Side effects of vaccination

Silent transmission

AI vaccines reduce the replication of HPAI viruses in the respiratory and gastrointestinal tracts and virus shedding after vaccination can still occur (Swayne et al., 1999; Capua et al., 2003). HPAI can still infect and replicate in vaccinated birds without clinical signs (Van der Goot et al., 2005) allowing them to act as silent carriers or excretors of virus

(Tiensin et al., 2005; Peyre et al., 2009). The study of Savill et al. (2006) demonstrated that if vaccination coverage is only sufficient to induce partial flock immunity, mortality of infected flocks may not rise above typical levels, while virus shedding and transmission still occurs. In this scenario, the presence of silent carriers can result in undetected transmission, which may increase the risk of disease spread among flocks.

Antigenic drift

Vaccination against AI may promote mutation in circulating virus strains. Furthermore, continued mass vaccination programs may produce faster antigenic drift of influenza viruses due to ongoing selection pressure (Lee et al., 2004; Escorcía et al., 2008; Steensels et al., 2009). For instance, the study of Lee et al. (2004) compared coding sequences of the HA1 subunit and NS gene of 52 Mexican H5N2 viruses isolated between 1993 and 2002. Results showed that the use of H5N2 vaccines in Mexico since 1995 appeared to have resulted in antigenic drift of the field virus away from the vaccine strain. More importantly, immunological pressure on circulating strains (arising from vaccination) might engender the emergence of drifted or shifted variants with enhanced pathogenicity in humans (Gambotto et al., 2008). Therefore, if not used appropriately, vaccination might result in the infection becoming endemic (Capua and Marangon, 2004; Savill et al., 2006). For these reasons it is essential to continuously monitor circulating viruses within vaccinated flocks, especially in the face of ongoing mass vaccinations. The time and effort involved to carry out monitoring effectively represents a major constraint for developing countries.

Emergence of vaccine resistant influenza viruses

The potential for the emergence of vaccine-resistant influenza viruses is a negative feature of vaccination as a long-term strategy to control HPAI in poultry populations (Lee et al., 2004; Smith, Naipospos, Nguyen, De Jong, Vijaykrishna, Usman, Hassan, Nguyen, Dao, Bui et al., 2006; Pasquato and Seidah, 2008; Peyre et al., 2009). A vaccination program that engenders the emergence of resistant strains might also promote the spread of resistant strains and undermine control efforts even if the vaccination itself protects against transmission of a vaccine-sensitive strain (Smith, Naipospos, Nguyen, De Jong, Vijaykrishna, Usman, Hassan, Nguyen, Dao, Bui et al., 2006; Peyre et al., 2009).

Furthermore, the spread of vaccine resistant strains may lead to a risk of generating a new pandemic virus with higher transmissibility between humans than the current H5N1 strains. The dynamics of competition between vaccine sensitive and vaccine-resistant strains is complex (Lipsitch et al., 2007; Moghadas et al., 2008). These dynamics might be influenced by several factors, such as a loss of protection effectiveness, a competitive advantage of vaccine-resistant strain, and the prevalence of vaccination. Understanding the dynamics of the spread of vaccine resistance is therefore crucial for implementation of effective mitigation strategies.

2.4.4 Practical aspects of vaccination

Campaign conduct

The first HPAI H5N1 epidemic in Vietnam occurred late 2003 (Ha Tay province, in the north) and early 2004 (Long An and Tien Giang provinces, in the south) (MARD, 2004). The epidemic then spread throughout the country and resulted in heavy poultry mortalities over a short period of time. The government of Vietnam applied various recommended control measures including stamping-out and restriction of animal movement to bring the epidemic under control. Stamping out was not only applied to infected flocks but also to those within a 5 kilometre zone around infected flocks (MARD, 2005*a*). As a result of these measures the epidemic was brought under control in late February 2004.

Vaccination was recommended as a useful tool to support the control of avian influenza, based on its documented success in countries such as Italy, Mexico and the USA (Swayne, 2003; Capua and Marangon, 2004). The primary justification for the use of vaccination as a tool for controlling HPAI was its effect on reducing the amount of virus shed from infected birds (Swayne, 2006) thereby reducing the level of environmental contamination. The government of Vietnam decided to use vaccine as a supporting tool in late 2005 (MARD, 2007). This decision was made following various technical consultations with international organisations (OIE and FAO) (FAO, 2004; FAO-OIE-WHO, 2005).

The first step in the Vietnamese vaccination program was the conduct of experiments under laboratory conditions in China (for the H5N1 vaccine) and The Netherlands (for the H5N2 vaccine) and Vietnam (for both the H5N1 and H5N2 vaccines) to determine vaccine efficacy. The vaccine was then administered to poultry under the field conditions

in two provinces (Nam Dinh, in the north and Tien Giang, in the south) in August 2005. In October and November 2005 poultry in 15 and 33 provinces, respectively received vaccination bringing the total number of provinces that had been vaccinated by the end of 2005 to 50 (MARD, 2007). Responses to vaccination were promising: no outbreaks occurred from late 2005 to December 2006 in both poultry and humans (MARD, 2007; Province, 2010).

The national HPAI H5N1 vaccination campaigns have been conducted intensively in two rounds each year. The first round occurs in between April to May one month before the second high risk period (between June and July). The second occurs in October and November one month before the first high risk period (between December and January) (Pfeiffer, 2005; MARD, 2007). Supplemental vaccination has also been conducted in other months for new and unvaccinated populations. Annually, these campaigns have used, on average, around 500 million vaccine doses (MARD, 2009). Chickens receive a single dose at 2 weeks of age. Ducks are given a first vaccination at two weeks of age and then a booster shot 4 weeks later (DAH, 2006). H5N2 vaccine is supplied to all semi-commercial and commercial flocks on a fee-for-service basis.

Under the national HPAI H5N1 vaccination campaigns, the central government provides the vaccine free of charge to all flocks comprised of less than 2000 birds. Local governments pay the vaccination fee. Farmers owning less than 2,000 birds do not pay any fee but they are requested to register their flocks and make their poultry available for vaccination. The central veterinary agency, the Department of Animal Health (DAH), takes the leading role in these vaccination campaigns and provides technical supervision to all provinces. The local veterinary agencies, the provincial Sub-Departments of Animal Health (SDAHs) directly supervise field veterinarians to carry out vaccination activities. SDAH personnel prepare budgets to the provincial authorities to cover vaccination fees. SDAH personnel transport vaccines to District Veterinary Stations (DVSs), which have refrigerators for storage of AI vaccines and other veterinary medicines. DVSs then provide their commune veterinarians or animal health workers with the required number of vaccine doses to cover the total number of registered poultry. Within communes, the lowest level of the administrative ladder, veterinary paraprofessionals (referred to as community animal health workers, AHWs) carry out the task of vaccination. Figure 2.6 summarises the delivery of vaccine from the central government to individual households.

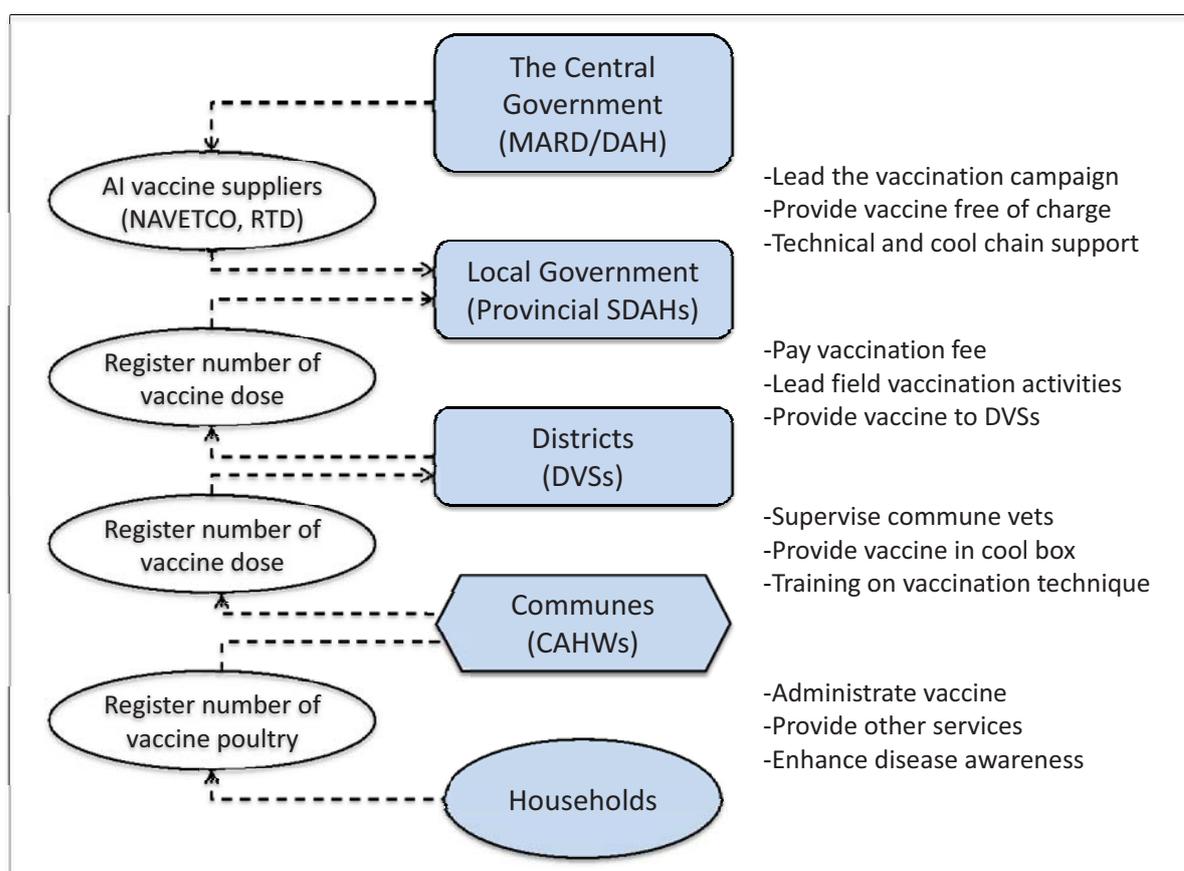


Figure 2.6: Diagram showing vaccine supply process for HPAI.

A reassortant H5N1 vaccine made in China has been used for all campaigns between 2005 and 2010 (inclusive). This vaccine was developed with the use of the H5N1 low pathogenic virus, A/Harbin/Re-1/2003 (referred to as Re-1), which was isolated from Guangdong province in China in 1996 (Tian et al., 2005). Both the HA and NA genes of this donor virus were used to generate the reassortant vaccine virus. Vaccine was firstly produced by the Weike Biological Company of the Harbin Veterinary Research Institute (Chinese Academy of Agricultural Sciences, Harbin, People's Republic of China). The patent has since been granted to several Chinese vaccine manufacturing companies. A recombinant vector-based vaccine, Trovac AVI H5 (TROVAC-H5), an avian influenza fowlpox vector vaccine, was used for chickens at one day old for a number of years. Use of this vaccine stopped in 2008 because it did not show good efficacy under both laboratory and field challenge experiments (DAH, 2008a).

2.4.5 Post-vaccination surveillance

Beside the vaccination campaigns, since September 2005 Vietnam has conducted post-vaccination monitoring, surveillance for circulation of HPAI H5N1 virus and community based surveillance for disease. The post-vaccination surveillance programmes have been designed and lead by DAH (DAH, 2007). The six Regional Animal Health Offices (RAHO1, 2, 3, 4, 6 and 7), the NCVD and SIVR (Figure 2.4) coordinate sampling activities and carry out laboratory testing. Provincial SDAH and DVS staff are responsible for sample collection.

Sampling of vaccinated birds was carried out using a multistage cluster design. High risk provinces were selected and within those provinces districts were selected at random. Within each district communes were selected. Finally, flocks were selected from each commune and a sample of 30 birds taken from each selected flock. Sampling was coordinated to occur one month after the start of each vaccination round. Blood samples were collected to evaluate the level of H5N1 antibody response. Annually, around 50,000 birds were sampled from 33 high risk provinces and from a number of lower risk provinces, resulting in 33–42 provinces being selected each year. In addition, swab samples were taken from unvaccinated birds, including muscovies that were not vaccinated. Unvaccinated birds were selected from either poultry households or from live bird markets in each

sampled province.

Serum samples were tested for the presence of anti-H5 antibodies using the haemagglutination inhibition (HI) test, as documented in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2009). Reference antigen produced from the Scottish strain was provided by the Veterinary Laboratories Agency (Schmittgen et al., 2000), in the United Kingdom. A HI titre of $4 \log_2$ was considered as positive samples although ten dilutions (1-10) were recorded (DAH, 2007; Taylor and Dung, 2007; Long, 2010). On-going post-vaccination monitoring has shown that between 55% – 65% of the total number of tested birds were HI positive. This was lower than the expected target of 70% (DAH, 2007). Other epidemiological studies have identified similar protection rates (Henning et al., 2010; Hoa et al., 2010; Long, 2010). In contrast, the annual challenge experiments performed under the laboratory conditions have demonstrated that current vaccine has an efficacy of greater than 99% (Thanh, 2007; NCVD, 2009). Post-vaccination monitoring at all of the state breeding farms has shown high protection rates, varying between 75% and 90% (Long, 2007a, 2008, 2009).

Swabs taken at the same time as blood samples have been tested by RRT-PCR with the use of primers and probes specified for each clade of HPAI H5N1 virus, which predominantly reside in certain areas of the country (clade 2.3.4 in the north and clade 1 in the south) (Wan et al., 2008). These studies indicate that a high prevalence of HPAI H5N1 virus circulates in 4% to 12% of tested flocks (Long, 2007a, 2008; Hoa et al., 2010; Long, 2010). A difficulty with the current post-vaccination surveillance program is that the HI test cannot differentiate antibody produced by vaccination or natural infection (Long, 2008). This presents major difficulties when interpreting results in areas where vaccination and HPAI outbreaks are occurring at the same time.

DIVA testing

Vaccination of poultry with vaccines containing killed whole virus antigens result in the production of antibodies to both the HA and NA proteins in the vaccine antigen. Thus, once a bird is vaccinated, it is difficult to distinguish between vaccinated and naturally infected birds. The 'DIVA' (differentiating infected from vaccinated animals) strategy is used to avoid this problem, based on the use of an inactivated oil emulsion vaccine containing the same HA subtype as the challenge virus, but a different NA subtype.

Due to a number of reasons, Vietnam did initially not implement a routine DIVA system. Firstly, elimination of virus was not the initial objective of the vaccination campaign in Vietnam (MARD, 2005*b*). Secondly, the circulation of other influenza viruses was limited (Nguyen et al., 2005) whilst serological DIVA strategies depend on the absence of other circulating influenza viruses which might complicate interpretation of results (Capua et al., 2003; Sims and Dung, 2009). Another reason is that assays for anti-NA antibodies are not routinely carried out in Vietnamese laboratories. Tests for detection of anti-NA antibodies in ducks were not readily validated. Additionally, the capacity of these laboratories was insufficient to analyse thousands of serum samples at the introduction of vaccination. Furthermore, when vaccination was introduced pre-market testing of unvaccinated flocks was not routinely done for HPAI. Thus, infected flocks could possibly be sold. If a premarket testing system for all poultry had been in place then there would have been a strong case for introducing a DIVA testing system to reduce the likelihood of infected vaccinated poultry being sent to market. Rather than implementing a DIVA system, a decision was made to improve the overall surveillance system once vaccination was implemented in order to detect circulating viruses, but pre-market testing of all flocks was not possible (Sims and Dung, 2009). Moreover, the time between infection and sero-conversion of birds in a vaccinated flock to allow anti-NA antibodies to be detected in a small sample of birds is at least 3 weeks and possibly longer (allowing a minimum of one week for infection to spread in the flock and two weeks for enough infected birds to develop antibodies). This means that even if a negative serological DIVA test result is obtained, it provides no guarantee that infection has not occurred in the 3 week period before the samples were collected. For short lived broilers this represents almost half of their life span and therefore the capacity of such a system to detect infection for broilers was too low to justify the expense of introducing serological DIVA testing as a routine measure for all vaccinated broiler flocks.

Constraints of vaccination and post-vaccination surveillance

In theory, the vaccination campaign should be conducted in exactly the same manner in all areas of the country. In practice however, measures of the proportion of vaccinated birds showing serological evidence of protection varied markedly between provinces (Taylor and Dung, 2007; Long, 2007*a*, 2008, 2009, 2010). A number of reasons can be used to

explain these differences. The collaboration of poultry owners is a key factor to control HPAI successfully. In the first years of the vaccination campaign, farmers closely collaborated to support local authorities to conduct the various control measures, including vaccination since there was a great deal of concern about the disease. Farmers registered their birds and made them available for vaccination when requested by local veterinary staff. However, in later years of the campaigns, not all farmers retained the same attitude. Some neither registered nor kept their birds available for vaccination because of a belief that vaccination was the cause of weight loss and reduced egg production (Hui et al., 2009; Rudolf et al., 2010). Current policy dictates that farmers have to register the number of birds that they own for vaccination, but current estimates are that only around 50% of farmers actually comply with this regulation (DLD, 2009). A low prevalence of registration severely limits the ability to achieve high vaccination coverages. Millions of doses of vaccine were left unused in SDAHs in some provinces in 2009 (DAH, 2009). Recognising these problems Vietnamese authorities are currently working closely with the FAO in conducting a project to gather evidence for a transitional strategy (GETS) for HPAI H5N1 vaccination in Vietnam (FAO, 2009).

Local logistic preparation, including the amount animal health workers receive for vaccination presents an additional difficulty. While vaccine is provided to farmers free of charge by the central government vaccination fees vary according to individual provincial budgets. A minimum vaccination fee (around VND 50,000 per working day or VND 100 per vaccinated bird) has been set as a guideline, but payment of this level is likely to not be enough to encourage animal health workers to carry out vaccination campaigns with the intensity and attention to detail that is required. For vaccination to be successful, there needs to be close communication between animal health workers and poultry owners (to arrange, for example, yarding of birds) and if this does not occur the likelihood of the program being successful is reduced. This issue has been pointed out in a number of studies conducted in the Mekong River Delta (Henning et al., 2009).

Storage and handling of vaccine from the moment it is manufactured to the time it is administered to a live bird presents additional opportunity for vaccination failure. Vaccine is delivered in a cool chain by contracted providers to each target province (SDAH). Provincial SDAHs then deliver the vaccine to the final destination (the commune). Commune veterinarians or animal health workers then carry out vaccination. However, not all

SDAHs have good cool chain systems, especially in hot weather conditions. Although provincial veterinary agencies (SDAHs and DVSSs) are required to provide field animal health workers with training on correct vaccination techniques, there is circumstantial evidence to suggest that this is not always the case.

Finally, the high turnover of poultry populations in backyard production systems represents a threat to vaccine efficacy at the population level. For instance, in the Red and Mekong River Deltas the density of poultry populations, especially free ranging and broiler duck populations, is high. These populations are not always well managed by their owners and duck flocks are frequently moved out of their home areas to scavenge for feed in rice fields (Men et al., 2006; Minh et al., 2009) meaning that it is often difficult for animal health workers to coordinate the timing of vaccination events. Theoretically, ducks should receive their first vaccination at 14 days and then a second shot at 42 days (DAH, 2006). Vaccinated birds are permitted to be sold two weeks after their last vaccination (i.e. at 56 days). This protocol has the potential to present difficulties for broiler ducks which are slaughtered at 75 to 90 days, particularly if vaccination is delayed.

The design and implementation of post-vaccination surveillance is not straightforward. Although the documented technical guidelines require random selection of flocks from surveillance areas it is possible that staff in some areas may select flocks for testing on the basis of convenience. As a result, post-vaccination monitoring results may not represent actual vaccination performance. Poor technique when sampling birds and inappropriate handling of samples once they have been collected are additional problem areas. Technical guidelines specify that at least 30 birds should be sampled per flock, but many farmers, particularly those with layer flocks, do not support this and some do not allow their birds to be sampled at all. Anecdotal information from the field is that staff in some areas retrieve samples from nearby flocks to meet the required numbers for testing. Factors operating at the laboratory level present a further level of variability.

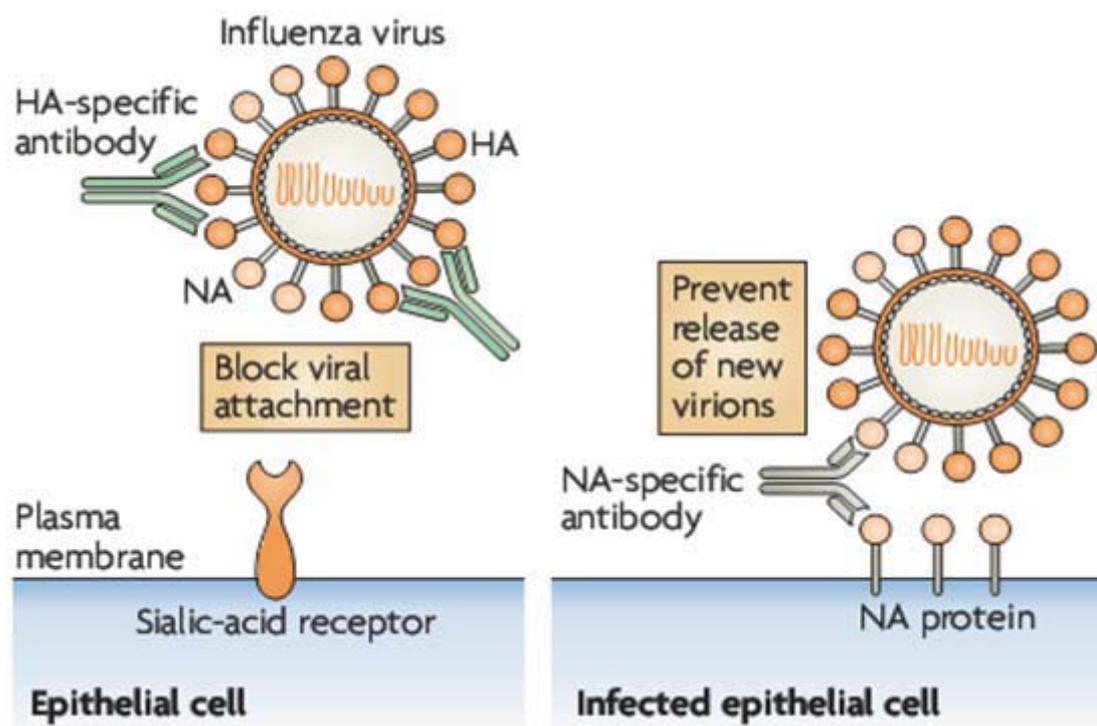
2.5 Molecular epidemiology of HPAI H5N1

2.5.1 HA and NA genes

As presented earlier the AI virus genome consists of eight negative-stranded RNA segments including six encoding for the internal proteins (the matrix proteins M, the nucleoprotein NP, the nonstructural protein NS, and the RNA polymerase proteins PA, PB1, PB2) and two coding for the surface glycoproteins HA and NA. The surface glycoproteins play an important role as targets of protective immune response (Figure 2.7). The HA gene, as a target of neutralising antibodies, is a classic example of an antigenically drifting protein (Webster et al., 1982). This gene accumulates an exceptional number of point mutations in the epitope regions or antibody combining regions (Wilson and Cox, 1990). NA is the second major antigenic determinant for neutralising antibodies. By catalysing the cleavage of glycosidic linkages to sialic acid on the host cell and virion surfaces, this glycoprotein prevents aggregation of virions, thus facilitating the release of progeny virus from infected cells. Understanding the evolution of HA and NA genes is important when conducting surveillance programmes and for selection of appropriate strains to include in vaccine to be used for vaccination campaigns.

