

**COMPARATIVE MOLECULAR EPIDEMIOLOGY AND
ANTIGENIC CHARACTERISATION OF
HIGHLY PATHOGENIC AVIAN INFLUENZA A(H5) VIRUS
IN VIETNAM**

by

Diep Nguyen Thi, DVM, MVS

Kellogg College – University of Oxford



**A thesis submitted for the degree of
Doctor of Philosophy**

Trinity 2017

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Declaration

I declare that the work contained in this thesis is the result of original research and has not been submitted to any other University or Institution.

The research I conducted and the papers published in this thesis are based on data collected and analysed from the national surveillance system and three research studies. I was principal researcher in all of the work and played a central role in research design, data collection, testing, analysis and interpretation of findings. I was solely responsible for overall project management, data management and analysis. I was also responsible for preparation of scientific manuscripts and coordination of co-author input for all papers published as part of this thesis.

The analyses in this thesis are my own work, except where indicated by references or acknowledgements in the text.

Signed: _____

A handwritten signature in blue ink, appearing to be 'May', is written over a horizontal line.

Abstract

Highly pathogenic avian influenza (HPAI) virus poses a significant economic disease burden to the poultry sector of Vietnam, and is a credible threat for emergence of novel zoonotic viruses. This thesis examines the molecular evolution and epidemiology of HPAI A(H5) viruses in Vietnam identified through the national poultry surveillance system. The investigations comprised analysis of detection rates and geographic distribution of A(H5) clade variants from samples collected in live bird markets (LBMs); comprehensive analysis of whole genome sequences of viruses collected from 2012 to 2015; development of laboratory protocols for production of reference chicken antisera; and in-depth antigenic characterisations of contemporary A(H5N1) isolates. The findings underscored the high prevalence and widespread distribution of HPAI A(H5) virus within apparently healthy poultry sampled in LBMs and the difficulties in designing appropriate and effective disease management strategies. Whole genome analysis revealed significant diversification and reassortment of A(H5) viruses, with substantial *in-situ* evolution of clade 1 and 2.3.4 viruses between 2010 and 2012 and lineage replacements involving clade 2.3.2.1 and 2.3.4.4 viruses during 2014-2015. Antigenic analysis of contemporary A(H5N1) clade variants using chicken antisera produced at the National Center for Veterinary Diagnostics (NCVD) proved to be feasible and reproducible, and recapitulated similar patterns of clade clustering as ferret antisera. Antigenic relationships between existing poultry vaccines and circulating field viruses also appeared aligned with *in-vivo* vaccine challenge studies, in terms of protection profiles. Given the co-circulation of multiple, antigenically distinct clade variants, there is a great need for vaccine strategies capable of inducing broadly

cross-reactive immunity. If traditional inactivated vaccines are used, bivalent or trivalent formulations may be required. The results strongly suggest that antigenic data could ultimately be used as a surrogate (or as a precursor) to challenge studies that take time and resources to conduct, thus potentially enabling more rapid assessments and decisions about poultry vaccine selection by Vietnamese animal health authorities.