2.5.2 Molecular classification of AI

HPAI and LPAI viruses can be separated based on their primary virulence characteristic. The HPAI viruses are able to be cleaved by ubiquitous proteases, which can be found in the most host cells. The HA0 precursor proteins of LPAI viruses have a single arginine at the cleavage site and another basic amino acid (arginine or lysine) at position ± 4 and its cleavage site is catalysed only by trypsin and trypsin-like host proteases. This restricts virus replication to locations where these proteases are found, namely, the respiratory and gastrointestinal tracts. In contrast, HPAI viruses possess multiple basic amino acids (arginine and lysine) at their HA0 cleavage sites either as a result of apparent insertion or apparent substitution (Capua and Alexander, 2001). HA0 cleavage in HPAI viruses is mediated by a poorly defined protease(s) that appears to be a protein processing subtilisin-related endoprotease (Stienekegrober et al., 1992). The ubiquitous nature of these proteases allows the HPAI virus to replicate systemically, damaging vital organs



Source: Horimoto T, 2005

Figure 2.7: Adaptive immune response to avian influenza virus.

and tissues, leading to disease and death (Rott, 1992).

2.5.3 Antigenic drift and antigenic shift

Influenza viruses have been isolated and used in vaccines. However, some vaccination campaigns have failed or have been less effective because the virus circulating in the population has continued to evolve. The term antigenic drift refers to minor genetic mutations to the virus, generated in the genome because of a lack of proofreading which occurs during viral replication. These subtle changes result from point mutations and may result in escape from host immunity or even changes in pathogenicity. Subsequently, new viral strains tend to replace older strains in a population. These altered strains may not be recognised by the bird's immune response to earlier influenza strains, and the host may become infected with new strains (Alexander and Brown, 2000). The impact of antigenic drift on vaccination with human influenza is a well characterised problem that requires the vaccine seed strain to be evaluated every year to try to achieve the best match with circulating strains (Smith, 2003). The serum antibody protection appears to be impacted less by antigenic drift in its ability to block viremia and prevent severe clinical disease, but it

has been shown previously that the level of virus shedding is correlated to the relatedness of the vaccine to the challenge strain (Swayne et al., 2000; Lee et al., 2004).

As well as antigenic drift, influenza viruses can undergo another type of genetic mutation, called ‘antigenic shift’, which occurs only occasionally. This antigenic mutation is a more substantial change and can cause the evolution of radically different viruses. AI viruses, including subtypes from different host species can simultaneously infect a host and exchange their genetic material via reassortment. This process may happen directly through poultry to human transmission or through mixing of a human subtype of influenza A virus with other animal A subtypes to build new subtypes. Subsequently, this may cause infection with a new serotype of virus that is not affected by the immune response generated by vaccination (CDC, 2004; WHO, 2004). As a result, pandemics may occur resulting in severe disease and case fatality rates. It is widely recognised that the medical communities (both human and veterinary) must prepare for flu pandemics before they actually occur (Lee, 2005).

2.5.4 Evolution of the HPAI H5N1

Phylogenetic analyses of amino acid changes show that AI viruses have a much lower evolutionary rate in comparison with those in mammalian species. Evolutionary rate can be used to estimate the date of emergence, or origin of new lineages of influenza viruses. The different rate of evolution that depends on the selective immune and viral adaptation in the host species can be evaluated by their mutation and replication rate (Suarez, 2000; Chen and Holmes, 2006). There is no convincing evidence that there has been net evolution of AI viruses for many decades. Some changes in their nucleotides have been identified and the rate of change in these nucleotides is similar among avian and mammalian viruses. Fortunately, many of the changes in the genome in all of the eight segments do not cause changes in the amino acid sequences (Webster, 2002). Nevertheless, influenza A virus pandemics in humans and animals can occur when new subtypes of HA genes are introduced from species such as aquatic birds, so that the evolution of HA genes is of critical importance. Although the avian influenza H5N1 viruses have caused serious disease in avian species and humans, there is no convincing evidence at the present time that this subtype has been directly transmitted from humans to humans (Halpin, 2005;

Juckett, 2006; Wong and Yuen, 2006).

While theoretically all combinations of gene subtypes can be generated via reassortment events of two different HA and NA influenza subtypes, only some combinations appear to be successful. In mixed infections of different virus strains, the gene segments of those strains can act like alleles of eukaryotic organisms. However, the reassortment of some genes may not be observed because the resultant protein-protein interactions prevent the independent evolution of the associated genes. Gene analysis has indicated that the HA surface protein has a much higher evolutionary rate compared with the internal protein genes such as M, NP, NS, PA, PB1, and PB2 (Webster et al., 1992).

At the present time HPAI H5N1 viruses circulating in Southeast Asia have their HA and NA genes derived from the prototype A/Gs/Gd/1/96 virus, whereas the genes encoding the six internal proteins (M1, M2, NP, PA, PB1, and PB2) are derived from several other sources. This diversity has allowed reassortment into various genotype groups, defined as unique gene constellations (Guan et al., 2002; Li et al., 2004; Duan et al., 2008). As each of the internal gene segments constituting these constellations, a neighbour-joining bootstrap support greater than 70% or a Bayesian posterior probability greater than 95% are the determinants for a distinct phylogenetic lineage. In some cases, the same unique gene constellation has led to the definition of two genotypes, which only differ by some molecular marker. Genotypes Z and Z+, for example, differ by the presence or absence of a multi-amino-acid deletion on the NA protein (Duan et al., 2008).

2.5.5 Emergence, circulation, and evolution of HPAI H5N1 virus IN VIETNAM

The genome sequence analysis of HPAI H5N1 viruses causing outbreaks in Vietnam in 2003 and 2004 indicated that they were genotype Z viruses (Province, 2010). These H5N1 viruses were identified as the same as those previously detected in Southern China (Nguyen et al., 2008). In addition, the continuing persistence of H5N1 genotype Z viruses in Vietnam since 2003 has resulted in the establishment of two geographically distinct groups. Whereas the group N viruses found in the Red River Delta in northern Vietnam are more closely related to viruses in Thailand (and Malaysia) the group S viruses found in the Mekong River Delta are more closely related to those from Cambodia (Smith, Fan,

Wang, Li, Qin, Zhang, Vijaykrishna, Cheung, Huang, Rayner et al., 2006).

In 2005 another H5N1 reassortant virus, designated as clade 2.3.2 (genotype G), was first detected in Vietnam. The gene segments of this virus were closely related to a virus isolated in Guangxi province in China in January 2005. This indicated the possibility of another introduction of virus from China in early 2005 (Chen et al., 2006). Similarly, in February 2006, H5N1 genotype G virus was identified (Nguyen et al., 2008).

Phylogenetic analyses of H5N1 influenza viruses that were isolated from outbreaks in Vietnam during 2005 to 2007 indicated a geographic distinction among the isolates and showed that multiple sublineages were present (Nguyen et al., 2008). It was found that isolates from samples taken in the northern and southern provinces belonged to different clades. The clade 1 viruses isolated in the southern provinces were more closely related to viral isolates from poultry in Cambodia in the same period, whereas the viral clades (2.3.2 and 2.3.4) isolated from the north were closely related to viruses isolated from poultry in Guangxi province in China. The study of multiple sublineages of influenza A virus (H5N1) in Vietnam (Nguyen et al., 2008) also showed evidence for co-circulation of these virus groups and evidence of the reassortment between different sublineages within Vietnamese influenza H5N1 isolates.

In 2007, a new sublineage (Fujian-like sublineage) was detected in the north of Vietnam (Le et al., 2005). This was classified as clade 2.3.4 which is thought to have emerged in China in 2005. Serological studies in China suggest that vaccination may have facilitated the selection of the Fujian-like sublineage (Smith, Naipospos, Nguyen, De Jong, Vijaykrishna, Usman, Hassan, Nguyen, Dao, Bui et al., 2006). Further systematic analysis of the evolution of HPAI H5N1 viruses isolated from the national surveillance program in Vietnam during 2001 and 2007 demonstrated that multiple viral introductions and reassortment events have occurred leading to the emergence of at least four novel genotypes (Wan et al., 2008). This study indicated that at least six clades or subclades of HPAI H5N1 were introduced into Vietnam during the previous seven years (clades 0, 1, 2.3.2, 2.3.4, 3 and 5) and nine reassortants of HPAI H5N1 virus emerged in Vietnam during this period.

It appears that H5N1 viruses have been introduced into Vietnam on multiple occasions. The majority of novel viruses were first detected in northern Vietnam, suggesting multiple introductions from China (Gutierrez et al., 2009). These viruses subsequently spread to

the south, often after reassorting with pre-existing local viruses (Wan et al., 2008; Wang et al., 2008). The apparent northern to southern spread of H5N1 may correspond to direct poultry trade routes between major population centres (Kilpatrick et al., 2006) or by trade routes along the Mekong River from Lao PDR to Vietnam. Some viruses may have spread back and forth between countries at different time points (Nguyen et al., 2008). Cross border poultry trade between Vietnam and China could have led to the introduction of clade 2.3.4 (presumably from Guangxi province, China) and other lineages into Vietnam (Nguyen et al., 2008).

Descriptive analysis of the national post-vaccination study in Vietnam (2007-2009)

Abstract – A repeated survey was conducted to describe spatial, temporal, and individual level factors influencing immunity in poultry vaccinated for HPAI in Vietnam from January 2007 to December 2009.

Our results show that protection risks varied between regions and provinces, with the southern and central regions of Vietnam having lower protection risks compared with the north. Temporal analyses show that the level of protection varied over the three year study period. Protection risks were highest in 2008 (76%) compared to 2007 (70%) and 2009 (68%). There was a strong correlation between vaccination round and protection risk. Protection risk following vaccinations carried out earlier in the year (April to June) were lower than those following vaccinations carried out later (October to December), except for 2009. Protection risks were 4% to 13% greater in chicken flocks compared with duck flocks. Protection risks were between 8% and 18% greater in layer flocks compared with broiler flocks. Flocks where the average age was less than three months had relatively low levels of protection, ranging from 52% to 54% across the three year study period. Protection risks were greater in larger flocks compared with smaller flocks, ranging from 71% to 80%.

Our findings indicate that vaccination coverage should be enhanced in young broiler duck flocks as these have been over represented in HPAI H5N1 outbreaks in recent years. Intensive efforts should be applied in those provinces identified as having low protection levels from year to year to improve vaccination delivery and administration.

3.1 Introduction

Highly pathogenic avian influenza (HPAI) H5N1 was first recognised in Hong Kong in 1996, when it caused the loss of approximately 1.5 million poultry and 18 human deaths (Claas et al., 1998; Saw et al., 1998; Subbarao et al., 1998). Since 1997 outbreaks of HPAI H5N1 have occurred in a number of other Asian countries, including Vietnam, where the first outbreak occurred in late 2003.

Vietnam has applied a number of measures to control HPAI H5N1 including stamping out, restriction of trade and vaccination (MARD, 2005a). During the first epidemic wave which started in December 2003 a stamping out policy was applied to infected places and flocks housed within a 5-kilometre buffer area around them. This resulted in the death of more than 45 million birds (Sims and Dung, 2009). This strategy was successful with control achieved by February 2004. Freedom from disease was short lived, with a second wave of outbreaks occurring between December 2004 and April 2005. In contrast to the first epidemic wave, the second epidemic wave involved a greater number of infected places and took longer to be brought under control. Hence, a number of concerns were raised over the efficiency of outbreak responses, the environmental effects of disposing large numbers of culled poultry, and the advantages and disadvantages of vaccination (MARD, 2007).

Although vaccination was considered as a means to support other control measures, it took more than a year to be adopted because of issues related to vaccine efficacy (MARD, 2007). To address these concerns, a number of laboratory challenge studies and field trials were undertaken at both international and national laboratories before vaccination was adopted for widespread use (MARD, 2007). The first round of vaccination for HPAI H5N1 commenced in September 2005 and involved a total of 18 provinces. Subsequently, vaccination coverage was extended to both the Mekong and Red River deltas and the lowland areas of Vietnam. As of August 2010, a total of nine vaccination rounds have been carried out between April and May and from October to November each year. The two vaccination rounds carried out each year involves administration of approximately 500 million doses of vaccine. At a cost of USD 0.02 per dose the drug cost alone of each vaccination year is estimated to be around USD 10 million (MARD, 2009; DAH, 2009).

To determine the efficacy of vaccination a series of serological surveillance surveys have been carried out by the Vietnamese Department of Animal Health, starting from the first vaccination campaign in 2007. In this paper we provide a descriptive epidemiological analysis of serological surveillance data collected in 2007, 2008 and 2009. Our aims were firstly to provide summary estimates of the proportions of the vaccinated poultry population with sufficient immunity against HPAI H5N1 and secondly to describe how the proportion protected varied by sampling round, location, species, production type, age and flock size. Identifying weak areas in the vaccination program, that is combi-

nations of spatial, temporal and individual bird-level factors associated with insufficient post vaccination immunity, should provide a starting point to the development of targeted strategies that will enhance the efficacy of Vietnam's vaccination program as a whole.

3.2 Materials and methods

The post-vaccination surveillance programmes were designed and lead by the central veterinary agency in Vietnam, the Department of Animal Health (DAH). DAH assigned the laboratories of the Regional Animal Health Offices (RAHO 1, 2, 3, 4, 6 and 7), the National Centre for Veterinary Diagnosis (NCVD) and the Sub-Institute of Veterinary Research (SIVR), located in three main regions of the country to coordinate sampling activities and to carry out laboratory testing. Samples were collected by staff of the Sub-Departments of Animal Health (SDAHs) with technical supervision provided by the eight veterinary diagnostic laboratories (DAH, 2007, 2008a).

This was a repeated survey¹ of the Vietnamese poultry population carried out during 2007, 2008 and 2009. Two sampling rounds were conducted in parallel with the national vaccination campaigns which occurred from April to May and October to November each year. One month post-vaccination, blood samples were collected from vaccinated poultry populations. The first round was carried out between June and July in 2007, 2008 and 2009. The second round occurred between November and December in 2007, 2008 and 2009.

Vaccinated poultry (chickens and ducks) were targeted in 42 provinces in 2007, 31 provinces in 2008 and 34 in 2009. These provinces were selected since they were identified as high risk areas based on the relatively high density of poultry and previous history of HPAI H5N1 outbreaks. The number of provinces decreased over the three year study period because of overlapping surveillance activities under different research projects.

A multi-stage sampling design was used. The first stage was the selection of districts within each province; the second stage was selection of communes within each district, and the third stage was selection of flocks within communes. At each stage a simple random sampling approach was applied. Within communes, information concerning the

¹A series of cross-sectional studies performed on the same study population over time.

vaccination status of flocks was derived from vaccination certificates kept by flock owners. All vaccinated flocks within a commune were numbered consecutively from 1 to n where n defined the total number of vaccinated flocks. A random sample of 20 (12 chicken and eight duck) flocks were selected on the basis of random numbers generated in a spreadsheet package. Thirty birds were sampled from each selected flock, resulting in 600 samples collected per commune per sampling round.

Between 2005 and 2010 the commercially available vaccine for HPAI H5N1 used in Vietnam was a genetically modified reassorted H5N1 low pathogenic virus, A/Harbin/Re-1/2003 (Qiao et al., 2006). This vaccine was produced by the Weike Biological Company of the Harbin Veterinary Research Institute (Chinese Academy of Agricultural Sciences, Harbin, People's Republic of China).

Blood samples were taken from either the wing or leg veins using 10 mL syringes. Blood samples were then kept in cooling boxes or refrigerators and shipped to the nearest diagnostic laboratory. At the laboratory sera were transferred to eppendorf tubes (2.5 mL), separated by centrifugation at 3000 rpm for 10 minutes, and stored at -20°C until testing. Collected sera were inactivated at 56°C for 30 minutes before testing.

Reference inactivated AIV haemagglutinin (H5) antigen and antiserum were produced and supplied by the OIE reference laboratory at the Veterinary Laboratories Agency, Weybridge, UK. The Scottish AI 1994 strain was used to produce antigens and antiserum.

Serum samples were tested for the presence of anti-H5 antibodies using the haemagglutination inhibition (HI) test as specified in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2009). The HI test was applied in U-shaped bottomed microwell plastic plates. Both positive and negative control antigens and antisera were used for each HI testing plate.

HI titres were classified as positive if there was inhibition at a serum dilution of greater than or equal to $1/16$ (or $4 \log_2$ when expressed as the reciprocal). In other words, a HI result of greater than or equal to $1/16$ represented a positive titre against four haemagglutination unit antigen. In this study, we classified birds with a titre of HI greater than or equal to $1/16$ as protected. As the vaccine does not allow one to distinguish between vaccination and infection antibodies, positive test results identified protected, vaccinated poultry or recovered (and immune) HPAI cases.

A spreadsheet was provided to each laboratory for entering sample and flock-level details. Data recorded for each flock included the flock identifier, the province, district and commune where the flock was located, species, average age, production type, the date of last vaccination, date of sampling, flock size, number of samples tested and the number of samples that returned a positive result, as outlined above.

The variables poultry age and flock size were stratified for descriptive analyses. Poultry were classified into the following age groups: < 3 months, 3-6 months, 6-10 months and > 10 months. Flock size was stratified according to FAO recommendations (Sims and Dung, 2009): < 100 birds, 100-500 birds, and > 500 birds. Results are presented as protection risk estimates, the proportion of birds protected against HPAI H5N1. Fleiss quadratic 95% confidence intervals (Fleiss, 1981) are provided assuming a design effect, due to the clustering of protection risk within flocks, of 12 (cluster size = 30 birds per flock; $\rho = 0.4$) (Taylor and Dung, 2007).

3.3 Results

A total of 148,765 serum samples were collected from 5,715 poultry flocks between 2007 and 2009. These included 51,840 samples from 2,053 flocks from 42 provinces in 2007, 38,385 samples from 1,501 flocks from 31 provinces in 2008, and 58,540 samples from 2,161 flocks from 34 provinces in 2009.

Table 3.1 shows that protection risk varied by region. Vaccinated poultry in the north had highest protection risk (80% to 90%) across all years. In contrast, protection risk in the central and southern region ranged from 62% to 74%, and 61% to 64%, respectively. In the north and the south protection risks were highest in 2008. There was a downward trend in the central region from 74% in 2007 to 62% in 2009. Protection risks were similar throughout the study period in the south. In general, protection risks were lower in the first sampling compared with the second in all three regions and years except for 2009.

The choropleth map shown in Figure 3.1a shows variation in protection risk by province. Figure 3.1b shows the point estimate of protection risk (and its 95% confidence interval) as a function of the latitude of each province's centroid. Only 25 of the 41 provinces took part in the surveillance program for all three years; the remaining provinces took part for one or two years only.

Table 3.1: Serological surveillance for HPAI H5N1 in Vietnam, 2007-2009. Details of the percentage of birds protected against HPAI H5N1 by region and year.

Region	2007			2008			2009		
	Tested	Protected	% (95% CI)	Tested	Protected	% (95% CI)	Tested	Protected	% (95% CI)
North	20779	16536	80 (78 - 81)	16792	15110	90 (88 - 92)	20428	16984	83 (81 - 85)
Central	8070	5967	74 (71 - 77)	10682	7230	68 (65 - 71)	15777	9742	62 (59 - 64)
South	22991	14039	61 (59 - 63)	10911	6943	64 (61 - 67)	27135	16701	62 (60 - 64)
Total	51840	36542	70 (69 - 72)	38385	29283	76 (75 - 78)	58540	39786	68 (67 - 70)

Protection risks at the provincial level for the combined three-year study period ranged from 39% to 93%. Protection risks of greater than 70% were achieved in 15 of 17 northern, 2 of 9 central and 3 of 18 southern provinces. In contrast, protection risks of less than 50% were observed in 1 of 17 northern, 1 of 9 central and 3 of 18 southern provinces (data not shown).

Vaccinated poultry had protection risks of greater than 70% in 42 surveillance provinces in 2007, 64% of 31 surveillance provinces in 2008 and 56% of 34 surveillance provinces in 2009 (data not shown). Particular patterns in protection risks were evident for individual provinces. For example, Ha Tinh in the centre and Dong Thap and Binh Phuoc in the south had consistently low protection risks (31% to 57%), whereas Nghe An (central) and Vinh Long (in the south) had greater than 70% of birds protected in 2007 and 2009, but less than 50% in 2008. Each year there were at least four or five provinces which had protection risks of less than 50%.

The analysis of flock-level characteristics stratified by year (Table 3.2) indicates that protection risks were 4% to 13% greater in chickens than in ducks. From 2007 to 2009 the protection risk in chickens decreased from 80% to 72%. Protection risks in ducks fluctuated, increasing from 67% in 2007 to 74% in 2008 and then decreasing to 64% in 2009. In addition, protection risks were between 8% and 18% greater in layers compared with broilers. A higher proportion of older birds were protected compared with younger birds. Birds younger than three months of age had low protection risks ranging from 52% to 54% across the three year study period. Protection risks were greater in larger flocks compared with smaller flocks.

When looking at flock-level characteristics stratified by year and sampling round protection risks were, in general, higher in the second sampling round compared with the first

Table 3.2: Serological surveillance for HPAI H5N1 in Vietnam 2007-2009. Details of the percentage of birds protected against HPAI H5N1 by sampling round, year, species, production type, age and flock size.

Variable	2007			2008			2009		
	Tested	Protected	% (95% CI)	Tested	Protected	% (95% CI)	Tested	Protected	% (95% CI)
Species:									
Chickens	23442	18963	81 (79 – 83)	17180	13423	78 (76 – 80)	28757	20734	72 (70 – 74)
Ducks	24540	16492	67 (65 – 69)	20485	15309	74 (73 – 77)	32863	21175	64 (63 – 66)
Other ^a	3858	1087	28 (23 – 33)	720	551	77 (66 – 87)	1720	1518	88 (83 – 94)
Type:									
Broilers	15803	9313	59 (56 – 62)	8249	5289	64 (61 – 68)	23036	15138	66 (64 – 68)
Layers	26034	19502	75 (73 – 77)	17214	14112	82 (80 – 84)	32904	24254	74 (72 – 75)
Unspecified	10003	7727	77 (74 – 80)	12922	9882	76 (74 – 79)	32904	24254	55 (51 – 58)
Age group:									
< 3 months	9495	4901	52 (48 – 55)	4300	2313	54 (49 – 59)	10565	5439	51 (48 – 55)
3-6 months	15151	10386	69 (66 – 71)	8612	6499	75 (72 – 79)	21431	16300	76 (74 – 78)
6-10 months	13330	10333	78 (75 – 80)	8930	7510	84 (81 – 87)	14545	10848	75 (72 – 77)
> 10 months	3318	2395	72 (67 – 77)	4120	3409	83 (79 – 87)	7960	5862	74 (70 – 77)
Flock size (n):									
< 100	9740	5451	56 (53 – 59)	5165	3579	69 (65 – 74)	10887	7758	71 (68 – 74)
100-500	15011	10292	69 (66 – 71)	11993	8553	71 (69 – 74)	29361	19866	68 (66 – 70)
> 500	17656	13038	74 (72 – 76)	12165	9678	80 (77 – 82)	20019	13302	66 (64 – 69)
Unspecified	9433	7761	82 (80 – 85)	9062	7473	82 (80 – 85)	3073	2501	81 (77 – 86)

^a Includes muscovy ducks and poultry of unspecified species type.

except in 2009 (Figures 3.2 to 3.5).

3.4 Discussion

The aim of the post-vaccination monitoring programmes between 2007 and 2009 was to determine the proportion of vaccinated birds that were protected against HPAI. It is stressed that the results presented here provide limited information on the proportion of birds that were actually vaccinated. This study builds on previous analyses of the post-vaccination surveillance programme in Vietnam, notably the reports of Taylor and Dung (2007). Samples were collected only from vaccinated flocks, so the results presented here do not provide an indication of overall vaccination coverage in the Vietnamese poultry population. A second issue is that we have considered immunity at the bird level only, ignoring individual flock-level effects. Unmeasured factors operating at the flock level (e.g. stresses related to nutrition, housing and general management) means that in some flocks

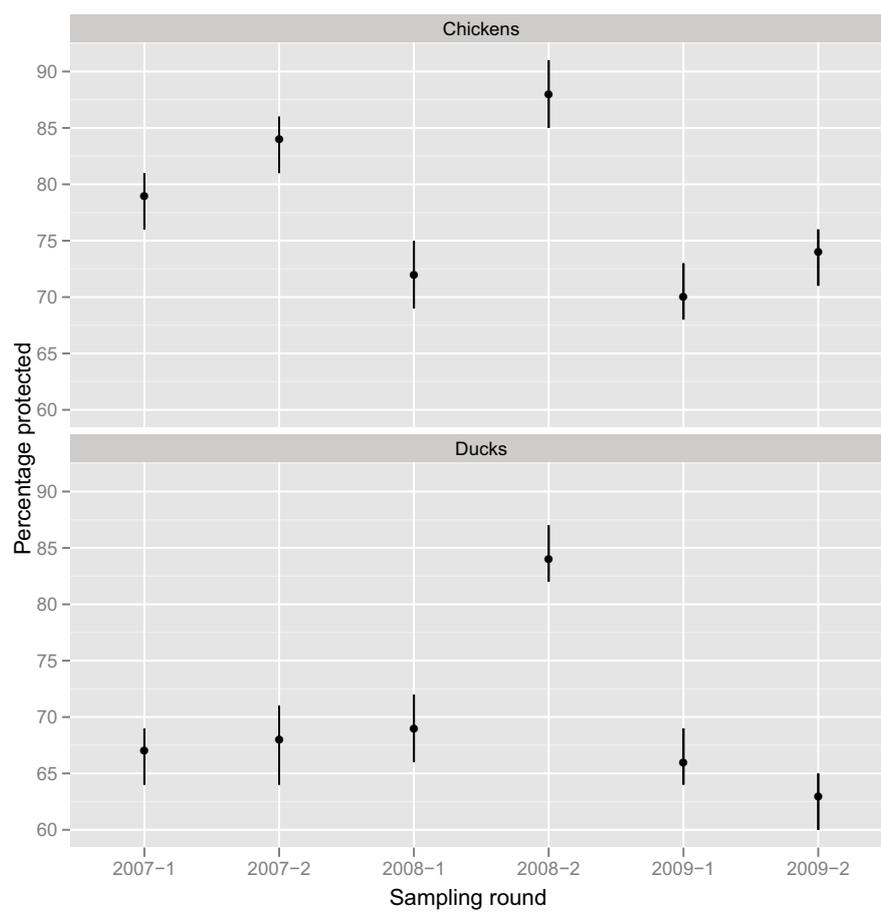


Figure 3.2: Serological surveillance for HPAI H5N1 in Vietnam 2007-2009. Trellis plot showing the percentage of birds protected against HPAI H5N1 by sampling round, year and species.

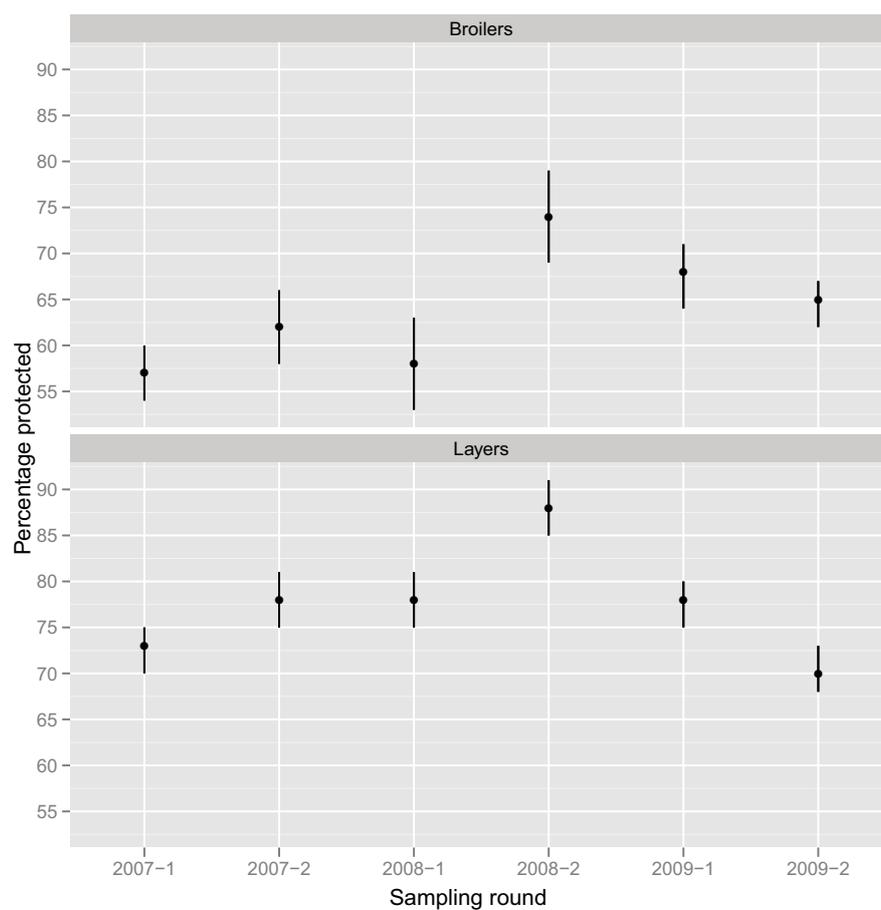


Figure 3.3: Serological surveillance for HPAI H5N1 in Vietnam 2007-2009. Trellis plot showing the percentage of birds protected against HPAI H5N1 by sampling round, year and production type.

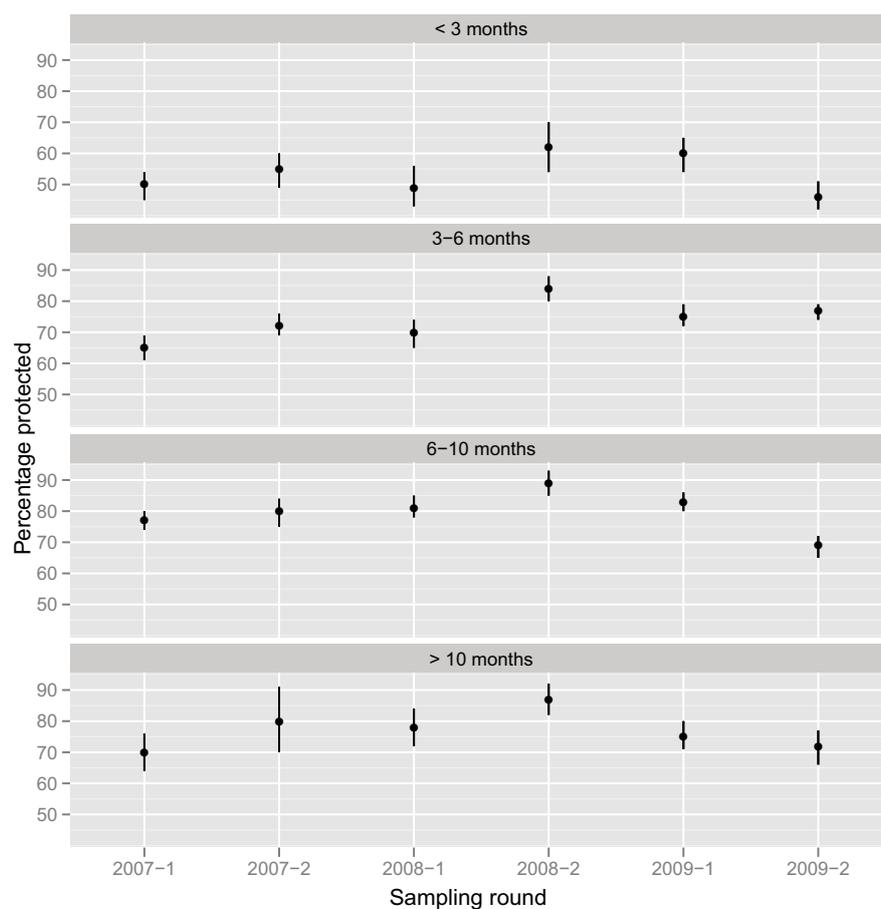


Figure 3.4: Serological surveillance for HPAI H5N1 in Vietnam 2007-2009. Trellis plot showing the percentage of birds protected against HPAI H5N1 by sampling round, year and age.

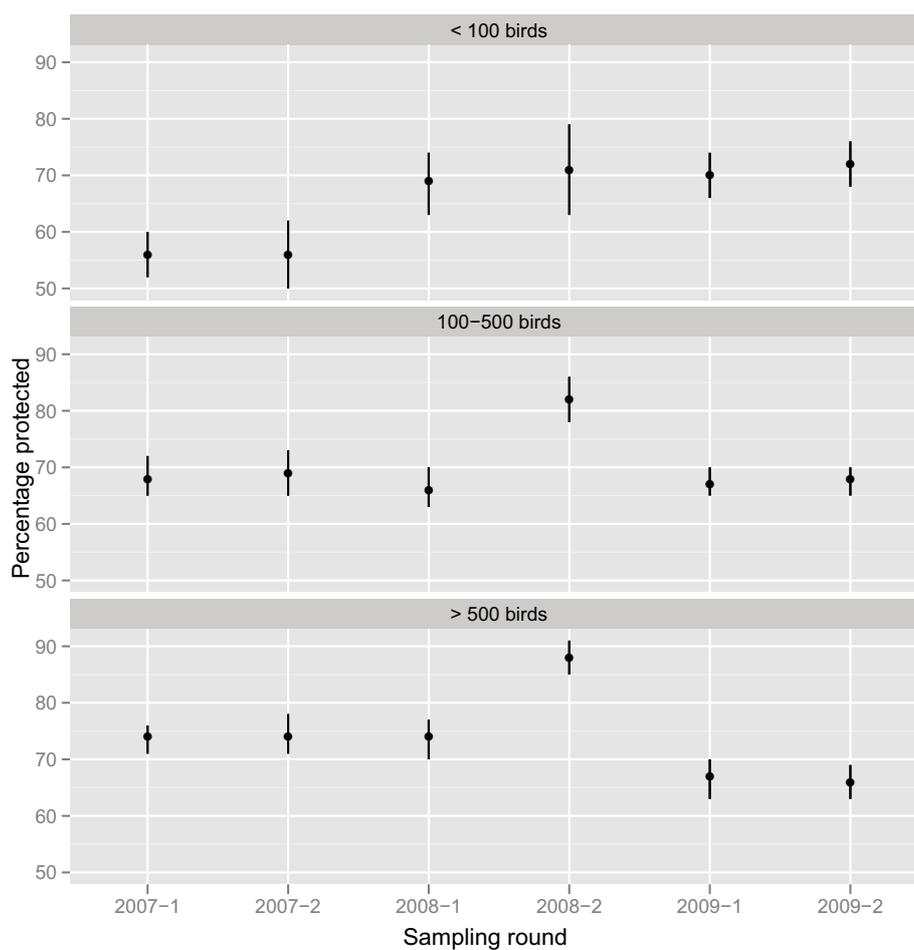


Figure 3.5: Serological surveillance for HPAI H5N1 in Vietnam 2007-2009. Trellis plot showing the percentage of birds protected against HPAI H5N1 by sampling round, year and flock size.

responses to vaccination will be greater than others. These effects will be investigated in detail in the second study in this series (Chapter 4).

3.4.1 Spatial factors

Our results show that protection risk varied between regions and provinces, with the southern and central provinces having lower protection risks compared with the north. These differences may be due to one or a combination of demographic, geographic and laboratory-level factors. For instance, in the south there are greater numbers of ducks compared with the north. The higher density of ducks in the Mekong River Delta could partly explain the lower protection risks in this area as ducks require two doses of vaccine to achieve maximum immunity (Sims and Dung, 2009). It is possible that the second dose of vaccine may not have been consistently given, especially for broiler duck populations because of their short life span of 2-4 months. According to national regulations, the first dose of vaccination should be given at two weeks of age and the second 28 days later (DAH, 2006). Moreover, some poultry owners may choose to vaccinate immediately prior to selling their flock in an effort to minimise perceived production losses following vaccination. In the Mekong River Delta field running duck flocks are frequently moved out of their home communes for feeding (Men et al., 2006; Minh, Stevenson, Schauer, Morris and Quy, 2010), which may have made it difficult for veterinary authorities stationed in a single area (e.g. commune) to locate individual flocks so that the second booster vaccination could be administered.

Laboratory effects may also have contributed to the observed regional differences as different laboratories may have used slightly different testing protocols. It is recommended that duck serum samples are treated by either receptor-destroying enzyme (RDE) or temperatures of 56 °C for 30 minutes to eliminate non-specific inhibitors. Duck serum samples analysed in the northern laboratories were not always treated at 56 °C which was routine practice in the southern laboratories. Differences in eliminating non-specific inhibitors and reading HI titres may have also contributed to differences in reported protection risks (Spackman, 2008). It is important that testing procedures are standardised across laboratories to minimise their potentially confounding effect on regional protection risk estimates.

Differences in weather and geographical conditions may be an additional reason for differences in protection risks across regions. For example, the southern and central provinces (Ha Tinh, Quang Tri, Thua Thien-Hue) are characterised by hot weather and remote flock locations, which may result in lower vaccination coverage as well as poorer vaccine quality due to poor cold chain management. Consequently, vaccinated poultry populations in this area could have lower levels of protection. Regional or provincial factors such as staff capacity (their number and level of training) could also account for differences in protection risks across regions.

The results also showed that some provinces had consistently low protection levels over the surveillance period, particularly Binh Phuoc, Long An and Ha Tinh. Further investigation is required to determine why these provinces had consistently low protection risks due to, for example, insufficient vaccine availability, poor vaccination technique, poor vaccine storage and/or delivery.

3.4.2 Temporal factors

Our temporal analyses show that the level of protection varied across the three year study period. Protection risks were highest in 2008 (76%) compared with 2007 (70%) and 2009 (68%) (Table 3.1), which could be explained by several factors. Firstly, changes in the severity of outbreaks across years are likely to have influenced disease awareness and thus the preparedness of poultry owners to cooperate with local authorities carrying out vaccination campaigns. Following the high number of poultry outbreaks ($n = 998$) and human cases ($n = 61$) during the previous two years in Vietnam the first vaccination round in late 2005 was carried out with strong cooperation between farmers and local authorities (MARD, 2009). HPAI appeared to be under control with no poultry outbreaks and no human cases for almost 12 months between late 2005 and November 2006. Despite the fact that vaccine was provided free of charge to farmers, it is likely that awareness decreased following the reduced incidence of outbreaks since late 2005, which may have resulted in reduced participation in the mass vaccination campaigns.

A second reason for the temporal fluctuation in protection risks relates to changes in the intensity of encouragement applied to poultry owners by DAH field staff. Prior to the second round of vaccination in 2008, several international and national meetings were or-

ganised to evaluate the success and limitations of the first vaccination campaigns, which were applied in the two periods of 2005-2006 and 2007-2008. These meetings were attended by national policy makers, local veterinary authorities and international experts. It was concluded that vaccination played an important role in the control of HPAI in Vietnam while the effect of alternative control measures such as improvement of biosecurity and restructuring the poultry production system remained limiting (FAO, 2008). An outcome from these meetings was that mass vaccination campaigns should be continued for the next two years (2009-2010). As a result of the strong messages arising from these meetings it is likely that the managers of local veterinary authorities boosted their field staff so that vaccination coverage could be improved. However, this encouragement could not be maintained over the following years, as indicated by the reduction in the number of vaccinated birds in 2009 (152 million) compared with that in 2007 (157 million) and 2008 in (260 million) (DAH, 2009).

Characteristics of the vaccine used by DAH could be a third factor that influenced protection levels, although it should be noted that the same vaccine strain was used for the three year study period. The vaccine used in 2007 and 2008 was produced by the Weike Biological Company of the Harbin Veterinary Research Institute, China. In 2009 vaccine was produced by Can Nguyen Hao Company, China although this new company used the same vaccine strain and protocol with authorisation from the original company. It is possible that this change could have had some influence on protection risks.

Our findings also show a strong correlation between vaccination round and level of protection. Protection risks following vaccinations carried out earlier in the year (April to May) were lower than that following vaccinations carried out later (October to November), except for 2009. The likely reason for this is that local staff exercised greater care with vaccination procedures in the second round since outbreaks occurred predominantly during the winter months, from December to February (Pfeiffer, 2005; Minh et al., 2009). This period also coincides with the Têt festival season when poultry trading and movement of poultry increases considerably. We hypothesise that poultry owners would be more cooperative at this time of the year because vaccination certifications are required to permit the movement of poultry.

3.4.3 Host factors

The analyses of protection level by species (Table 3.2 and Figure 3.2) show that chickens were more likely to be protected compared with ducks. This finding may be explained by the fact that ducks require two doses of vaccine to achieve full immunity as discussed earlier.

Birds classified as layers were more likely to be protected compared with broilers (Figure 3.3). It is likely that the longer life span and the greater economic value of layers may have caused owners to vaccinate layer flocks more readily. Older birds were more likely to be protected compared with younger birds (Figure 3.4), an observation also made by Henning et al. (2010) in a study of farm- and flock-level risk factors associated with HPAI outbreaks on small holder duck and chicken farms in the Mekong River Delta of Vietnam.

It is difficult to distinguish between the individual effects of age and production type on immunity as broiler flocks are generally comprised of younger birds only. Hence, age will confound the observed association between production type and protection risk. Age may affect the likelihood of immunity not just because older birds are more likely to receive booster vaccinations, but also because of their greater likelihood of being exposed to high- and/or low-pathogenic H5N1 virus. It has been shown that exposure to highly pathogenic H5N1 virus may result in subclinical infection, particularly in ducks (Sturm-Ramirez et al., 2005). Low pathogenic H5N1 viruses have been detected in domestic waterfowl at live-bird markets in Vietnam (Jadhao et al., 2009) and natural exposure to these viruses may induce an immune response. One shortcoming of post-vaccination surveillance is the inability to distinguish serologically between vaccination and natural exposure to field viruses.

Our results showed that birds in larger flocks were more likely to be protected compared with birds from smaller flocks (Figure 3.5). This is supported by outbreak data showing a lower occurrence of AI in large flocks (DAH, 2009). Higher protection risks in larger flocks could be related to their economic value, resulting in owners of larger flocks taking more care with vaccination campaign directives in order to protect their flocks from infection and culling. In addition, larger flocks are likely to be moved over longer distances (Men et al., 2006; Minh, Stevenson, Schauer, Morris and Quy, 2010) which means that farmers need vaccination certifications to allow them to move their poultry freely.

A greater likelihood of long-distance movements for poultry grazing in rice paddy fields may also increase the chance of natural exposure to HPAI H5N1 viruses, which are widely circulating in Vietnam (Hoa et al., 2010; Long, 2010; Nomura et al., 2010).

3.4.4 Limitations of this study

A limitation of this study relates to selection bias of study subjects. Although the documented technical guidelines specified a random selection of flocks from the surveillance areas, it is possible that staff in some provinces may have selected flocks based on convenience. For instance, the percentage of overlapping surveillance communes in two sampling rounds in 2009 was about 22% of the total number of communes selected in Vietnam (Long, 2010). The biological plausibility of our results provides some (albeit limited) support to the argument that this bias had a relatively minor influence on the overall findings of this study.

Another limitation was that the vaccine used in Vietnam does not allow us to distinguish between vaccinated and infected birds. This means that birds that had previously experienced infection and had recovered were mistakenly counted as having a successful vaccination event. One potential strategy to distinguish between vaccinated and infected birds would be to apply a DIVA strategy, which relies on the use of vaccines containing antigens with a different N subtype to the field strain (e.g. H5N2 antigen but H5N1 field virus). For instance, if birds seropositive to the H5 antigen have been exposed to the field virus, they should have N1 antibody. If birds seropositive to the H5 vaccine strain have been exposed to the field virus, they should have anti-N2 antibody. At present, there are several factors limiting the application of DIVA technology in Vietnam. An assay for anti-NA antibodies is not currently available in Vietnamese diagnostic laboratories. Also, an anti-NA antibody test in ducks is not available (Sims and Dung, 2009). It would be prudent to continue evaluating alternative vaccination strategies in the future, which allows distinction between vaccinated and infected animals, so as to maximise the usefulness of post-vaccination surveillance results and allow the use of serological monitoring to detect natural (subclinical) infection.

3.5 Conclusions

This study has identified spatial, temporal and individual bird-level factors associated with HPAI H5N1 protection risk in vaccinated poultry in Vietnam for the period 2007 to 2009. Our findings indicate that vaccination coverage should be enhanced in young broiler duck flocks as these have been over represented in HPAI H5N1 outbreaks in recent years. The importance of other poultry groups such as those within smaller flocks should be carefully evaluated. Risk-based vaccination approaches may be effective in reducing the overall vaccination workload, allowing animal health staff to achieve optimum vaccination coverage and protection in high-risk groups. Our findings indicate that intensive efforts should be applied in those provinces with low protection levels from year to year to improve vaccination delivery and administration.

Determinants of HPAI vaccination success in the Mekong River Delta, Vietnam

Abstract – A cross-sectional study was conducted between January and December 2009 in the Mekong River Delta of Vietnam to identify characteristics that influenced the probability that a flock of poultry were protected against HPAI H5N1 following a vaccination event. Seven provinces, comprised of 64 districts where no outbreaks of HPAI H5N1 had been recorded previously were included in the study. A multi-stage sampling design was used to select vaccinated flocks. Sera were tested for the presence of anti-H5 antibodies using the haemagglutination inhibition test. A flock was defined as being protected if equal or greater than 70% of birds had HI titres of greater than 1/16. The outcome for these analyses was protection risk, defined as the number of flocks protected against HPAI H5N1 divided by the total number of flocks that were vaccinated.

A total of 13,180 serum samples were analysed. A mixed-effects logistic regression model was fitted to the data to assess the contribution of province, district, commune and flock level effects on the probability of being protected against HPAI H5N1. Explanatory variables contributing to protection risk included the production type of the flock, flock age and species. The proportions of variance occurring at the province, district, commune and flock level were 8%, 4%, 3% and 85%, respectively. Individual flock-level effects were the main contributor to variation in protection risk.

Our findings indicate that interventions to improve HPAI vaccination efficacy should be focussed at the individual flock (i.e. household) level. Careful review of all vaccination-related activities carried out by provincial authorities would also be a useful strategy. Compared with interventions targeted at the individual flock level, the likelihood of success of interventions applied at the provincial level should have a greater likelihood of success because of the smaller number of individual stakeholders involved. Although the effect of district and commune were relatively minor contributors to the variation in protection risk, reiteration of the importance of vaccination procedures to district-level and commune-level authorities is still advised because of the close association that these groups have with individual flock owners.

4.1 Introduction

Highly pathogenic avian influenza H5N1 virus has caused tremendous losses in poultry in Vietnam since late 2003. The disease also has brought epidemics to several countries including Indonesia, Egypt, and China. Animal health authorities in these countries

have applied various control measures included stamping out, movement restriction and vaccination, but the disease currently remains endemic in many areas (OIE, 2009). Viral mutation is of concern, especially under the pressure of vaccination. This concern is more acute in countries such as Indonesia, Vietnam and China where several types of vaccine have been used at the same time while the prevalence of virus circulating in the population remains relatively high.

Vietnam has a large poultry population with the majority of animals reared under backyard conditions. Meat from poultry comprises an important component of the diet and, as a result, broiler flocks (comprised of large numbers of young birds) comprise a large component of the poultry population. A highly dynamic population contributes to the maintenance of HPAI H5N1 in the environment facilitated by the presence of domestic ducks which are a natural reservoir for infection (Sturm-Ramirez et al., 2005). Although Vietnam has used vaccination as a tool to control the disease implemented in the form of two massive campaigns per year since late 2005 (MARD, 2009) the disease has not been eradicated completely. Outbreaks still occur sporadically and routine surveillance activities has shown that asymptomatic infection in poultry is relatively high (Hoa et al., 2010; Long, 2010).

This study focused on the Mekong River Delta because of its relatively high density of poultry, particularly ducks. Poultry production in this area of Vietnam has unique characteristics, for example field running ducks are regularly moved from one location to another for feeding and broiler duck production is characterised by large flocks reared for a short period of time before being slaughtered at 2-3 months of age (DAH, 2006; Minh, Stevenson, Morris and Schauer, 2010). HPAI H5N1 outbreaks have occurred repeatedly in this region since late 2003. The objectives of this study were to quantify the effect of provincial, district, commune and household-level risk factors on the probability of a flock being protected following vaccination. Identifying the relative importance of factors influencing vaccination efficacy is necessary if Vietnam is to take steps to further improve the overall effectiveness of vaccination as a control measure for HPAI H5N1.

4.2 Materials and methods

A cross-sectional study was conducted between January and December 2009 in the Mekong River Delta of Vietnam to identify characteristics that influenced the probability that a flock of poultry were protected against HPAI H5N1 following a vaccination event. Seven out of the thirteen provinces in this region took part (Dong Thap, Vinh Long, Tra Vinh, Soc Trang, Bac Lieu, Ca Mau, and Kien Giang, Figure 4.1). These provinces were purposively selected. Currently, there are no diagnostic tests that can be used to distinguish antibody produced by vaccination and that produced by natural (subclinical) infection. Therefore, to estimate the true proportion of poultry flocks with sufficient immunity against HPAI H5N1 following vaccination, this study concentrated on only those communes within selected provinces where there was no prior history of HPAI H5N1 outbreaks.

The post-vaccination surveillance programmes were designed and lead by the central veterinary agency, the Department of Animal Health (DAH). DAH assigned the Regional Animal Health Office 7 (RAHO 7) in the Mekong River Delta to coordinate sampling activities and to carry out laboratory testing. Samples were collected by staff of the seven Sub-Departments of Animal Health (SDAHs) with technical supervision provided by staff of RAHO 7 (DAH, 2007, 2008a).

Vaccinated poultry populations (chickens and ducks) were targeted in the seven study provinces in 2009 (Figure 4.1). Two sampling rounds were conducted in parallel with the national vaccination campaigns (April to May and October to November). One month post-vaccination, blood samples were collected from vaccinated poultry populations. The first round was carried out between June and July in 2009. The second round occurred between November and December in 2009.

A multi-stage sampling design was used. The first stage was the selection of districts within each selected province. The second stage was selection of communes within each district, the third stage was selection of flocks within communes and the final stage was the selection of birds within flocks. At each stage a simple random sampling approach was applied. All vaccinated flocks in each commune were numbered consecutively from 1 to n where n defined the total number of commune flocks. A random sample of 20 (12 chicken and eight duck) flocks were selected on the basis of random numbers generated

in a spreadsheet package. Thirty birds were sampled from each selected flock, resulting in 600 samples collected per commune per sampling round.

Between 2005 and 2010 the commercially available vaccine for HPAI H5N1 used in Vietnam was a genetically modified reassorted H5N1 low pathogenic virus, A/Harbin/ Re-1/2003 (Qiao et al., 2006). This vaccine was produced by the Weike Biological Company of the Harbin Veterinary Research Institute (Chinese Academy of Agricultural Sciences, Harbin, People's Republic of China).

Blood samples were taken from either the wing or leg veins using 10 mL syringes. Blood samples were then kept in cooling boxes or refrigerators and shipped to the RAHO 7 diagnostic laboratory. At the laboratory sera were transferred to eppendorf tubes (2.5 mL), separated by centrifugation at 3000 rpm for 10 minutes, and stored at -20 °C until testing. Collected sera were inactivated at 56 °C for 30 minutes before testing.

Reference inactivated AIV haemagglutinin (H5) antigen and antiserum were produced and supplied by the OIE reference laboratory at the Veterinary Laboratories Agency, Weybridge, UK. The Scottish AI 1994 strain was used to produce antigens and antiserum.

Serum samples were tested for the presence of anti-H5 antibodies using the haemagglutination inhibition (HI) test as specified in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2009). The HI test was applied in U-shaped bottomed microwell plastic plates. Both positive and negative control antigens and antisera were used for each HI testing plate.

HI titres were classified as positive if there was inhibition at a serum dilution of greater than or equal to 1/16 (or $4 \log_2$ when expressed as the reciprocal). In other words, a HI result of greater than or equal to 1/16 represented a positive titre against four haemagglutination unit antigen. A flock was defined as being protected if greater than 70% of birds within a flock had HI titres greater than or equal to 1/16. The outcome for these analyses was protection risk, defined as the number of flocks classified as protected against HPAI H5N1 (using the criteria provided above) divided by the total number of flocks that were vaccinated.

Data recorded for each flock included the flock identifier, the province, district and commune where the flock was located, the predominant species present in each flock (duck or chicken), average age, production type (broiler or layer), the date of last vaccination, the

date of sampling, flock size, the number of samples tested and the number of samples that returned a positive result.

Variables thought to explain flock-level protection risk included the predominant species in the flock (duck or chicken), production type (broiler or layer), and average age (a continuous variable expressed in months). Descriptive analyses were carried out to summarise the main features of the data. Bivariate analyses using the chi-squared test and the Kruskal-Wallis test were undertaken to select, for multivariable modelling, variables associated with protection risk.

To select variables that best explained the probability of a flock being protected against HPAI H5N1 following vaccination a backward stepwise approach was used. All variables associated with a flock being protected at an alpha level less than 0.2 at the bivariate level were entered into the model. The significance of each explanatory variable was tested using the Wald test. Explanatory variables that were not statistically significant were removed from the model one at a time, beginning with the least significant, until the estimated regression coefficients for all the variables retained were significant at an alpha level of less than 0.05. Having developed a model that only included flock-level determinants of protection risk (a fixed-effects model), we included random effect terms to account for the effect of unmeasured variables operating at the province, district and commune level (a mixed-effects model). Using this approach the logit transform of the probability that a flock i was protected against HPAI H5N1, p_{ijkl} , was modelled as a linear function of a set of fixed, flock-level effects $\beta_1 \dots \beta_m$ and unmeasured influences at the province P_j , district D_k and commune C_l level:

$$\log \left[\frac{p_{ijkl}}{1 - p_{ijkl}} \right] = \beta_0 + \sum_{i=1}^m \beta_m x_{mijkl} + P_j + D_k + C_l + \epsilon_{ijkl}. \quad (4.1)$$

The results of the final model are reported in terms of adjusted odds ratios for each explanatory variable. An adjusted odds ratio (and its 95 per cent confidence interval [CI]) of greater than 1 indicates that, after adjusting for other variables in the model, exposure to the explanatory variable increased the risk of a flock being protected. An adjusted odds ratio (and its 95 per cent CI) of less than 1 indicates that exposure to the explanatory variable was protective, and an odds ratio of 1 indicates that the variable had no influence on protection risk. A Receiver Operating Characteristic (ROC) curve were constructed on the

basis of the protection status of flocks predicted by the model. The area under the ROC curve, which ranges from zero to one, provided a measure of the model's ability to discriminate between protected and unprotected flocks. The greater the area under the ROC curve the better the models discriminatory power (Hosmer and Lemeshow, 2000). Statistical analyses were performed using the MASS package (Venables and Ripley, 2002) in R version 2.11.1 (R Development Core Team, 2010).

4.3 Results

The data was comprised of records for 464 flocks representing a total of 14,525 individual birds. Since one of our study design criteria was to analyse data from communes that had no reported outbreaks of HPAI H5N1, 13 communes comprised of 43 flocks (a total of 1345 birds) were excluded. The final data set was comprised of details for 421 flocks (a total of 13,180 individual birds). Table 4.1 provides descriptive statistics of the flock-level data. Table 4.2 provides a summary of the hierarchical structure of the data. Table 4.3 provides details of the descriptive analyses of each of the explanatory variables included in the fixed-effects and mixed-effects models.

Regression coefficients and their standard errors for each of the fixed effects and the variance estimates for the province, district and commune level random effect terms are shown in Table 4.4. After adjusting for the effect of production type and age and provincial, district, and commune level effects, the odds of protection in chicken flocks was 2.39 (95% CI 1.65 - 3.48) times the odds of protection in duck flocks. The odds of protection in layer flocks was 2.32 (95% CI 1.55 - 3.47) times the odds of protection in broiler flocks. The odds of protection in flocks where the average age of birds was greater than or equal to 6 months was 2.58 (95% CI = 1.85 - 3.59) times that of flocks where average age was less than 6 months.

In the mixed-effects model, the level 1 (flock level) variance was constrained to unity. The estimates of the proportion of variance at the province, district, commune and flock level were computed by assuming the level 1 variance on the logit scale was $\pi^2/3$. The variance estimates at the province, district and commune level were 0.2884, 0.1165 and 0.2208 (respectively) giving rise to the total variance in the data as 3.9157 ($0.2884 + 0.1165 + 0.2208 + \pi^2/3$). The proportions of variance at the province, district, commune and flock

level were 8% ($0.2906 \div 3.8761$), 4% ($0.1663 \div 3.8761$), 3% ($0.1293 \div 3.8761$) and 85% ($\pi^2/3 \div 3.8761$), respectively. Figure 4.2 is a box and whisker plot of the point estimates and 95% confidence intervals for each of the fixed, flock-level determinants of protection risk for the fixed-effect and mixed-effect models. Accounting for the hierarchical structure of the data in the mixed-effects model shifted the effect of species towards the null (i.e. closer to one) and increased the uncertainty around each of the estimated odds ratios. The point estimate of the odds ratios for age and production type were largely unchanged, but the confidence intervals around each were increased.

The predictive power of the mixed-effects model, as measured by the area under Receiver Operating Characteristic curve was 0.79 (Figure 4.3).

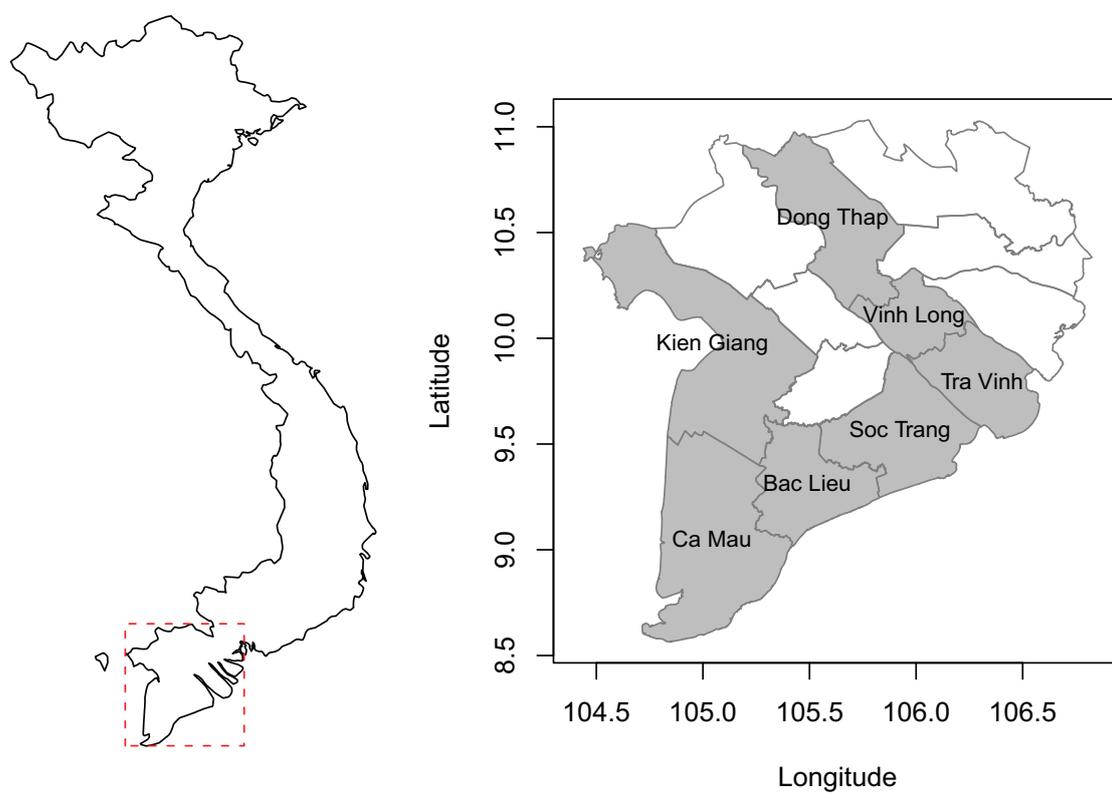


Figure 4.1: Risk factors for HPAI H5N1 vaccination failure in the Mekong River Delta of Vietnam in 2009. Location of the seven provinces included in this study: Dong Thap, Vinh Long, Tra Vinh, Soc Trang, Bac Lieu, Ca Mau, and Kien Giang.

Table 4.1: Risk factors for HPAI H5N1 vaccination failure in the Mekong River Delta of Vietnam in 2009. Descriptive statistics of flock-level data.

Variable	<i>n</i> flocks	Mean (SD)	Median (Q1, Q3)	Range	Missing
Average age (months)	421	8 (6)	6 (4, 12)	1, 36	0
Flock size	421	980 (4700)	350 (150, 700)	17, 62000	0
Date of vaccination (round 1)	225	21 Apr (-)	23 Apr (4 Apr, 14 May)	2 Jan, 11 Nov	25
Date of vaccination (round 2)	196	9 Jul (-)	6 Jun (21 Apr, 16 Oct)	2 Jan, 3 Dec	36
Vaccination-sampling interval (days)	414	50 (27)	44 (30, 60)	11, 156	197
Samples tested per flock	421	31 (7)	30 (30, 30)	25, 60	0

Table 4.2: Risk factors for HPAI H5N1 vaccination failure in the Mekong River Delta of Vietnam in 2009. Structure of the data for the mixed-effects model.

Level	Number	Average number per unit at next-higher level ^a	Range
Province	7	-	-
District	64	9	7 – 11
Commune	185	3	1 – 9
Flock	421	2	1 – 10

^a Each province had, on average, 9 (range 7-11) districts. Each district had, on average, 3 (range 1-9) communes. Each commune had, on average, 2 (range 1-10) households.

Table 4.3: Risk factors for HPAI H5N1 vaccination failure in the Mekong River Delta of Vietnam in 2009. Descriptive statistics of the explanatory variables included in the fixed-effects and mixed-effects models.

Variable	Levels	<i>n</i> flocks	<i>n</i> birds
Species	Chicken	161	4796
	Duck	260	8384
Production type	Broiler	166	5003
	Layer	255	8177
Age ^a	≤ 6 months	236	7114
	> 6 months	185	6066

^a Median age 6 months.

Table 4.4: Risk factors for HPAI H5N1 vaccination failure in the Mekong River Delta of Vietnam in 2009. Regression coefficients and their standard errors for the final mixed-effects logistic regression model.

Variables	Coefficient (SE)	t-value	P-value	OR (95% CI)
Fixed effects:				
Intercept	-0.4578 (0.2731)	-1.67	0.09	
Species:				
Duck				Reference
Chicken	0.8732 (0.1910)	4.57	< 0.01	2.39 (1.65 - 3.48) ^a
Production type:				
Broiler				Reference
Layer	0.8406 (0.2063)	4.07	< 0.01	2.32 (1.55 - 3.47)
Average age:				
≤ 6 months				Reference
> 6 months	0.9464 (0.1699)	5.57	< 0.01	2.58 (1.85 - 3.59)
Mixed effects:				
	Variance			
Province	0.2906			
District	0.1663			
Commune	0.1293			

^a Interpretation: After adjusting for the effect of production type, flock age and provincial, district, and commune level effects, the odds of protection in chicken flocks was 2.39 (95% CI 1.65 - 3.48) times greater than the odds of protection in duck flocks.

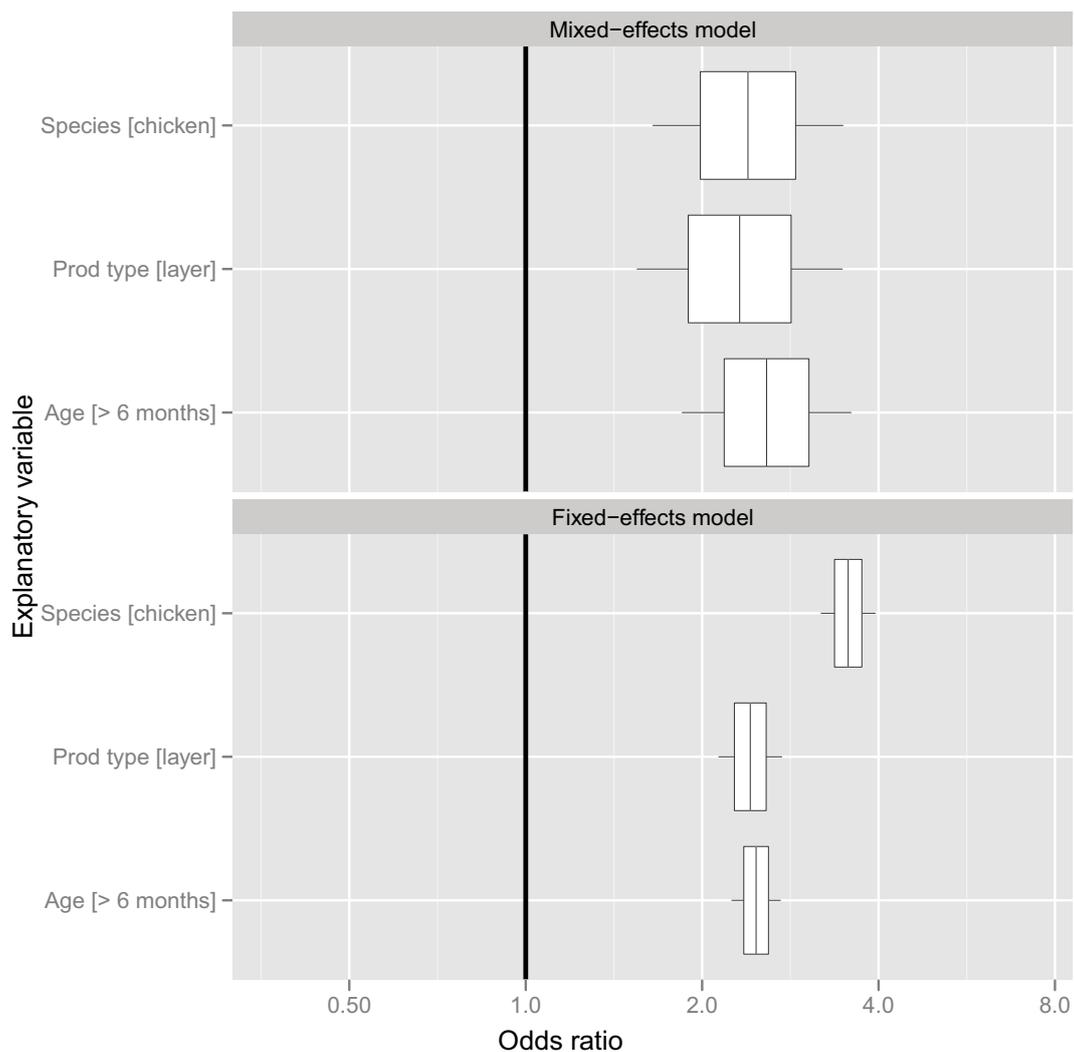


Figure 4.2: Risk factors for HPAI H5N1 vaccination failure in the Mekong River Delta of Vietnam in 2009. Box and whisker plot showing the point estimate and lower and upper bounds of the odds ratios for each of the explanatory variables included in the fixed- and mixed-effects models.

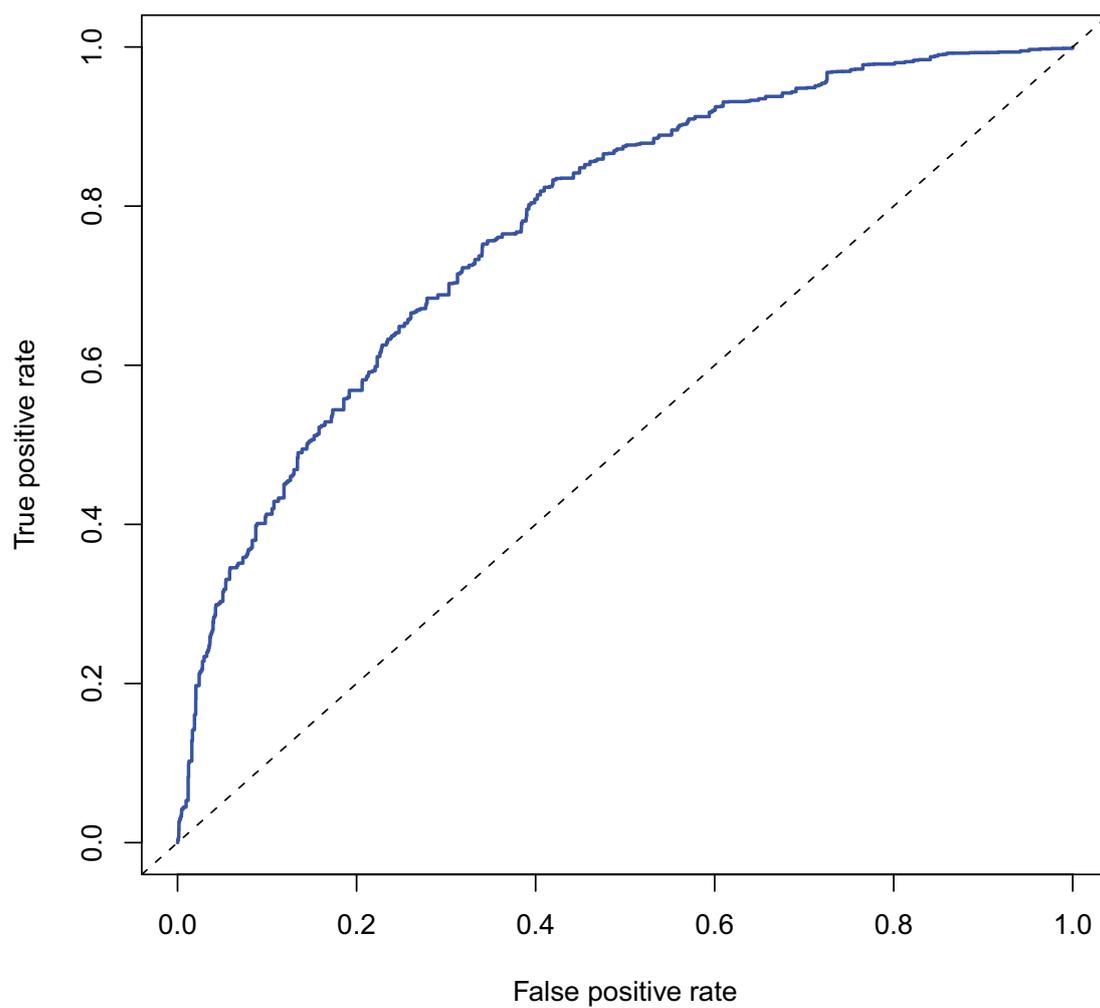


Figure 4.3: Risk factors for HPAI H5N1 vaccination failure in the Mekong River Delta of Vietnam in 2009. Receiver operating characteristic (ROC) curve showing the predictive ability of the mixed-effects model shown in Table 4.4. The area under the ROC curve was 0.79.

4.4 Discussion

The findings presented here support the conclusions of our descriptive analysis of the national post-vaccination study for 2007-2009 (Chapter 3). Vaccinated chicken flocks were 2.39 (95% CI 1.65 - 3.48) times more likely to be protected against HPAI H5N1 than vaccinated duck flocks. Layer flocks were 2.32 (95% CI 1.55 – 3.47) times more likely to be protected against HPAI H5N1 than broiler flocks and flocks greater than or equal to 6 months of age were 2.58 (95% CI = 1.85 – 3.59) times more likely to be protected than younger flocks. In short, the likelihood of a flock being protected was greater for flocks comprised of older, layer birds.

Inclusion of the random effect terms in the model provided the opportunity to assess the contribution of unmeasured provincial, district, commune and flock-level influences on protection risk. Our findings were that most of the variation in protection risk occurred at the individual flock level (84%). This means that the actions taken by individual flock owners are critical determinants of the success of the Vietnamese vaccination program (MARD, 2009). There are several reasons that might explain why this would be the case. The first relates to awareness of the importance of HPAI H5N1 as a disease, which in turn influences how closely flock owners cooperate with field staff who carry out the field vaccination programs. Duck farmers are of particular concern here. It is known that many owners of duck flocks choose to vaccinate their flocks immediately prior to sale to minimise perceived production losses from production. This means that flocks are often moved prior to the onset of full immunity from vaccination, increasing their risk of being infected and then transmitting infection onto other flocks. In the Mekong River Delta field running duck flocks are moved away from their home communes for feeding (Men et al., 2006; Minh, Stevenson, Schauer, Morris and Quy, 2010) which makes it difficult for DAH staff stationed in a single area (e.g. commune) to locate a flock so that vaccinations (particularly the second booster shot) can be administered. Many poultry flocks in the Delta are raised under backyard conditions. In these systems poultry are reared continuously and continuous mixing occurs between young and older birds. Groups of mixed age birds makes it difficult for field staff to distinguish between birds requiring primary and booster vaccinations. This is particularly a problem when young birds arrive or are just hatched after a vaccination round (Henning et al., 2009). Additional flock-level influences on

protection risk include environmental conditions (temperature, relative humidity), plane of nutrition, level of parasitism, and the presence of other endemic disease conditions in the flock at the time of vaccination. These factors combine to negatively impact on an animal's ability to mount an effective immune response to vaccination (Davison et al., 2008). To minimise the effect of individual flock-level effects on protection risk alternative vaccination strategies might be considered. For example, rather than conducting two vaccination rounds per year, vaccine might be made available throughout the year for use in specified age groups. Such a strategy would be most practical if vaccine was administered by individual flock owners. In adopting this strategy, care would need to be taken to ensure that flock owners received adequate instruction on correct vaccination technique.

HPAI H5N1 vaccination campaigns in Vietnam have been conducted with the involvement of a number of government authorities (Figure 2.6). The central government provides vaccine free of charge while local governments cover vaccination fees. Farmers are not required to pay any fee for vaccination but they are requested to make their birds available when veterinary staff come to their farms or households for vaccination (MARD, 2009). Critical to the success of the vaccination campaigns has been the contributions made by provincial, district and commune-level authorities. The role of provincial authorities (in the form of SDAHs) is to supervise the payment of vaccination fees, play a primary role in leading vaccination activities and to provide vaccine to field staff stationed in district offices. The role of district authorities is to store and deliver vaccine to commune staff, improve awareness among poultry owners about HPAI control, provide direct technical supervision to field veterinarians, organise the operation of vaccination campaigns within their respective areas and to take samples for the post-vaccination surveillance studies. Commune staff are responsible for travelling to individual households to administer vaccine to individual birds. Of these three levels, our analyses show that provincial-level effects had a greater contribution to individual flock variation in protection risk (8%) compared with districts (4%) and communes (3%). This implies that a review and tightening of all provincial-level activities related to vaccination (for example, attention to storage facilities and cold chain maintenance) should have the greatest impact on vaccine efficacy. Compared with interventions targeted at the individual flock level, the likelihood of success of interventions applied at the provincial level should have a greater likelihood of success because of the smaller number of individual stakeholders

involved. Although the effect of district and commune were relatively minor contributors to the variation in protection risk, reiteration of the importance of vaccination procedures to district-level and commune-level authorities is still advised because of the close association that these groups have with individual flock owners.

Molecular epidemiology of HPAI H5N1 in Vietnam between 2008 and early 2010

Abstract – The continuous monitoring of viral changes is important for Vietnam where multiple clades of highly pathogenic avian influenza (HPAI) virus have been circulating and routine mass vaccination campaigns against HPAI H5N1 have been carried out since 2005. Furthermore, the potential for subclinical infection has been a concern, representing a major constraint to effective and ongoing passive surveillance. This study investigates the molecular characteristics of the HA and NA genes of 18 HPAI H5N1 viruses isolated from 2008 to early 2010 from clinical outbreaks in the north ($n = 4$) and south of Vietnam ($n = 9$) as well as from two flocks with no clinical signs ($n = 5$).

Our findings support the observation that two HA clades (2.3.4 and 1) previously reported in the north (clade 2.3.4) and south (clade 1) are the predominant viral clades in Vietnam. Furthermore, this study indicates that the motif of multiple basic amino acids at the HA cleavage site is maintained in viruses characterised not only in diseased birds, but also in apparently healthy birds, particularly in field running and muscovy ducks. This suggests that a virus with high pathogenic potential for poultry could be maintained in ducks. This finding provides evidence for the need to further study the pathogenesis of current H5N1 viruses in different species. Additionally, the evidence of amino acid substitution or insertion at the pathogenic site of the HA genes of the two H5N1 viruses circulating in Vietnam may indicate antigenic drift. The co-evolution of segments within HA and NA genomes provides evidence of reassortment between different sublineages within Vietnam H5N1 isolates. This reassortment suggests a high level of genetic compatibility between viruses with diverse parental genotypes. Continued monitoring of viral change is important, particularly if mass vaccination programs are continued to be used as a control measure for HPAI H5N1 in Vietnam.

5.1 Introduction

Outbreaks caused by the highly pathogenic avian influenza (HPAI) H5N1 virus were first reported in southern China in 1996 and 1997 (Claas et al., 1998; Subbarao et al., 1998). The virus became widespread and endemic in poultry in south-east Asian countries throughout 2003 and 2004 and then spread to Europe and Africa in 2005. The disease

has caused outbreaks in birds in over 60 countries (Alexander, 2007a; OIE, 2009). Consequently, millions of chickens and ducks have been culled in an effort to stop disease spread (Knobler et al., 2005). More alarmingly, H5N1 viruses have been sporadically transmitted to and have caused severe disease in humans. By 31 August 2010, there had been 505 human cases of H5N1 infection in 15 countries, 300 (59%) of which were fatal (WHO, 2010).

HPAI H5N1 viruses are pleomorphic, enveloped RNA viruses belonging to the family of Orthomyxoviridae. The genome of the virus consists of eight unique segments (Swayne, 2008). Antigenic diversity of HPAI H5N1 viruses occurs primarily at two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). The surface structural protein HA is the main determinant factor of avian influenza virus (AIV) pathogenicity. The cleavage of HA into HA1 and HA2 is a prerequisite for generalised infections and the amino acid sequence in this area is the main determinant of AIV virulence (Bosch et al., 1979; Senne et al., 1996). Deletion of 20 amino acid sequence in the stalk region of the NA has been proposed as an important factor in increased virulence and pathogenicity (Zhou et al., 2009). Whilst HPAI viruses are characterised by the presence of multiple alkaline amino acids at the cleavage site of HA gene, LPAI isolates contain no, or only one alkaline amino acid (Swayne, 2008). HA virus surface glycoprotein, as a target for neutralising antibodies, is a classic example of an antigenically drifting protein (Webster et al., 1982). The HA gene accumulates an exceptional number of point mutations in the epitope or antibody combining regions of the protein (Wilson and Cox, 1990). The NA is the second major antigenic determinant for neutralising antibodies. By catalysing the cleavage of glycosidic linkages to sialic acid on host cell and virion surfaces, this glycoprotein prevents aggregation of virions, facilitating the release of progeny virus from infected cells (De Jong et al., 2006). Hence, understanding the evolution of HA and NA genes is important for surveillance and vaccine strain selection.

Although HPAI H5N1 virus were isolated from live bird markets in Vietnam as early as 2001 (Nguyen et al., 2005), the first poultry epidemic occurred in late 2003 (NIHE, 2005), resulting in the death of 45 million poultry from either infection or culling. The next epidemic wave occurred in 2004-2005 with the loss of over two million poultry (Sims and Dung, 2009). Thereafter, the incidence of outbreaks has decreased considerably following the implementation of multiple control strategies including biannual mass vaccination

campaigns. Despite the reduced incidence of outbreaks, the virus has become endemic in Vietnam and continues to cause small epidemics with intermittent sporadic outbreaks. As of 1 October 2010, a total of 119 human cases of H5N1 influenza (the second highest number after Indonesia, $n = 165$) have been identified in Vietnam since the start of the epidemic (WHO, 2010). Similar to the reduced disease incidence in poultry, the annual number of human cases has decreased from 29 and 65 in 2004 and 2005, respectively, to zero to eight cases per year since 2006.

Multiple virus introductions have occurred in Vietnam between 2001 and 2007 (Wan et al., 2008). The first HPAI H5N1 outbreaks in Vietnam in 2003 and 2004 were caused by clade 1 genotype Z viruses (Province, 2010). These viruses have the same genetic origin as the H5N1 viruses first detected in southern China in 2002 which suggests that they originated in this region (Smith, Fan, Wang, Li, Qin, Zhang, Vijaykrishna, Cheung, Huang, Rayner et al., 2006). In 2005 clade 2.3.2 (genotype G) viruses were first identified and in 2007 clade 2.3.4 (Fujian-like) viruses were first detected in Vietnam (Nguyen et al., 2008). Gene segments of both these viruses were closely related to viruses isolated in Guangxi province in China (Chen et al., 2006), suggestive of further virus introductions from China. Furthermore, the study by Tung (2008) first detected clade 7 viruses in the north of Vietnam in 2007, which were most closely related to viruses detected in Shanxi, China.

Reassortment events of viruses within Vietnam or between Vietnam and China have further contributed to the presence of mixed genotypes. The most notable reassortment event occurred between clade 2.3.4 and clade 1 viruses, leading to one of the four genotypes in Vietnam that have not been reported elsewhere (Wan et al., 2008). Clade 2.3.4 viruses replaced the previously circulating clade 1 and clade 2.3.2 viruses in the north in 2007 (Nguyen et al., 2008). In contrast, clade 1 viruses, closely related to viruses found in Cambodia, continued to circulate in the south of Vietnam, leading to a geographical distinction between isolates since 2007 (Nguyen et al., 2008). In conclusion, the presence of mixed genotypes complicates the epidemiological situation in Vietnam as it is associated with an increased risk of reassortment events and potentially gives rise to differences in epidemiological features of disease such as clinical expression and immune responses to vaccine.

Vaccination of poultry in high risk areas since 2005 is thought to be one of the key reasons

behind the reduced incidence of disease that has been observed in poultry and humans. However, continuing mass vaccinations may produce faster antigenic drift of influenza viruses due to ongoing selection pressure (Lee et al., 2004; Suarez et al., 2006; Escorcia et al., 2008). Experimental studies conducted in Vietnam indicate that the GS/GD/96-based vaccine in use has good efficacy against all currently circulating clades (Thanh, 2007; Tung, 2008; NCVD, 2009). This is supported by results from Tian et al. (2010), which showed that the vaccine used in Vietnam induced complete protection of chickens against clades 1, 2.2 and 2.3.4 with no virus shedding or clinical disease. However, levels of HI titres to these viruses (HI titres: 3.9 to 6.0.) were 8- to 32-fold lower compared to titres to the homologous GS/GD/1/96 virus (HI titres: 8.7 to 9.3). Furthermore, protection of vaccinated chickens against clade 7 viruses was incomplete with virus shedding and mortality being observed in 25% of study birds. Another risk associated with vaccination is insufficient flock protection, which may lead to subclinical disease combined with virus shedding and thus increased likelihood of antigenic drift (Lee et al., 2004). Previous analyses have identified risk factors influencing vaccination failure in poultry based on post-vaccination surveillance data (Chapter 4). Given the circulation of mixed genotypes and the difficulties in achieving optimum vaccination coverage under field conditions, there is a need to continuously monitor molecular changes to ensure the early detection of changes in virus characteristics.

The aim of this study was to investigate molecular characteristics of recent AIV isolates from clinically diseased and apparently healthy birds, the results of which can be used to inform future surveillance and control strategies for HPAI H5N1 in Vietnam.

5.2 Materials and methods

Eighteen H5N1 viruses, isolated between February 2008 and January 2010 were used for this study (Table 5.1). These samples originated from two sources. Five surveillance samples were selected from a longitudinal study in the Mekong River Delta (NZAID, 2009). The remaining samples were collected from outbreak investigations (four samples from the north and nine samples from the south). Outbreak samples from the south were selected to represent a mixture of provinces and years, representing 25% of the total number of outbreaks from February 2008 to January 2010. The four outbreak samples

from the north represented 17% of the total number of outbreaks that occurred between January and June 2009.

Diagnostic procedures differed according to the source of the virus. Samples from the longitudinal study were firstly screened by RRT-PCR to detect matrix gene of influenza viruses. Individual samples from all M genes positive and 10% of M gene negative pools were subjected for virus isolation. In contrast, outbreak samples were directly tested for H5 and N1 by RRT-PCR virus according to the routine diagnostic procedure for outbreak confirmation. Selected H5N1 positive outbreak samples were subsequently subjected to virus isolation.

Mixed cloacal and faecal specimens were collected under the longitudinal study and during outbreak investigations in northern provinces. These swabs were placed in transport medium, consisting of phosphate-buffered saline (PBS) containing 50% glycerol, penicillin (2,000 IU/mL), gentamicin (250 µg/mL), polymixin B (2,000 IU/mL), nystatin (500 IU/mL), ofloxacin HCl (60 µg/mL) and sulfamethoxazole (200 µg/mL). Tissue samples were collected from outbreak investigations in the south. Specimens were chilled until arrival at the laboratory, where they were stored at -80 °C for long term storage.

Isolation of influenza virus was performed in embryonated chicken eggs sourced from breeding farms vaccinated for HPAI H5N1. Two 9- to 11-day old chicken embryos were inoculated via the allantoic cavity with 200 µL of sample with antimicrobials. The eggs were incubated at 37 °C for three (outbreak samples) or four days (longitudinal study samples) and candled daily for viability. Embryos that died within 24 hours of inoculation were discarded as nonspecific. Allantoic fluids were harvested from eggs and screened for the presence of virus by conventional hemagglutination assay (HA) using chicken red blood cells (OIE, 2009). All negative samples were passaged a second time and negative duck samples a third time (longitudinal study samples only).

Virus isolation was conducted with the conventional procedures under biosafety level 3 containment at the National Centre for Veterinary Diagnostic (NCVD) in Hanoi and at the Regional Animal Health Office No. 6 in Ho Chi Minh City, Vietnam. Isolates were then sent to Australian Animal Health Laboratory (AAHL), Geelong, Australia for sequencing. Viral RNA was extracted from virus-infected allantoic fluids using the MagMax Viral Isolation kit (Ambion) following manufacturer's instructions. Full length sequencing of the

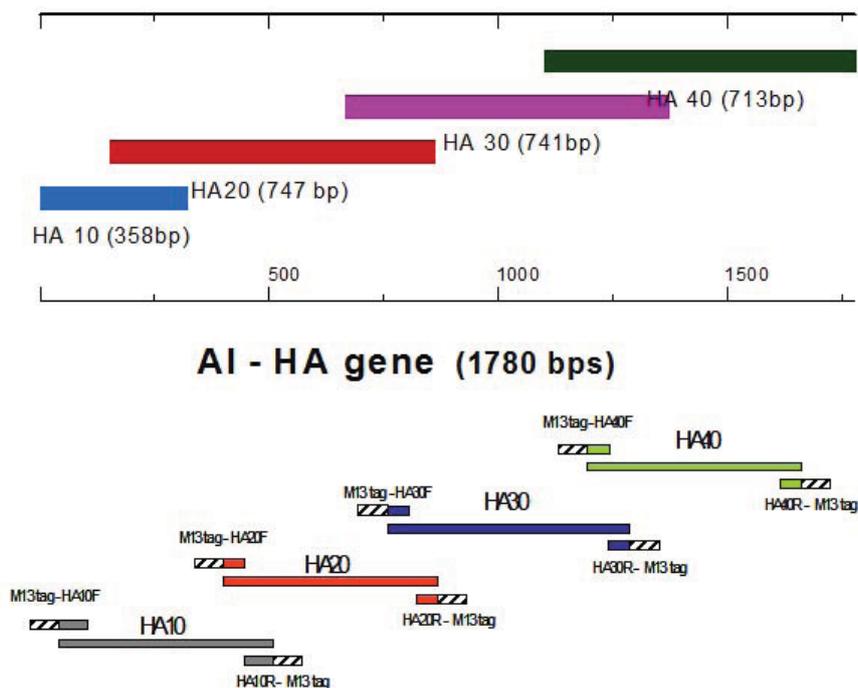


Figure 5.1: Amplification strategy used for determination of the full length coding sequence of the H5N1 HA gene (segment 4). Source: Sequencing Laboratory, Diagnostic Surveillance and Response Group at the AAHL, Geelong Australia.

H5N1 HA and NA genes followed the protocols of AAHL with minor modifications. RT-PCR was performed to amplify overlapping gene segments as used for post-sequencing assembly into the full length sequence, using Superscript III with Platinum Taq One-Step RT-PCR kit (Invitrogen). The combination of M13-tagged primers were designed to span the entire length of each gene with overlapping fragments (Figure 5.1). Cycling conditions included reverse transcription (48 °C for 30 minutes) followed by inactivation at 94 °C for 2 minutes, 40 cycles of denaturation at 94 °C for 30 seconds, annealing 50 °C for 40 seconds, extension at 68 °C for 40 seconds with final elongation for 5 minutes at 68 °C.

RT-PCR products were recovered using agarose gel electrophoresis. The sizes of each amplification product were as follows: HA 10 = 358 bp, HA 20 = 747 bp, HA 30 = 741 bp, and HA 40 = 713 bp (Figure 5.2). For the NA gene the amplification product sizes were: NA 10 = 607 bp, NA 20 = 752 bp, NA 30 = 741 bp, and NA 40 = 367 bp.

PCR products were then purified using the QIAquick Gel Extraction Kit (Qiagen). Direct PCR product sequencing was carried out using Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction (ABI, FosterCity, CA) and the ABI-Prism 310 Genetic Analyzer

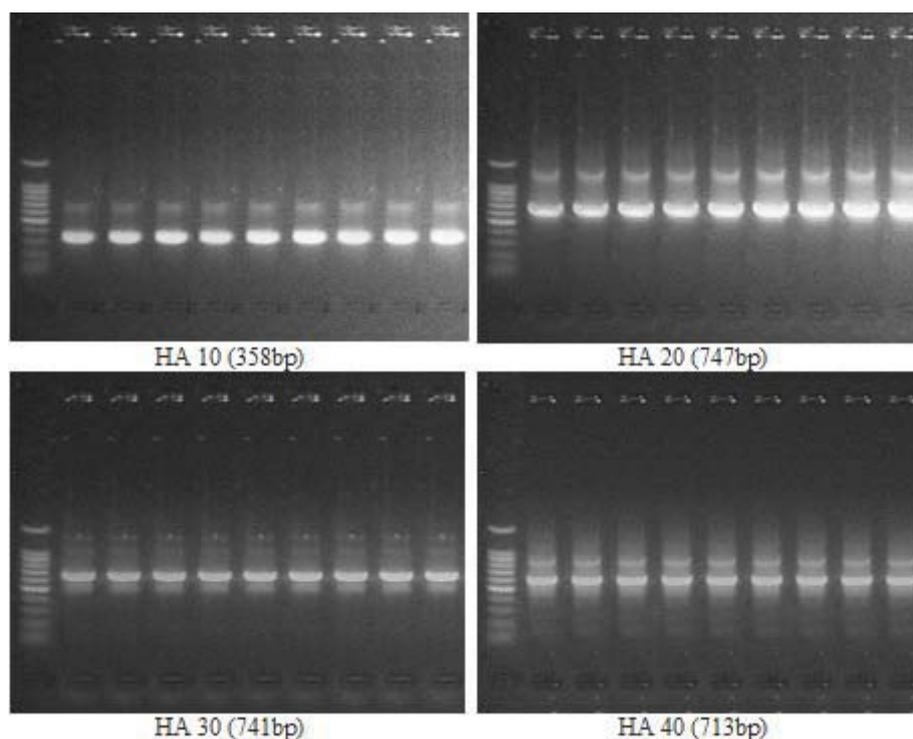


Figure 5.2: Analysis of PCR products generated by RT-PCR for the HA gene to amplify full-lengths of the HA gene in four separate RT-PCR reactions visualised on agarose gel electrophoresis. Lane 1: DNA ladder; Lanes 2-8: avian influenza H5N1 isolates; Lane 9: avian influenza H5N1 positive control. The expected size of the RT-PCR products are stated under each gel.

(Perkin-Elmer, Norwalk, CT).

Raw sequence outputs (sequence chromatograms) were analysed with the SeqMan module of the Lasergene software (DNASTar Inc., Madison, WI). Consensus nucleotide contiguous sequences were aligned by the Clustal W algorithm using the MegAlign 5.07 module of the Lasergene molecular biology software. Phylogenetic analyses were performed using the MegAlign software package (DNASTAR, Madison, WI).

Phylogenetic trees generated for this study were based on near full length HA gene (the length in nucleotides) and NA (the length in nucleotides) coding sequences. Phylogenetic inferences relied on neighbour-joining (NJ) methods with Kimura 2-distance parameters using MEGA 4.¹ Support for the consensus tree topology was evaluated by performing 1,000 replicates. Trees were rooted at A/goose/Guangdong/1/1996 for both HA and NA genes (Wallace et al., 2007).

For the HA gene, nucleotide and amino acid sequence relatedness among these and other H5N1 clade viruses, as well as several H5N1 prototype strains representing the major

¹URL: <http://www.megasoftware.net/>

Table 5.1: Details of the avian influenza H5N1 virus isolates from the north and south of Vietnam between 2008 and early 2010, and used for the molecular analyses described in this paper.

No.	Strain (HPAI H5N1)	Sample date	Species	Province	District	Signs
1	A/Chicken/Vietnam/VP_NCVD_279/09 ^a	28/01/2009	Chicken	Vinh Phuc	Tam Duong	Yes
2	A/Chicken/Vietnam/VP_NCVD_281/09 ^a	09/02/2009	Chicken	Vinh Phuc	Tam Duong	Yes
3	A/Chicken/Vietnam/TB_NCVD_287/09 ^a	09/06/2009	Chicken	Thai Binh	Quynh Phu	Yes
4	A/Chicken/Vietnam/DB_NCVD_292/09 ^a	23/02/2009	Chicken	Dien Bien	Dien Bien City	Yes
5	A/Duck/Vietnam/LA_1047/08 ^b	13/02/2008	Duck	Long An	Ben Luc	Yes
6	A/Duck/Vietnam/TG_2289/08 ^b	05/04/2008	Duck	Tien Giang	Chau Thanh	Yes
7	A/Duck/Vietnam/CM_T0907/09 ^b	03/02/2009	Duck	Ca Mau	Tran Van Thoi	Yes
8	A/Ck/Vietnam/CM_T0912/09 ^b	05/02/2009	Chicken	Ca Mau	Thoi Binh	Yes
9	A/Duck/Vietnam/HG_T0916/09 ^b	07/02/2009	Duck	Hau Giang	Vi Thuy	Yes
10	A/Muscovy duck/Vietnam/CM_T0928/09 ^b	12/02/2009	Muscovy	Ca Mau	Nam Can	Yes
11	A/Duck/Vietnam /ST_T0931/09 ^b	13/02/2009	Duck	Soc Trang	Nga Nam	Yes
12	A/Muscovy duck/Vietnam/CM_T0934/09 ^b	13/02/2009	Muscovy	Ca Mau	Phu Tan	Yes
13	A/Duck/Vietnam/BD_914/09 ^b	17/02/2009	Duck	Binh Duong	Thu Dau Mot	Yes
14	A/Duck/Vietnam/CT_S2_195/09 ^c	14/01/2009	Duck	Can Tho	Co Do	No
15	A/Muscovy duck/Vietnam/BL_S11_1026/10 ^{c,d}	11/01/2010	Muscovy	Bac Lieu	Vinh Loi	No
16	A/Muscovy duck/Vietnam/BL_S11_1027/10 ^{c,d}	11/01/2010	Muscovy	Bac Lieu	Vinh Loi	No
17	A/Muscovy duck/Vietnam/BL_S11_1029/10 ^{c,d}	11/01/2010	Muscovy	Bac Lieu	Vinh Loi	No
18	A/Muscovy duck/Vietnam/BL_S11_1030/10 ^{c,d}	11/01/2010	Muscovy	Bac Lieu	Vinh Loi	No

^a Outbreak samples collected in the north of Vietnam.

^b Outbreak samples collected in the south of Vietnam.

^c Samples from the longitudinal study in the south of Vietnam.

^d Isolates from one flock.

circulating H5N1 clades, were calculated by pair wise comparisons of the near full length (the length in nucleotides) HA coding sequence.

5.3 Results

Table 5.1 contains information related to origin, time, host species and clinical signs associated with the 18 H5N1 isolates, derived from 13 outbreak samples (north: numbers 1 to 4; south: numbers 5 to 13) and five longitudinal study samples (numbers 14 to 18). The latter five samples originated from two flocks with no obvious clinical signs of disease.

To determine the phylogenetic relationships of the H5N1 viruses included in this study, we constructed a phylogenetic tree of the H5N1 HA gene with the use of reference viruses representing four relevant H5-HA clades (Table 5.2). These HA reference clades were identified and designated according to the recently described nomenclature system for

the HPAI H5N1 viruses as well as potential precursors for viruses currently circulating in Viet Nam (Wan et al., 2008). The phylogenetic tree of the HA gene (Figure 5.3) showed that all 18 Vietnamese isolates were derived from the Gs/GD-like lineage. However, two distinct H5N1 clade lineages were evident, clade 2.3.4 and clade 1, which showed geographical separation (Figure 5.4). Four H5N1 viruses isolated from outbreaks in the north of Vietnam in 2009 (A/Chicken/Vietnam /VP_NCVD_279/09; A/Chicken /Vietnam /VP_NCVD_281/09; A/Chicken /Vietnam /TB_NCVD_287/09 and A/Chicken /Vietnam /DB_NCVD_292/09) were identified as clade 2.3.4 virus, whereas all of the 14 remaining H5N1 viruses collected from the south of the country were identified as clade 1. In addition, four of the southern isolates collected from the same flock of Muscovy ducks (A/Muscovy duck/Vietnam /BL_S11_1026/10; A/Muscovy duck/Vietnam /BL_S11_1027/10; A/Muscovy duck /Vietnam /BL_S11_1029/10; and A/Muscovy duck /Vietnam /BL_S11_1030/10) were identical.

Analyses indicated that these Vietnamese HA sequences shared 93% to 98% nucleotide sequence identity with A/Goose/Guangdong/1/1996 virus, while percentage amino acid sequence similarities to A/Goose/Guangdong/1/1996 virus ranged from 93% to 95% (Table 5.3). The five H5N1 isolates collected from two flocks of the longitudinal study were 98% to 99% similar to other isolates from the same Mekong region, although these flocks did not exhibit obvious clinical signs of disease.

Analysis of H5-HA0 sequences showed that all of the isolates possessed HA cleavage site motifs characteristic of HPAI virus (Figure 5.5). The HA cleavage site of A/Duck/Vietnam/HG_T0916/2009 H5N1 virus isolated in the south (Hau Giang province) contained two amino acids 'PQREERRRKKRGLF', which differed from other southern H5N1 viruses, with 'PQRE-GRRKKRGLF' (arginine (R) to glycine (G) and a single amino acid insertion of E). Furthermore, the HA cleavage site of A/Chicken/Vietnam/VP_NCVD281/09 virus isolated in the north (Vinh Phuc province) contained one amino acid 'PLRE-KRR-KRGLF' differing from other northern H5N1 viruses 'PLRE-RRR-KRGLF' (arginine (R) to lysine (K)). The HA cleavage site of the remaining H5N1 viruses was unchanged compared to sequencing data of previous H5N1 viruses in the same areas (Figure 5.5). Notably, the HA cleavage site 'SPQRE-GRRKKRGLF' was also identified in the five H5N1 viruses which were isolated from apparently healthy ducks and muscovy ducks in the Mekong River Delta.

Table 5.2: Putative precursor viruses of HPAI H5N1 from Vietnam (Wan et al., 2008).

Clade	Precursor virus	Abbreviation
0	A/Goose/Guangdong/1/96	GD96-like
1	A/Duck/Hongkong/821/02	HK821-like
2.3.2	A/Duck/China/E319-2/03	E319-like
	A/goose/Guangxi/3316/05	GX3316-like
2.3.4	A/Japanese white-eye/Hongkong/1038/06	HK1038-like
	A/Chicken/Fujian/584/06	FJ584-like

Table 5.3: Sequence similarities between A/Goose/Guangdong/1/1996 and the 18 Vietnamese sequences described in this paper.

Sequences	HA gene		NA gene	
	Nucleotide (bps %)	Amino acid (aas %)	Nucleotide (bps %)	Amino acid (aas %)
A/Chicken/Vietnam /VP_NCVD_279/09	1654 (97)	538 (95)	1273 (90)	426 (90)
A/Chicken /Vietnam /VP_NCVD_281/09	1660 (98)	538 (95)	1273 (90)	429 (91)
A/Chicken /Vietnam /TB_NCVD_287/09	1651 (97)	538 (95)	1280 (91)	434 (92)
A/Chicken /Vietnam /DB_NCVD_292/09	1655 (97)	538 (95)	1280 (91)	434 (92)
A/Duck/Vietnam/LA_1047/08	1603 (94)	534 (94)	1274 (90)	427 (91)
A/Duck/ Vietnam /TG_2289/08	1599 (94)	537 (94)	1275 (90)	427 (91)
A/Duck/Vietnam /CM_T0907/09	1600 (94)	536 (94)	1303 (92)	435 (92)
A/Ck/Vietnam /CM_T0912/09	1590 (93)	536 (94)	1270 (90)	427 (91)
A/Duck/Vietnam /HG_T0916/09	1599 (94)	535 (94)	1273 (90)	426 (90)
A/Mus. Duck/Vietnam /CM_T0928/09	1599 (94)	536 (94)	1273 (90)	426 (90)
A/Duck/Vietnam /ST_T0931/09	1598 (94)	538 (94)	1272 (90)	422 (89)
A/Mus. Duck/Vietnam /CM_T0934/09	1591 (93)	535 (94)	1269 (90)	424 (90)
A/Duck/Vietnam /BD_914/09	1597 (94)	534 (94)	1270 (90)	421 (89)
A/Duck/Vietnam /CT_S2_195/09	1594 (94)	534 (94)	1268 (90)	423 (90)
A/Mus. Duck/Vietnam /BL_S11_1026/10	1590 (93)	533 (93)	1264 (89)	420 (89)
A/Mus. Duck /Vietnam /BL_S11_1027/10	1590 (93)	533 (93)	1264 (89)	420 (89)
A/Mus. Duck /Vietnam /BL_S11_1029/10	1590 (93)	533 (93)	1264 (89)	420 (89)
A/Mus. Duck /Vietnam /BL_S11_1030/10	1590 (93)	533 (93)	1264 (89)	420 (89)

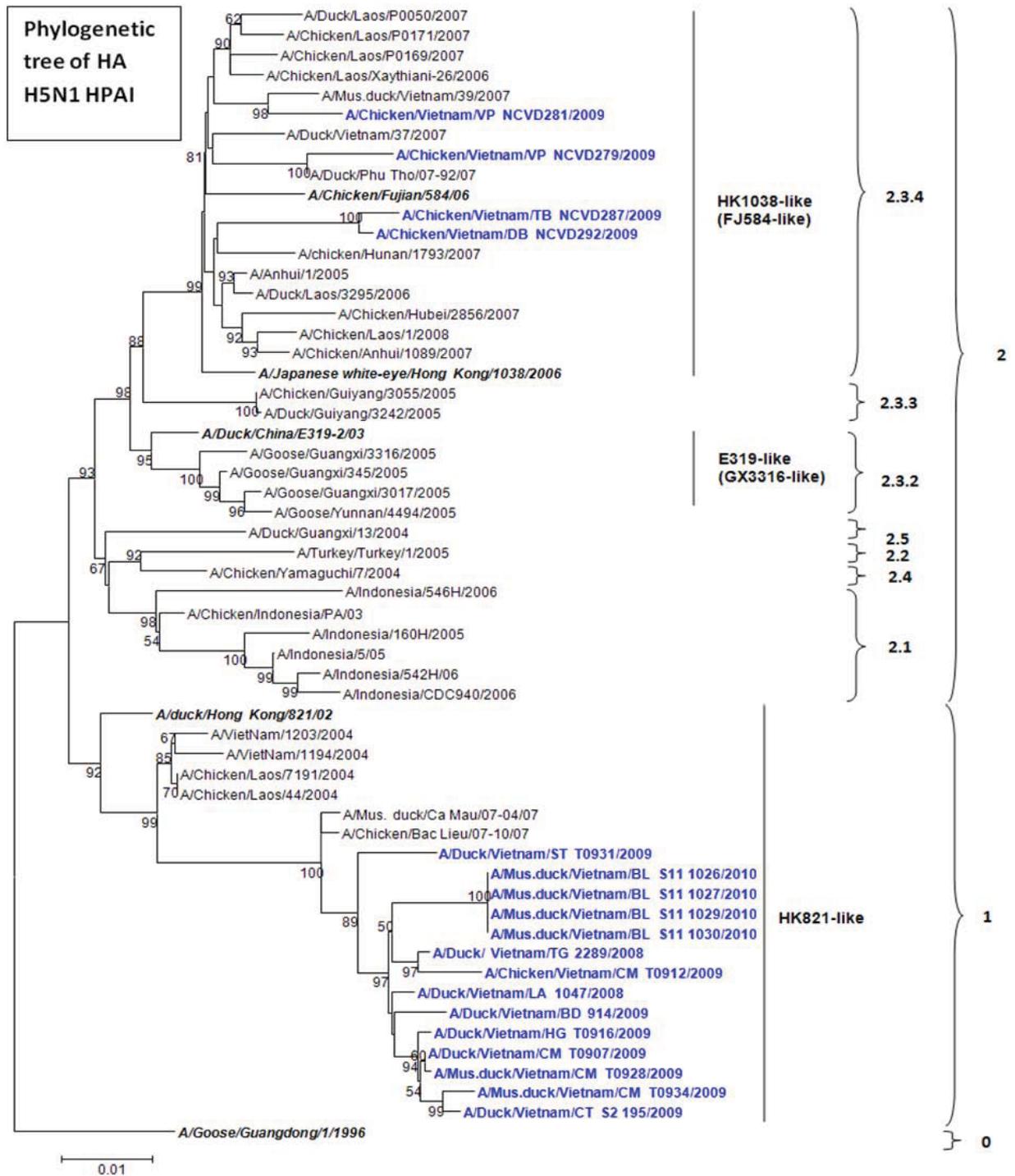


Figure 5.3: Phylogenetic tree of viral HA sequences generated by neighbour-joining analysis. Bootstrap values at each node represent 1000 replicates. Values less than 50% are not shown. Scale bar represents 10 nucleotide substitutions. The 18 Vietnamese HPAI H5N1 isolates are shown in blue text.

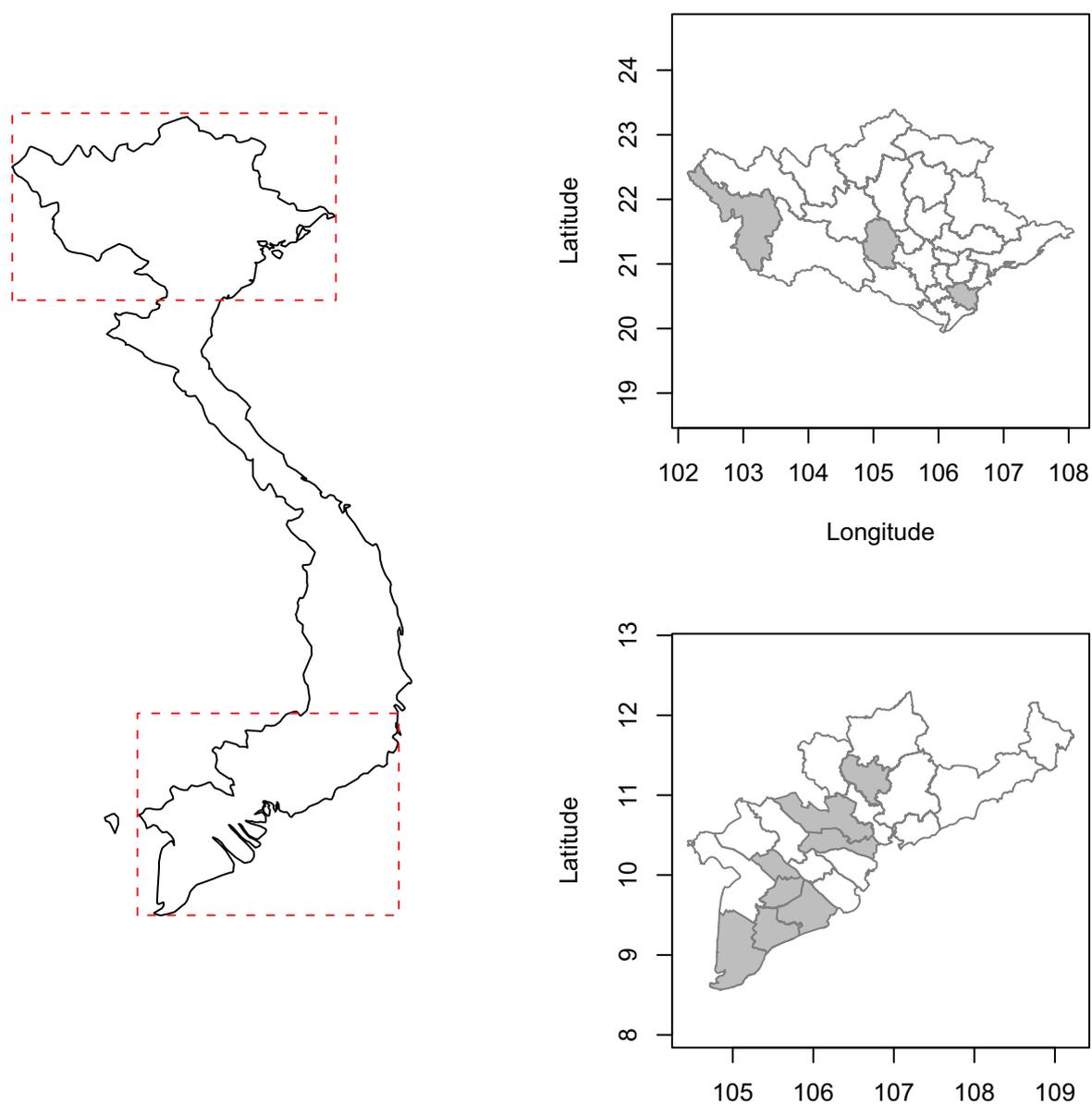


Figure 5.4: Map of Vietnam showing the province of origin of the 18 H5N1 isolates collected between 2008 and 2010.



Figure 5.5: Amino acid residue analysis of the H5-HA cleavage site region (highlighted in yellow) of the viruses described in this study.

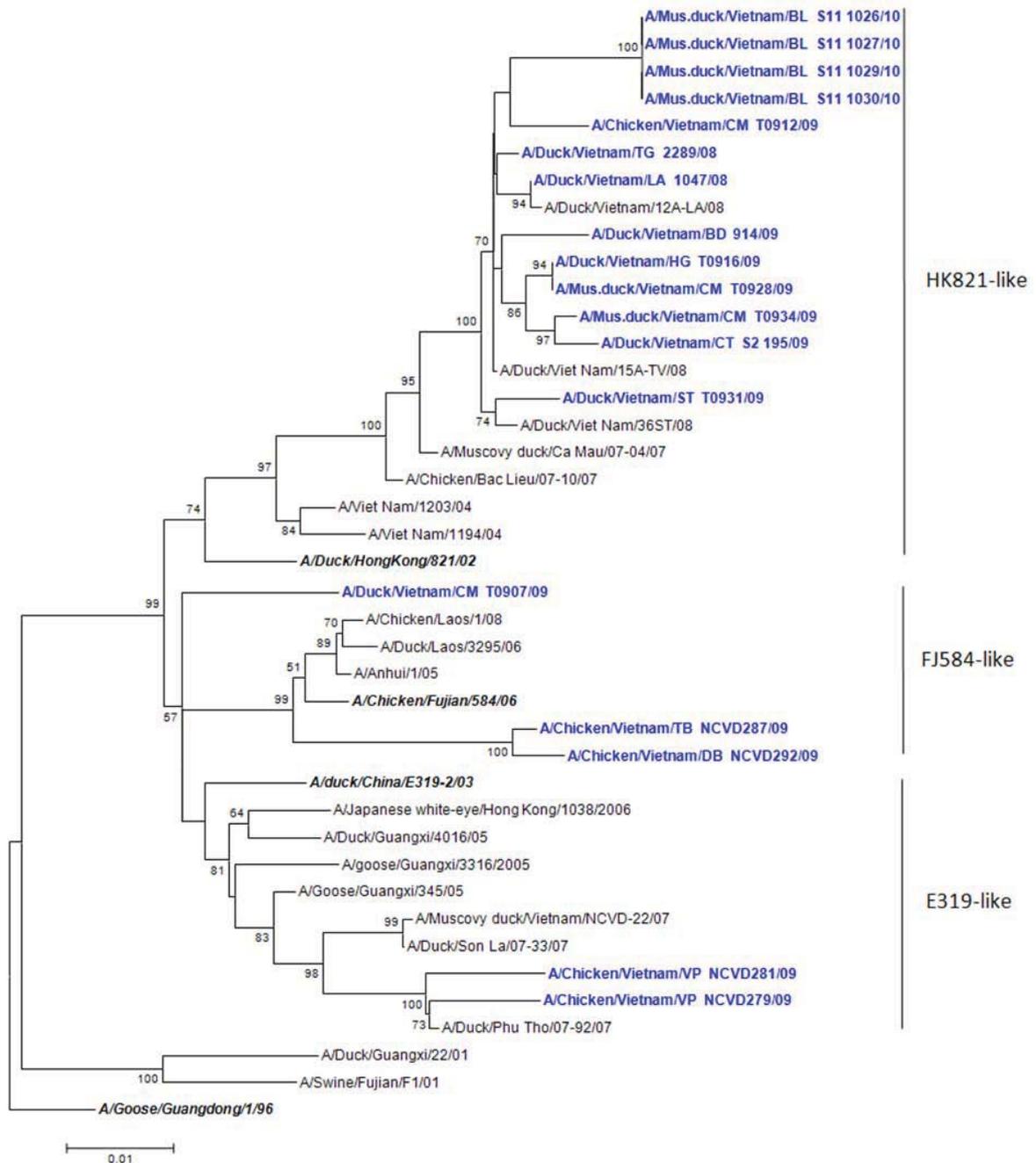


Figure 5.6: Phylogenetic tree of viral NA sequences generated by neighbour-joining analysis. Bootstrap values at each node represent 1000 replicates. Values less than 50% are not shown. Scale bar represents 10 nucleotide substitutions. The 18 Vietnamese HPAI H5N1 isolates are shown in blue text.

Figure 5.6 shows that the neuraminidase gene of the H5N1 viruses isolated in the south of Vietnam clustered with N1 NA genes of HK821-like viruses with the exception of A/Duck/Vietnam/CM_T0907/09 virus. In contrast, the NA gene of the H5N1 viruses isolated in the north was related to avian N1 NA genes of E319-like and FJ584-like viruses. Similar to the HA gene, the NA gene of the four H5N1 viruses collected from the same flock (A/Muscovy duck/Vietnam/BL_S11_1026/10; A/Muscovy duck/Vietnam/BL_S11_1027/10; A/Muscovy duck/Vietnam/BL_S11_1029/10; A/Muscovy duck/Vietnam/BL_S11_1030/10) were identical to each other. In addition, two other H5N1 isolates from two different southern provinces (A/Duck/Vietnam/HG_T0916/09 and A/Muscovy duck/Vietnam/CM_T0928/09) were closely related to each other.

Analyses indicated that the NA sequences of the viruses in this study were derived from A/Goose/Guangdong/1/1996 virus, with nucleotide sequence identities ranging from 89% to 92% (Table 5.3). Similarities of amino acid sequence to A/Goose/Guangdong/1/1996 virus also ranged from 89% to 92%.

Phylogenetic analyses also indicated that all NA sequences in this study were most closely related to A/Goose/Guangdong/1/1996 virus, with nucleotide sequence identity ranging from 89% - 92% (Table 5.3). Similarities of amino acid sequence to A/Goose/Guangdong/1/1996 virus also ranged from 89% to 92%.

In this study, the 20-amino acid deletion at the NA stalk region (positions 49 to 68) was present in all the 2008-2010 H5N1 isolates. Known mutations associated with oseltamivir resistance were observed among the conserved residues (E119V, H274Y, R292K and N294S) at the NA active site of all H5N1 isolates from this study (data not presented).

The co-evolution of segments within the HA and NA gene segments was analysed by comparing the relative position of each isolate in the respective phylogenetic trees (tree topology). The individual gene segment trees revealed that gene segments of Vietnam isolates from a given clade did not always co-evolve with other members of that clade. For instance, A/Chicken/Vietnam/VP_NCVD_279/09 and A/Chicken/Vietnam/VP_NCVD_281/09 had an HA gene derived from FJ584-like or clade 2.3.4 viruses but an NA gene from E319-like viruses, whose HAs belonged to clade 2.3.2 viruses (Figures 5.3 and 5.6).

5.4 Discussion

The first H5N1 viruses that were isolated from live bird markets in Vietnam in 2001 did not cause clinical disease either in field or experimentally infected birds (Nguyen et al., 2005). However, other genetically distinct H5N1 viruses resulted in a series of epidemics characterised by high levels of mortality since late 2003 (Nguyen et al., 2008; Wan et al., 2008). This shows that both low and highly virulent avian influenza H5N1 viruses can be maintained in poultry populations in Vietnam. Since the start of mass vaccination, the incidence of poultry outbreaks and human cases has reduced considerably, but the disease appears to be endemic with ongoing small-scale poultry outbreaks and sporadic cases of disease in humans. An important question that needs to be answered is whether or not subclinical infection contributes to the maintenance of HPAI viruses within poultry populations between epidemics. This study focused on identifying molecular changes of HPAI H5N1 viruses using viruses isolated from outbreak samples, i.e. birds showing clinical signs, and samples collected from apparently healthy birds from a longitudinal study.

5.4.1 Molecular analyses of H5N1 HA genes

Phylogenetic analyses of HA genes showed a geographic distinction among the isolates characterised in this study (Figure 5.3). The four northern isolates belonged to clade 2.3.4, while all southern isolates belonged to clade 1 (Figure 5.3). This geographical distinction has been reported in Vietnam since 2007 (Nguyen et al., 2008; Wan et al., 2008; Inui, 2009). Results by Wan et al. (2008) suggest that the majority of viruses containing new genes were first detected in the north of Vietnam and then spread to the south, probably as a result of long distance movement of poultry. Despite the relatively small sample size of this study, a persistent ongoing geographical distinction of clades would be somewhat surprising.

The apparent persistence of this geographic distinction of two major clades causing outbreaks in Vietnam might be explained by factors restricting the co-circulation of these virus clades such as separate poultry trading routes or geographical boundaries. In 2006, outbreaks re-emerged mainly in backyard poultry populations, where virus spread is likely

to be more localised. This hypothesis is supported by the results of Minh, Stevenson, Morris and Schauer (2010) who showed that in the 2009 epidemic in the Mekong River Delta, local spread of virus was more predominant than long-distance spread, with household-to-household infection rate within communes being in the order of 50 times greater than the household-to-household infection rate between communes. This may indicate that over recent years long-distance spread has been curbed due to the control of disease in commercial flocks and measures to control long distance spread such as movement restrictions of live birds, closure of live bird markets in large cities, and a two-year ban on waterfowl hatching (MARD, 2007).

Sequence similarities between genes of each of the Vietnam isolates and putative precursor viral genes showed a high degree of similarity, which suggests that the isolates studied here are likely to be descendents from the same putative precursor virus or a closely related virus already present in Vietnam. This finding has previously been reported in Vietnam (Wan et al., 2008).

The motif of multiple basic amino acids at the HA cleavage site, which is characteristic for HPAI viruses, was maintained in all isolates. Similar to the phylogenetic tree analysis, three out of four isolates from northern Vietnam had the same basis cleavage site 'PLRE-RRR-KRGLF' as that of viruses found in Hong Kong and Anhui, China (Figure 5.5), which are all related to clade 2.3.4. In contrast, the basis cleavage site of southern isolates were genetically most identical to the H5N1 viruses found in Hong Kong (one amino acid difference R to G, 'PQREGRRKKRGLF'), which is closely related to clade 1. This amino acid replacement from R to G has been observed in Vietnam isolates since 2006 (Anonymous, 2011).

One isolate (A/Chicken/Vietnam/VP_NCVD_281/09) obtained from the north contained a single amino acid that differed from the other northern isolates ('PLRE-RRR-KRGLF', changed from R to K). This finding provides evidence of an amino acid substitution at the pathogenic site of the HA gene of H5N1 viruses circulating in Vietnam which was previously confirmed in Thailand (Amonsin et al., 2006). Similarly, one isolate (A/Duck/Vietnam/HG_T0916/2009) obtained in the south added one amino acid (E), but mutated one amino acid (G to R) compared to the cleavage site of viruses previously detected in the south of Vietnam ('PQREERRRKKRGLF'). This additional basic amino acid has not been observed previously. Such amino acid changes may make H5N1 viruses

more virulent and expand tissue tropism as the cleavage is facilitated by tissue specific proteases, and the extended cleavage site may offer additional specificities (Swayne, 2008). Such genetic mutations are also of concern for public health as they can significantly alter the ability of viral HA proteins to bind to receptors on the surface of host cells (Gambaryan et al., 2006), thus possibly leading to higher ability of the virus to be transmitted amongst humans.

Subclinical infection

Importantly, our phylogenetic analysis indicated that those isolates obtained from the longitudinal study where birds did not show obvious clinical signs of disease were similar to isolates obtained from clinical outbreaks in both ducks and chickens. All these isolates shared the same amino acid sequence at the cleavage site with isolates obtained from clinical outbreaks (GRRRKRG). The presence of multiple basic amino acids at the connecting peptide between HA1 and HA2 is considered to be a characteristic of viruses that are highly pathogenic in chickens (Senne et al., 1996). This suggests that the H5N1 viruses isolated from flocks without obvious clinical signs may be highly virulent to chickens but may not always produce clinical disease in waterfowl such as ducks and muscovy ducks (Nguyen et al., 2005). Although the HA genes of the five isolates of the longitudinal study possessed multiple basic amino acid motifs at the cleavage site, experimental proof of differences in pathogenicity of these viruses between species and age groups would be valuable.

Despite an increased virulence observed in ducks since 2005 in both experimental (Chen et al., 2004; Pantin-Jackwood and Swayner, 2007) and field studies (Takakuwa et al., 2010), the virulence of HPAI viruses in ducks also may depend on other factors such as age (Pantin-Jackwood and Swayner, 2007; Londt et al., 2010) and immune status (Savill et al., 2006). For instance, vaccination and/or previous infection with low pathogenic H5N1 viruses may cause infection and virus shedding, but no clinical disease. Questionnaire details collected from owners of the two longitudinal study flocks showed that birds were one to one and a half months of age and unvaccinated at the time of the outbreak. All birds were sero-negative at the time H5N1 testing was carried out and seroconverted in the following month, indicating that an immune response had occurred following natural infection. Hence, neither age nor pre-existing immunity can explain the lack of clinical

signs in these birds. To further investigate factors contributing to subclinical infection in domestic waterfowl, it is recommended to carry out additional experimental and longitudinal field studies to determine additional risk factors for silent infection. Silent infection is a major constraint to effective passive surveillance, which is currently the predominant routine surveillance system applied in Vietnam.

In a case-control study conducted in the Mekong River Delta in 2009, 10% ($n = 16$) of control flocks randomly selected from the same or neighbouring villages within an outbreak commune within 7 days of a reported outbreak tested H5N1 positive by H5 and N1 gene RRT-PCR (Minh, Stevenson, Schauer, Morris and Quy, 2010). For five of eleven control flocks (45%), for which the presence of clinical signs could be assessed after the positive test result, no clinical signs were detected by the flock owner up to 9 days after sample collection (unpublished data). Similar to the longitudinal study, four of these five flocks were less than two months of age and vaccinated less than one week prior to sample collection, thus making full immune response to vaccination unlikely. Virus isolation was attempted on these samples, but no isolate could be obtained, so that no inferences can be made on whether these isolates were LPAI or HPAI H5N1 viruses. However, results of this molecular study and the fact that these isolates were obtained from outbreak areas suggest that these viruses isolated from flocks with no clinical signs may have been HPAI viruses. This would provide further evidence that subclinical infection with highly pathogenic H5N1 virus may occur under field conditions in Vietnam, both in outbreak and non-outbreak areas. Subclinical infection of poultry may be one of the main drivers for the maintenance of H5N1 viruses in between epidemics.

5.4.2 Molecular analyses of H5N1 NA genes

Reassortment

Analyses of the Vietnamese H5N1 gene segments showed evidence of reassortment compared with H5N1 viruses detected earlier in neighbouring China and Hong Kong. Phylogenetic analyses of the neuraminidase (NA) gene show a similar phylogenetic relationship to the HA tree. However, reassortment of viruses from distinct clades was identified in two northern isolates, A/Chicken/Vietnam/VP_NCVD_279/09 and A/Chicken/Vietnam/VP_NCVD_281/09, which shared the H5 HA gene derived from FJ584-like (clade 2.3.4 viruses) with the N1

NA gene from E319-like viruses, whose HAs belonged to clade 2.3.2 viruses. Previous studies (Nguyen et al., 2008; Wan et al., 2008) also reported evidence of reassortment between different sublineages within Vietnam HPAI H5N1 isolates. This suggests a high level of genetic compatibility between viruses with diverse parental genotypes.

20-amino acid deletion and Oseltamivir resistance

The NA stalk plays a critical role in virulence of H5N1 avian influenza virus. The 20 amino acid deletion from amino acids 49-68 is associated with high virulence, although similar pathogenicity was observed for viruses with different stalk deletions (Zhou et al., 2009). Our analyses show that this stalk deletion was present in all H5N1 isolates. This shortening of the NA stalk region has been previously observed (Zhao et al., 2007) and presumably represents an adaptation of the H5N1 viruses to domestic poultry (Matrosovich et al., 1999). Amino acids conveying oseltamivir resistance were detected among the conserved residues (E119V, H274Y, R292K, and N294S) at the NA active site of all H5N1 isolates from this study. Oseltamivir resistant H5N1 isolates have been reported previously in humans in Vietnam (Le et al., 2005).

5.5 Conclusions

Our findings support the observation that two HA clades (2.3.4 and 1) previously reported in the north (clade 2.3.4) and south (clade 1) are predominant viral clades in Vietnam. Furthermore, this study indicates that the motif of multiple basic amino acids at the HA cleavage site is maintained in viruses characterised not only in diseased birds, but also in apparently healthy birds, particularly in field running and muscovy ducks. This suggests that a virus with high pathogenic potential for poultry could be maintained in ducks. This finding provides evidence for the need to further study the pathogenesis of current H5N1 viruses in different species. Additionally, the evidence of amino acid substitution or insertion at the pathogenic site of the HA genes of the two H5N1 viruses circulating in Vietnam may indicate antigenic drift. The co-evolution of segments within HA and NA genomes provides evidence of reassortment between different sublineages within Vietnam influenza (H5N1) isolates. This reassortment suggests a high level of genetic

compatibility between viruses with diverse parental genotypes. Continued monitoring of viral change is important, especially if mass vaccination programs are continued to be used as a control measure for HPAI H5N1 in Vietnam.

General discussion

Although mass vaccination has been a useful tool for controlling HPAI H5N1 in Vietnam it is a strategy that is both expensive and time consuming to implement effectively. Recognising these issues the overall objective of this thesis has been to evaluate aspects of the Vietnamese mass vaccination program for HPAI H5N1 that has been in place since 2005.

To address these objectives a range of analytical approaches have been used. Chapter 3 used data from the national post-vaccination surveillance program in Vietnam collected between 2007 and 2009. A descriptive analysis of these data was conducted to document spatial, temporal, and individual-level factors influencing immunity in poultry vaccinated for HPAI H5N1. The key findings here were that protection risks varied between regions and provinces, with the southern and central regions of Vietnam having lower protection risks compared with the north. Some provinces had consistently low protection risks over the surveillance period and further investigation is required to determine more precisely the reasons for this.

In Chapter 4, a mixed effects logistic regression model was used to identify factors influencing the probability of vaccination success at the flock level. A specific aim in this chapter, through the inclusion of random effect terms at the province, district, and commune level was to provide greater insight into the relative contribution of these influences on protection risk. This information is important, because it provides decision makers with a better idea of where to target interventions aimed at improving the vaccination program as a whole. The findings in Chapter 4 were that the proportions of variance occurring at the province, district, commune and flock level were 8%, 4%, 3% and 85%, respectively. Individual flock-level effects were the main contributor to variation in protection

risk which means that interventions to improve vaccination efficacy should be focussed at the individual flock (i.e. household) level. Careful review of all vaccination-related activities carried out by provincial authorities would also be a useful strategy. Compared with interventions targeted at the individual flock level, the likelihood of success of interventions applied at the provincial level should have a greater likelihood of success because provincial animal health authorities are centrally managed and the smaller number of individual parties involved would enhance acceptance of suggested changes to facilities and procedures.

Chapter 5 presented details of the molecular characteristics of HPAI H5N1 using 18 isolates from clinical outbreaks in the north and south of Vietnam as well as from flocks with no clinical signs. The findings reported here indicate that two HA clades, previously reported in the north (clade 2.3.4) and south (clade 1), are still present in these two regions. This indicates that subclinical infection with HPAI H5N1 virus may occur under field conditions in both outbreak and non-outbreak areas, particularly in duck flocks. Subclinical infection and under reporting both limit the sensitivity of passive surveillance, which is the predominant means of outbreak detection used in Vietnam. To more effectively control HPAI H5N1 in Vietnam, risk based active surveillance strategies need to be applied together with passive surveillance to enhance detection.

Although this thesis has focused on HPAI H5N1, it should be stressed that the epidemiological principles that underline the analytical approaches are applicable to most, if not all, infectious diseases of livestock. In this respect, the lessons learnt here are likely to assist Vietnamese animal health authorities to implement the necessary systems and infrastructure to allow adequate prompt and efficient investigations of novel and emerging diseases which are likely to emerge in the future. In particular, an approach for analysing post-vaccination surveillance data (Chapter 4) has been developed. These methods could easily be applied to other diseases such as foot-and-mouth disease (FMD), classical swine fever (CSF), and porcine reproductive and respiratory syndrome (PRRS), that are currently controlled in Vietnam by regular mass vaccination programs.

In conclusion, although vaccination has been an important tool to the control of HPAI H5N1 in Vietnam, regular review of vaccination as a method for disease control of disease needs to be undertaken. This will allow current programs to be fine-tuned, with a long term strategy being to reduce vaccination coverage to such a point such that at some stage

in the future it might be phased out entirely. The findings reported here can be seen as a necessary first step in this process.

Bibliography

- Alexander, D. (2000), 'A review of avian influenza in different bird species', *Veterinary Microbiology* **74**, 3–13.
- Alexander, D. (2007a), 'An overview of the epidemiology of avian influenza', *Vaccine* **25**(30), 5637–5644.
- Alexander, D. (2007b), 'Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006', *Avian Diseases* **51**(s1), 161–166.
- Alexander, D. and Brown, I. (2000), 'Recent zoonoses caused by influenza A viruses', *Revue Scientifique et Technique de l'Office International des Epizooties* **19**(1), 197–225.
- Amonsin, A., Chutinimitkul, S., Pariyothorn, N., Songserm, T., Damrongwantanapokin, S., Puranaveja, S., Jam-on, R., Sae-Heng, N., Payungporn, S., Theamboonlers, A. et al. (2006), 'Genetic characterization of influenza A viruses (H5N1) isolated from 3rd wave of Thailand AI outbreaks', *Virus Research* **122**(1-2), 194–199.
- Anonymous (2005), *WHO Manual on Animal Influenza Diagnosis and Surveillance*, World Health Organization, Geneva, Switzerland.
- Anonymous (2011), 'Genbank'. Available as: <http://www.ncbi.nlm.nih.gov/genbank/>. Accessed 1 January 2011.
- Ausvetplan (2007), *Disease Strategy Avian Influenza*, Technical report, Australian Veterinary Emergency Plan, Department of Primary Industries, Australia.
- Barefoot, B., Athearn, K., Sample, C. and Ramsburg, E. (2009), 'Intramuscular immunization with a vesicular stomatitis virus recombinant expressing the influenza hemag-

- glutinin provides post-exposure protection against lethal influenza challenge', *Vaccine* **28**(1), 79–89.
- Bosch, F., Orlich, M., Klenk, H. and Rott, R. (1979), 'The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses', *Virology* **95**(1), 197–207.
- Broor, S. (2005), 'Recent avian influenza outbreaks: A pandemic in the waiting', *Indian Journal of Medical Microbiology* **23**(2), 72–73.
- Butcher, G. and Miles, R. (1994), 'Vaccine Failure in Poultry: Factors to Consider'. Available as: <http://edis.ifas.ufl.edu/pdf/files/VM/VM06200.pdf>. Accessed 20 January 2011.
- Capua, I. and Alexander, D. (2001), *Avian Influenza and Newcastle Disease*, Springer Verlag, Rome, Italy.
- Capua, I. and Alexander, D. (2009), *Avian Influenza and Newcastle Disease: A Field and Laboratory Manual*, Springer Verlag, Rome, Italy.
- Capua, I. and Marangon, S. (2004), 'Vaccination for avian influenza in Asia', *Vaccine* **22**(31-32), 4137–4138.
- Capua, I., Terregino, C., Cattoli, G., Mutinelli, F. and Rodriguez, J. (2003), 'Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza', *Avian Pathology* **32**(1), 47–55.
- Capua, I., Terregino, C., Cattoli, G. and Toffan, A. (2004), 'Increased resistance of vaccinated turkeys to experimental infection with an H7N3 low-pathogenicity avian influenza virus', *Avian Pathology* **33**(2), 158–163.
- CDC (2004), 'The influenza (flu) viruses'. Available as: <http://www.cdc.gov/flu/about/fluviruses.htm>. Accessed 20 January 2011.
- Chen, H., Deng, G., Li, Z., Tian, G., Li, Y., Jiao, P., Zhang, L., Liu, Z., Webster, R. and Yu, K. (2004), 'The evolution of H5N1 influenza viruses in ducks in southern China', *Proceedings of the National Academy of Sciences of the United States of America* **101**(28), 104–152.

- Chen, H., Smith, G., Li, K., Wang, J., Fan, X., Rayner, J., Vijaykrishna, D., Zhang, J., Zhang, L., Guo, C. et al. (2006), 'Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control', *Proceedings of the National Academy of Sciences of the United States of America* **103**(8), 2845–2850.
- Chen, R. and Holmes, E. (2006), 'Avian influenza virus exhibits rapid evolutionary dynamics', *Molecular Biology and Evolution* **23**(12), 2336–2341.
- Choi, Y., Nguyen, T., Ozaki, H., Webby, R., Puthavathana, P., Buranathal, C., Chaisingh, A., Auewarakul, P., Hanh, N., Ma, S. et al. (2005), 'Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004', *Journal of Virology* **79**(16), 10821–10825.
- Claas, E., Osterhaus, A., van Beek, R., De Jong, J., Rimmelzwaan, G., Senne, D., Krauss, S., Shortridge, K. and Webster, R. (1998), 'Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus', *The Lancet* **351**(9101), 472–477.
- DAH (2006), Guidelines for post-vaccination monitoring and surveillance of avian influenza, Technical Report 1181/TY-DT, Department of Animal Health, Hanoi, Vietnam.
- DAH (2007), Guidelines for post-vaccination monitoring and surveillance of avian influenza, Technical Report 623/TY-DT, Department of Animal Health, Hanoi, Vietnam.
- DAH (2008a), Guidelines for post-vaccination monitoring and surveillance of avian influenza, Technical Report 431/TY-DT, Department of Animal Health, Hanoi, Vietnam.
- DAH (2008b), Reevaluation of the Trovac TM AVI H5 (TROVAC-H5) vaccine, Technical report, Department of Animal Health, Hanoi, Vietnam.
- DAH (2009), Annual report for animal diseases and control strategies, Technical report, Department of Animal Health, Hanoi, Vietnam.
- Davison, F., Kaspers, B. and Schat, K. A. (2008), *Avian Immunology*, Elsevier, San Diego, USA.
- De Jong, M., Simmons, C., Thanh, T., Hien, V., Smith, G., Chau, T., Hoang, D., Chau, N., Khanh, T., Dong, V. et al. (2006), 'Fatal outcome of human influenza A (H5N1) is

- associated with high viral load and hypercytokinemia', *Nature Medicine* **12**(10), 1203–1207.
- Desvaux, S., Do, P., Carles, M. and Brioude, A. (2007), Field surveillance model for HPAI in Vietnam in a vaccination context: Methodology and preliminary results, *in* 'Proceedings of the 12th International Conference of the Association of Institutions of Tropical Veterinary Medicine', Centre de Coopération Internationale en Recherche Agronomique pour le Développement, pp. 151–155.
- DLD (2009), Report for the implementation of the regulation on the poultry production and management, Technical Report 92/BNN-TT, Department of Livestock Development, Bangkok, Thailand.
- Duan, L., Bahl, J., Smith, G., Wang, J., Vijaykrishna, D., Zhang, L., Zhang, J., Li, K., Fan, X., Cheung, C. et al. (2008), 'The development and genetic diversity of H5N1 influenza virus in China, 1996-2006', *Virology* **380**(2), 243–254.
- Escorcia, M., Vazquez, L., Mendez, S., Rodriguez-Rojo, A., Lucio, E. and Nava, G. (2008), 'Avian influenza: genetic evolution under vaccination pressure', *Virology Journal* **5**(15). Available as: <http://www.virologyj.com/content/5/1/15>. Accessed 20 January 2011.
- FAO (2004), Recommendations on the Prevention, Control and Eradication of Highly Pathogenic Avian Influenza in Asia, Technical report, Food and Agriculture Organization of the United Nations, Hanoi, Vietnam.
- FAO (2007), 'The global strategies for prevention and control of H5N1 highly pathogenic avian influenza'. Available as: http://www.fao.org/docs/eims/upload/210745/glob_strat_HPAI_apr07_en.pdf. Accessed 20 January 2011.
- FAO (2008), 'Situation update', *FAO AIDE News* **51**, 1–12.
- FAO (2009), Gathering Evidence for a Transitional Strategy (GETS) for HPAI H5N1 Vaccination in Vietnam. Technical Report, Technical report, Food and Agriculture Organization of the United Nations, Hanoi, Vietnam.

- FAO-OIE-WHO (2005), Recommendations on the Prevention, Control and Eradication of Highly Pathogenic Avian Influenza in Asia, Technical report, Food and Agriculture Organization of the United Nations, Hanoi, Vietnam.
- Fleiss, J. (1981), *Statistical Methods for Rates and Proportions*, John Wiley Sons.
- Gabriel, G., Dauber, B., Wolff, T., Planz, O., Klenk, H. and Stech, J. (2005), 'The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host', *Proceedings of the National Academy of Sciences of the United States of America* **102**(51), 18590–18595.
- Gambaryan, A., Tuzikov, A., Pazynina, G., Bovin, N., Balish, A. and Klimov, A. (2006), 'Evolution of the receptor binding phenotype of influenza A (H5) viruses', *Virology* **344**(2), 432–438.
- Gambotto, A., Barratt-Boyes, S., de Jong, M., Neumann, G. and Kawaoka, Y. (2008), 'Human infection with highly pathogenic H5N1 influenza virus', *The Lancet* **371**(9622), 1464–1475.
- Gilbert, M., Xiao, X., Pfeiffer, D., Epprecht, M., Boles, S., Czarnecki, C., Chaitaweesub, P., Kalpravidh, W., Minh, P., Otte, M. et al. (2008), 'Mapping H5N1 highly pathogenic avian influenza risk in Southeast Asia', *Proceedings of the National Academy of Sciences* **105**(12), 4769–4774.
- Govorkova, E., Rehg, J., Krauss, S., Yen, H., Guan, Y., Peiris, M., Nguyen, T., Hanh, T., Puthavathana, P., Long, H. et al. (2005), 'Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004', *Journal of Virology* **79**(4), 2191–2198.
- Guan, Y., Peiris, M., Kong, K., Dyrting, K., Ellis, T., Sit, T., Zhang, L. and Shortridge, K. (2002), 'H5N1 influenza viruses isolated from geese in Southeastern China: evidence for genetic reassortment and interspecies transmission to ducks', *Virology* **292**(1), 16–23.
- Gutierrez, R., Naughtin, M., Horm, S., San, S. and Buchy, P. (2009), 'A (H5N1) virus evolution in South East Asia', *Viruses* **1**(3), 335–361.
- Halpin, J. (2005), 'Avian flu from an occupational health perspective', *Archives of Environmental and Occupational Health* **60**(2), 59–69.

- Henning, J., Henning, K., Morton, J., Long, N., Ha, N., Vu, L., Vu, P., Hoa, D. and Meers, J. (2010), 'Highly pathogenic avian influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Vietnam', *Preventive Veterinary Medicine* p. In press.
- Henning, K., Henning, J., Morton, J., Long, N., Ha, N. and Meers, J. (2009), 'Farm-and flock-level risk factors associated with Highly Pathogenic Avian Influenza outbreaks on small holder duck and chicken farms in the Mekong Delta of Vietnam', *Preventive Veterinary Medicine* **91**(2-4), 179–188.
- Hoa, N., Lan, L., Weaver, J., Tam, L. and Anh, T. (2010), Surveillance for avian influenza under the VAHIP programme, Technical report, Vietnamese Avian and Human Influenza Control and Preparedness Project, Hanoi, Vietnam.
- Hosmer, D. and Lemeshow, S. (2000), *Applied Logistic Regression*, John Wiley and Sons, New York, USA.
- Hui, Z., Yang, J., Yuan, J., Ling, Y. and He, C. (2009), 'Reduced egg production in hens associated with avian influenza vaccines and formalin levels', *Journal of Information* **53**(1), 16–20.
- Humberd, J., Guan, Y. and Webster, R. (2006), 'Comparison of the replication of influenza A viruses in Chinese ring-necked pheasants and chukar partridges', *Journal of Virology* **80**(5), 2151–2161.
- Inui, K. (2009), Evolution of HPAI H5N1 Virus in Vietnam. Technical Report, Technical report, Food and Agriculture Organisation of the United Nations and the National Centre for Veterinary Diagnostics, Hanoi, Vietnam.
- Jadhao, S., Nguyen, D., Uyeki, T., Shaw, M., Maines, T., Rowe, T., Smith, C., Huynh, L., Nghiem, H., Nguyen, D., Nguyen, H., Nguyen, H. T., Hoang, L., Nguyen, T., Phuong, L., Klimov, A., Tumpey, T., Cox, N., Donis, R., Matsuoka, Y. and Katz, J. (2009), 'Genetic analysis of avian influenza A viruses isolated from domestic waterfowl in live-bird markets of Hanoi, Vietnam, preceding fatal H5N1 human infections in 2004', *Archives of Virology* **154**, 1249–1261.

- Juckett, G. (2006), 'Avian influenza: preparing for a pandemic', *American Family Physician* **74**(5), 783–790.
- Ka-Oudt, H., Zakia, M. and Kamelt, M. (2008), 'Evaluation of the immune response in AI vaccinated broiler chickens: Effect of biosecurity faults on immune response', *International Journal of Poultry Science* **7**(4), 390–396.
- Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K., Kawaoka, Y. and Webster, R. (1994), 'Potential for transmission of avian influenza viruses to pigs', *Journal of General Virology* **75**(9), 2183–2189.
- Kilpatrick, A., Chmura, A., Gibbons, D., Fleischer, R., Marra, P. and Daszak, P. (2006), 'Predicting the global spread of H5N1 avian influenza', *Proceedings of the National Academy of Sciences* **103**(51), 19368–19373.
- Knobler, S., A., M., A., M. and S.M, L. (2005), *The Threat of Pandemic Influenza: Are We Ready?*, Washington D.C., USA.
- Le, Q., Kiso, M., Someya, K., Sakai, Y., Nguyen, T., Nguyen, K., Pham, N., Ngyen, H., Yamada, S., Muramoto, Y. et al. (2005), 'Avian flu: isolation of drug-resistant H5N1 virus', *Nature* **437**, 1108–1108.
- Lee, C., Senne, D. and Suarez, D. (2004), 'Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus', *Journal of Virology* **78**(15), 8372–8381.
- Lee, J. (2005), 'Flu pandemic preparedness media briefing'. Available as: http://www.who.int/dg/lee/speeches/2005/flupandemic_mediabriefing/en/index.html. Accessed 20 January 2011.
- Li, K., Guan, Y., Wang, J., Smith, G., Xu, K., Duan, L., Rahardjo, A., Puthavathana, P., Buranathai, C., Nguyen, T. et al. (2004), 'Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia', *Nature* **430**(6996), 209–213.
- Lipatov, A., Kwon, Y., Sarmiento, L., Lager, K., Spackman, E., Suarez, D. and Swayne, D. (2008), 'Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses', *PLoS Pathogens* **4**(7), 1–10.

- Lipsitch, M., Cohen, T., Murray, M. and Levin, B. (2007), 'Antiviral resistance and the control of pandemic influenza', *PLoS Medicine* **4**(1), 111–115.
- Lo, C., Wu, Z., Misplon, J., Price, G., Pappas, C., Kong, W., Tumpey, T. and Epstein, S. (2008), 'Comparison of vaccines for induction of heterosubtypic immunity to influenza A virus: cold-adapted vaccine versus DNA prime-adenovirus boost strategies', *Vaccine* **26**(17), 2062–2072.
- Londt, B., Nunez, A., Banks, J., Alexander, D., Russell, C., Richard-Londt, A. and Brown, I. (2010), 'The effect of age on the pathogenesis of a highly pathogenic avian influenza (HPAI) H5N1 virus in Pekin ducks infected experimentally', *Influenza and Other Respiratory Viruses* **4**(1), 17–25.
- Long, N. (2007a), An analysis of data generated by post-vaccination sero-monitoring and surveillance activities, following HPAI vaccination in Vietnam in 2007, Technical report, Food and Agriculture Organisation of the United Nations and the Department of Animal Health, Vietnam.
- Long, N. (2007b), Final report on TADinfo training workshops, Technical report, Food and Agriculture Organisation of the United Nations and the Department of Animal Health, Hanoi, Vietnam.
- Long, N. (2008), An analysis of data generated by post-vaccination sero-monitoring and surveillance activities, following HPAI vaccination in Vietnam in 2008, Technical report, Food and Agriculture Organisation of the United Nations and the Department of Animal Health, Vietnam.
- Long, N. (2009), An analysis of data generated by post-vaccination sero-monitoring and surveillance activities, following HPAI vaccination in Vietnam in 2009, Technical report, Food and Agriculture Organisation of the United Nations and the Department of Animal Health, Vietnam.
- Long, N. (2010), An analysis of data generated by post-vaccination sero-monitoring and surveillance activities, following HPAI vaccination in Vietnam in 2009, Technical report, Food and Agriculture Organisation of the United Nations and the Department of Animal Health, Vietnam.

- Louis, Y. (2006), *Bird flu: a rising pandemic in Asia and beyond?*, World Scientific Publishing Co. Pte. Ltd., chapter Avian influenza: basic science, potential for mutation, transmission, illness symptomatology and vaccines.
- MARD (2004), Prevention and control of avian influenza. a report submitted to the prime minister (translated title), Technical report, Ministry of Agriculture and Rural Development, Hanoi, Vietnam.
- MARD (2005a), Circular for Implementing Urgent Strategies for the Prevention and Control of HPAI/H5N1 in Vietnam, Technical Report 69/2005/TT-BNN, Ministry of Agriculture and Rural Development, Hanoi, Vietnam.
- MARD (2005b), The use of vaccination for control and then eradicate avian influenza in Vietnam, phase I (2005 – 2006), Technical report, Ministry of Agriculture and Rural Development, Hanoi, Vietnam.
- MARD (2007), The situation of HPAI/H5N1, vaccination strategy and management and other control measures, Technical report, Ministry of Agriculture and Rural Development, Hanoi, Vietnam.
- MARD (2009), The use of vaccination for control and then eradicate avian influenza in Vietnam, phase III (2009 – 2010), Technical report, Ministry of Agriculture and Rural Development, Hanoi, Vietnam.
- Matrosovich, M., Zhou, N., Kawaoka, Y. and Webster, R. (1999), 'The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties', *Journal of Virology* **73**(2), 1146–1155.
- Men, B., B. O. and Preston, T. (2006), Studies on Duck Production in the Mekong Delta, Vietnam, Technical report, Faculty of Agriculture, Cantho University, Vietnam.
- Minh, P., Morris, R., Schauer, B., Stevenson, M., Benschop, J., Nam, H. and Jackson, R. (2009), 'Spatio-temporal epidemiology of highly pathogenic avian influenza outbreaks in the two deltas of Vietnam during 2003-2007', *Preventive Veterinary Medicine* **89**(1-2), 16–24.

- Minh, P., Stevenson, M., Morris, R. and Schauer, B. (2010), 'Risk factors associated with outbreaks of highly pathogenic avian influenza H5N1 in the Mekong River Delta of Vietnam', *Preventive Veterinary Medicine* p. Under review.
- Minh, P., Stevenson, M., Schauer, B., Morris, R. and Quy, T. (2010), 'A description of the management of itinerant grazing ducks in the Mekong River Delta of Vietnam', *Preventive Veterinary Medicine* **94**(1-2), 101–107.
- Moghadas, S., Bowman, C., Rost, G. and Wu, J. (2008), 'Population-wide emergence of antiviral resistance during pandemic influenza', *PLoS One* **3**(3), 1839–1849.
- Monto, A. (2000), 'Epidemiology and virology of influenza illness', *The American Journal of Managed Care* **6**(5), 255–264.
- NCVD (2009), Early vaccination scheme for duck (H5N1 vaccine), Technical report, National Centre for Veterinary Diagnostics, Hanoi, Vietnam.
- Nguyen, D., Uyeki, T., Jadhao, S., Maines, T., Shaw, M., Matsuoka, Y., Smith, C., Rowe, T., Lu, X., Hall, H. et al. (2005), 'Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001', *Journal of Virology* **79**, 4201–4212.
- Nguyen, T., Nguyen, V., Vijaykrishna, D., Webster, R., Guan, Y., Peiris, J. and Smith, G. (2008), 'Multiple sublineages of influenza A virus (H5N1), Vietnam, 2005-2007', *Emerging Infectious Diseases* **14**, 632–636.
- NIHE (2005), Avian influenza (A/H5N1) situation in Vietnam 2003-2005, Technical report, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam.
- Nomura, N., Sakoda, Y., Nam, H., Long, N., Huy, C., Tien, T., Endo, M., Yoshida, H., Yamamoto, N., Okamatsu, M., Yamage, M., Sakurai, K. and Kida, H. (2010), 'Characterization of influenza viruses isolated from domestic and feral birds in Vietnam (under review)'.
- NZAID (2009), 'Development of epidemiological skills and technical information to support the management and control of Highly Pathogenic Avian Influenza in Vietnam 2007 – 2011'.

- OIE (2009), 'Update on highly pathogenic avian influenza in animals (type H5)'. Available as: http://www.oie.int/download/AVIANINFLUENZA/A2009_AI.php. Accessed 20 January 2011.
- Pantin-Jackwood, M. and Swayne, D. (2007), 'Pathogenicity of Asian highly pathogenic avian influenza H5N1 virus infections in ducks', *Avian Diseases* **51**, 250–259.
- Pasquato, A. and Seidah, N. (2008), 'The H5N1 influenza variant Fujian-like hemagglutinin selected following vaccination exhibits a compromised furin cleavage', *Journal of Molecular Neuroscience* **35**(3), 339–343.
- Peyre, M., Fusheng, G., Desvaux, S. and Roger, F. (2009), 'Avian influenza vaccines: a practical review in relation to their application in the field with a focus on the Asian experience', *Epidemiology and Infection* **137**(01), 1–21.
- Pfeiffer, D. (2005), Assistance in the geospatial analysis of HPAI outbreaks in Vietnam, Technical report, Royal Veterinary College, London.
- Province, C. (2010), 'H5N1 avian influenza: Timeline of major events'. Available as: http://www.who.int/csr/disease/avian_influenza/ai_timeline/en/index.html. Accessed 20 January 2011.
- Qi, X., Li, X., Rider, P., Fan, W., Gu, H., Xu, L., Yang, Y., Lu, S., Wang, H. and Liu, F. (2009), 'Molecular characterization of highly pathogenic H5N1 avian influenza A viruses isolated from raccoon dogs in China', *PloS One* **4**(3), 4682–4690.
- Qiao, C., Tian, G., Jiang, Y., LI, Y., Shi, J., YU, K. and Chen, H. (2006), 'Vaccines developed for H5 highly pathogenic avian influenza in China', *Annals of the New York Academy of Sciences* **1081**(1), 182–192.
- R Development Core Team (2010), *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing, Vienna, Austria. Available as: <http://www.R-project.org>. Accessed 20 January 2011.
- Rott, R. (1992), 'The pathogenic determinant of influenza virus', *Veterinary Microbiology* **33**(1-4), 303–310.

- Rudolf, M., Poppel, M., Frohlich, A., Breithaupt, A., Teifke, J., Blohm, U., Mettenleiter, T., Beer, M. and Harder, T. (2010), 'Longitudinal 2 years field study of conventional vaccination against highly pathogenic avian influenza H5N1 in layer hens', *Vaccine* **28**, 6832–6840.
- Savill, N., St Rose, S., Keeling, M. and Woolhouse, M. (2006), 'Silent spread of H5N1 in vaccinated poultry', *Nature* **442**(7104), 757–757.
- Saw, T., Lim, W., Shortridge, K., Tam, J., Liu, K. and Mak, K., Tsang, T., Ho, Y., Lee, F. and Kwong, H. (1998), 'Isolation of avian influenza A (H5N1) viruses from humans - Hong Kong', *Journal of the American Medical Association* **279**, 263–264.
- Schmittgen, T., Zakrajsek, B., Mills, A., Gorn, V., Singer, M. and Reed, M. (2000), 'Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: Comparison of endpoint and real-time methods', *Analytical Biochemistry* **285**(2), 194–204.
- Senne, D. (2007), 'Avian influenza in North and South America, 2002–2005', *Journal of Information* **51**, 167–173.
- Senne, D., Panigrahy, B., Kawaoka, Y., Pearson, J., Suss, J., Lipkind, M., Kida, H. and Webster, R. (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**(2), 425–437.
- Sims, L. (2007), 'Experience in control of avian influenza in Asia', *Developments in Biologicals* **130**, 39–43.
- Sims, L. and Dung, D. (2009), Vaccination of poultry in Vietnam against H5N1 highly pathogenic avian influenza, Technical report, The Department of Agriculture, Fisheries and Forestry, Australia. Available as: http://www.aitoolkit.org/site/DefaultSite/filesystem/documents/CASE%20STUDY_07-09-09%20final.pdf. Accessed 20 January 2011.
- Smith, D. (2003), 'Applications of bioinformatics and computational biology to influenza surveillance and vaccine strain selection', *Vaccine* **21**(16), 1758–1761.

- Smith, G., Fan, X., Wang, J., Li, K., Qin, K., Zhang, J., Vijaykrishna, D., Cheung, C., Huang, K., Rayner, J. et al. (2006), 'Emergence and predominance of an H5N1 influenza variant in China', *Proceedings of the National Academy of Sciences* **103**(45), 16936.
- Smith, G., Naipospos, T., Nguyen, T., De Jong, M., Vijaykrishna, D., Usman, T., Hassan, S., Nguyen, T., Dao, T., Bui, N. et al. (2006), 'Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam', *Virology* **350**(2), 258–268.
- Spackman, E. (2008), *Avian Influenza Virus*, Humana Press, Athens, Greece.
- Steensels, M., Bublot, M., Van Borm, S., De Vriese, J., Lambrecht, B., Richard-Mazet, A., Chanavat-Bizzini, S., Duboeuf, M., Le Gros, F. and van den Berg, T. (2009), 'Prime-boost vaccination with a fowlpox vector and an inactivated avian influenza vaccine is highly immunogenic in Pekin ducks challenged with Asian H5N1 HPAI', *Vaccine* **27**(5), 646–654.
- Stevenson, D. (1990), 'Mortality caused by improper vaccination techniques in chickens', *The Canadian Veterinary Journal* **31**(2), 118–118.
- Stienekegrober, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H. and Garten, W. (1992), 'Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease', *The European Molecular Biology Organization Journal* **11**(7), 2407–2414.
- Sturm-Ramirez, K., Hulse-Post, D., Govorkova, E., Humberd, J., Seiler, P., Puthavathana, P., Buranathai, C., Nguyen, T., Chaisingh, A., Long, H. et al. (2005), 'Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia?', *Journal of Virology* **79**(17), 11269–11279.
- Suarez, D. (2000), 'Evolution of avian influenza viruses', *Veterinary Microbiology* **74**(1-2), 15–27.
- Suarez, D., Lee, C. and Swayne, D. (2006), 'Avian influenza vaccination in North America: strategies and difficulties', *Developments in Biologicals* **124**, 117–124.

- Suarez, D. and Schultz-Cherry, S. (2000), 'Immunology of avian influenza virus: a review', *Developmental and Comparative Immunology* **24**(2-3), 269–283.
- Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J. et al. (1998), 'Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness', *Science* **279**(5349), 393–399.
- Swayne, D. (2003), 'Vaccines for list A poultry diseases: emphasis on avian influenza', *Developments in Biologicals* **114**, 201–212.
- Swayne, D. (2006), 'Principles for vaccine protection in chickens and domestic waterfowl against avian influenza', *Annals of the New York Academy of Sciences* **1081**(1), 174–181.
- Swayne, D. (2008), *Avian Influenza*, Blackwell Publishing, Iowa, USA.
- Swayne, D., Beck, J., Garcia, M. and Stone, H. (1999), 'Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines', *Avian Pathology* **28**(3), 245–255.
- Swayne, D., Perdue, M., Beck, J., Garcia, M. and Suarez, D. (2000), 'Vaccines protect chickens against H5 highly pathogenic avian influenza in the face of genetic changes in field viruses over multiple years', *Veterinary Microbiology* **74**(1-2), 165–172.
- Takakuwa, H., Yamashiro, T., Le, M., Phuong, L., Ozaki, H., Tsunekuni, R., Usui, T., Ito, H., Yamaguchi, T., Ito, T. et al. (2010), 'Possible circulation of H5N1 avian influenza viruses in healthy ducks on farms in northern Vietnam', *Microbiology and Immunology* **54**(1), 58–62.
- Taylor, N. and Dung, D. (2007), An analysis of data generated by post-vaccination sero-monitoring and surveillance activities, following HPAI vaccination in Vietnam (2005 – 2006), Technical Report OSRO/RAS/604/USA (VIE) Epi Project Viet Nam, Food and Agriculture Organisation of the United Nations and the Department of Animal Health, Hanoi, Vietnam.

- Thanh, T. (2007), Protective efficacy of the H5N1 (strain Re-1) vaccine against HPAI H5N1 viruses isolated in Vietnam between 2006 and 2007, Technical report, National Centre for Veterinary Diagnostics, Hanoi, Vietnam.
- Tian, G., Zeng, X., Li, Y., Shi, J. and Chen, H. (2010), 'Protective efficacy of the H5 inactivated vaccine against different highly pathogenic H5N1 avian influenza viruses isolated in China and Vietnam', *Avian Diseases* **54**(s1), 287–289.
- Tian, G., Zhang, S., Li, Y., Bu, Z., Liu, P., Zhou, J., Li, C., Shi, J., Yu, K. and Chen, H. (2005), 'Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics', *Virology* **341**(1), 153–162.
- Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., Parakamawongsa, T., Premashthira, S., Amonsin, A., Gilbert, M. et al. (2005), 'Highly pathogenic avian influenza H5N1, Thailand, 2004', *Emerging Infectious Diseases* **11**(11), 1664–1672.
- Tumpey, T., Kapczynski, D. and Swayne, D. (2004), 'Comparative susceptibility of chickens and turkeys to avian influenza A H7N2 virus infection and protective efficacy of a commercial avian influenza H7N2 virus vaccine', *Avian Diseases* pp. 167–176.
- Tung, N. (2008), Avian Influenza vaccine efficacy test for recent isolates (2007-2008) in Vietnam. Technical Report, Technical report, National Centre for Veterinary Diagnostic (NCVD), Hanoi, Vietnam.
- Van der Goot, J., Koch, G., De Jong, M. and Van Boven, M. (2005), 'Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens', *Proceedings of the National Academy of Sciences of the United States of America* **102**(50), 18141–18146.
- Venables, W. and Ripley, B. (2002), *Modern Applied Statistics with S*, Springer Verlag, New York, USA. Available as: <http://www.stats.ox.ac.uk/pub/MASS4>. Accessed 20 January 2011.
- Wallace, R., HoDac, H., Lathrop, R. and Fitch, W. (2007), 'A statistical phylogeography of influenza A H5N1', *Proceedings of the National Academy of Sciences* **104**(11), 4473–4478.

- Wan, X., Nguyen, T., Davis, C., Smith, C., Zhao, Z., Carrel, M., Inui, K., Do, H., Mai, D., Jadhao, S. et al. (2008), 'Evolution of highly pathogenic H5N1 avian influenza viruses in Vietnam between 2001 and 2007', *PLoS One* **3**(10), 3462–3474.
- Wang, J., Vijaykrishna, D., Duan, L., Bahl, J., Zhang, J., Webster, R., Peiris, J., Chen, H., Smith, G. and Guan, Y. (2008), 'Identification of the progenitors of Indonesia and Vietnam avian influenza A (H5N1) viruses from southern China', *Journal of Virology* **82**, 3405–3414.
- Webster, R. (2002), 'The importance of animal influenza for human disease', *Vaccine* **20**, S16–S20.
- Webster, R., Bean, W., Gorman, O., Chambers, T. and Kawaoka, Y. (1992), 'Evolution and ecology of influenza A viruses', *Microbiology and Molecular Biology Reviews* **56**(1), 152–179.
- Webster, R. and Hulse, D. (2004), 'Microbial adaptation and change: avian influenza', *Revue Scientifique et Technique de l'Office International des Epizooties* **23**(2), 453–466.
- Webster, R., Laver, W., Air, G. and Schild, G. (1982), 'Molecular mechanisms of variation in influenza viruses', *Nature* **296**, 115–121.
- Webster, R., Sharp, G. and Claas, E. (1995), 'Interspecies transmission of influenza viruses.', *American Journal of Respiratory and Critical Care Medicine* **152**(4 Pt 2), S25–S30.
- WHO (2004), 'Avian Influenza — Fact Sheet'. Available as: http://www.who.int/csr/don/2004_01_15/en/. Accessed 20 January 2011.
- WHO (2010), 'Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO'. Available as: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_08_31/en/index.html. Accessed 20 January 2011.
- Widjaja, L., Krauss, S., Webby, R., Xie, T. and Webster, R. (2004), 'Matrix gene of influenza A viruses isolated from wild aquatic birds: ecology and emergence of influenza A viruses', *Journal of Virology* **78**(16), 8771–8779.

- Wilson, I. and Cox, N. (1990), 'Structural basis of immune recognition of influenza virus hemagglutinin', *Annual Review of Immunology* **8**(1), 737–787.
- Wong, S. and Yuen, K. (2006), 'Avian influenza virus infections in humans', *Chest* **129**(1), 156–168.
- Zhao, W., Wan, C., Cao, H., Lai, P., Liu, Y., Zhu, L. and Zhang, L. (2007), A comparison of genome sequences among the H5N1 strains of Hong Kong, Thailand, Vietnam and China, *in* N. Callaos, W. Lesso, C. Zinn, D. Tominaga, S. Hashimoto, S. Huang and M. Truta, eds, 'WMSCI 2007: 11th World Multi-Conference on Systemics, Cybernetics and Informatics, Vol IV, Proceedings', pp. 83–87.
- Zhou, H., Yu, Z., Hu, Y., Tu, J., Zou, W., Peng, Y., Zhu, J., Li, Y., Zhang, A., Yu, Z. et al. (2009), 'The special neuraminidase stalk-motif responsible for increased virulence and pathogenesis of H5N1 influenza A virus', *PloS One* **4**(7), 6277–6285.