List of Publications

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Nomenclature

AI: Avian Influenza

ADU: Antigenic Distance Measure

AHW: Animal Health Worker

APHIS: Animal and Plant Health Inspection Service

BEAST: Bayesian Evolution Analysis Sampling Trees

BSL: Biosafety laboratory

BPL: Beta-propiolactone

CCs: Collaborating Centres

CI: Confidence Interval

CDC: Centers for Disease Control and Prevention

C-ECE: Chicken Embryonated Chicken Egg

CRBC: Chicken Red Blood Cell

CVV: Candidate Vaccine Virus

DAH: Department of Animal Health

DVS: District Veterinary Services

IFN: Interferon

EID: Egg Infective Dose

ELISA: Enzyme-labelled Immunosorbent

ERLs: Essential Regulatory Laboratories

ESS: Effective Sample Size

FAO: Food and Agriculture Organization of the United Nations

FMD: Foot and Mouth Disease

GISN: Global Influenza Surveillance Network

GISRS: Global Influenza Surveillance and Response System

GLEWS: Global Early Warning and Response System

GTR: General Time-Reversible

HA: Haemagglutinin

HAU: Haemagglutination Unit

HI: Haemagglutination Inhibition

HPAI: Highly Pathogenic Avian Influenza

HPD: Highest Posterior Density

Kg: Kilogram

LBM: Live Bird Market

LIFSAP: Livestock Competitiveness and Food Safety Project

M: Matrix

MARD: Ministry of Agriculture and Rural Development

MCC: Maximum Clade Credibility

MCMC: Markov chain Monte Carlo

MDCK: Madin-Darby Canine Kidney

MDS: Multidimensional scaling

MN: Microneutralisation

MP: Matrix Protein

MRD: Mekong River Delta

NA: Neuraminidase

NAI: Neuraminidase Inhibitors

NCVD: National Centre for Veterinary Diagnostics

NIBSC: National Institute for Biological Standards and Control

NIC: National Influenza Centre

NEP: Nuclear Export Protein

NP: Nucleoprotein

NS: Non-structural

NVSL: National Veterinary Services Laboratories

OIE: International Office of Epizootics

OP: Oropharyngeal

PA: Polymerase acidic

PB: Polymerase Basic

PCR: Polymerase Chain Reaction

RAHO: Regional Animal Health Office

RBC: Red Blood Cell

RRD: Red River Delta

PRRS: Porcine Reproductive and Respiratory Syndrome

RT- PCR: Reverse-Transcription-Polymerase Chain Reaction

rRT- PCR: real-time Reverse-Transcription-Polymerase Chain Reaction

sDAH: sub-Department of Animal Health

tMRCA: Time to Most Recent Common Ancestor

VAHIP: Vietnam - Avian and Human Influenza Control and Preparedness Project

VE: Vaccine Efficacy

VTM: Virus Transport Media

WGS: Whole Genome Sequence

WHO: World Health Organization

USA: United States Agency for International Development

US: United States

USAID: United States Agency for International Development

USDA: United States Department of Agriculture

CHAPTER 1

Introduction

Vietnam is considered a hotspot for influenza virus evolution, both for human seasonal influenza strains and animal influenza viruses with pandemic potential (Gilbert et al., 2008; Russell et al., 2008). HPAI A(H5) viruses have been endemic in Vietnamese poultry ever since the first major epizootic waves in 2003, that caused losses of >20% of the standing poultry population (45 million dead or destroyed). The government responded to the first epizootic A(H5N1) waves through stamping out campaigns (depopulation), which was enormously disruptive to local economies and livelihoods. Poultry vaccination against A(H5N1) influenza was introduced in 2005, when the number of human cases of A(H5N1) was increasing and stamping out measures were not controlling the spread of the disease. Since then, poultry vaccination against HPAI A(H5) has remained a major component of government intervention strategies, both as part of animal disease control and as part of national pandemic preparedness. Implementation of HPAI vaccination programmes has been repeatedly revised and modified over the years, and ongoing evaluation of these programmes remains a significant challenge.

Vietnam is a rapidly developing country, with one of the fastest growing economies in the world. The country has undergone extraordinary development in the last two decades, from ranking amongst the poorest of countries in the world in 1990, to being recognised as a middle-income country by the United Nations in 2011 (The Government of Vietnam,

2012). The transformations underway include rapid urbanisation, intensification of agriculture and livestock production, modernisation of retail marketing and food distribution systems, and considerable changes in consumer and dietary habits; these factors have both direct and indirect influences on the future trajectory of avian influenza endemicity, spread and evolution, and on the associated impacts on human and animal health. Despite the rapid modernisation of many aspects of agricultural production in Vietnam, the poultry sector remains dominated by disaggregated smallholder ‘backyard’ producers with mixed species and poor biosecurity, which poses challenges for surveillance and intervention programmes.

This thesis explores the genetic diversity of HPAI in Vietnam using samples and virus isolates generated through national poultry surveillance systems, as well as poultry outbreak responses. I have investigated the relationship between phylogenetic measures of genetic diversity and biologically significant antigenic variation. Within the context of endemic circulation of both HPAI as well as low pathogenic avian influenza (LPAI) in Vietnam, there is a critical need for thorough characterisation of field virus variants in order to perform risk assessment, update poultry vaccines and to consider alternative control methods. In this introduction chapter, I review basic aspects of avian influenza virology and epidemiology, present a brief history of HPAI in Vietnam and describe the Vietnamese government systems for poultry disease surveillance, outbreak response and poultry vaccination practices.

1.1. Avian influenza

1.1.1. Viral structural and genome organisation

Avian influenza (AI) is an infectious disease of poultry caused by influenza A viruses within the *Orthomyxoviridae* family. The influenza A virus is a negative sense, single-

stranded RNA virus with eight gene segments (basic polymerase 2 [PB2], basic polymerase 1 [PB1], acid polymerase [PA], haemagglutinin [HA], nucleoprotein [NP], neuraminidase [NA], matrix [M], non-structural [NS]) (Rabadan *et al.*, 2006; Palese and Shaw, 2007) (Figure 1.1). These eight gene segments encode at least 15 different viral proteins, including PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NS2, and supplementary proteins recently identified with little or unknown functions (Sonnberg *et al.*, 2013), i.e., PB1-F2 (Krumbholz *et al.*, 2011), PB1-N40 (Helen M. Wise, 2009), PA-X (Wise *et al.*, 2009), PA-N155 and PA-N182 (Muramoto *et al.*, 2013). Each viral RNA segment is folded into a double-helical hairpin structure (Tao and Zheng, 2012). Table 1.1 summarises the length of each viral segment and the function of protein(s) encoded by each segment.

All influenza virus RNA segments contain non-coding sequences at their 5' and 3' ends that flank the coding region (Palese and Shaw, 2007). The two viral surface glycoproteins HA and NA contain the major antigenic determinants (Horimoto and Kawaoka, 2001; Palese and Shaw, 2007) and form the basis of the classification system into HA/NA viral subtypes (Fouchier *et al.*, 2005).

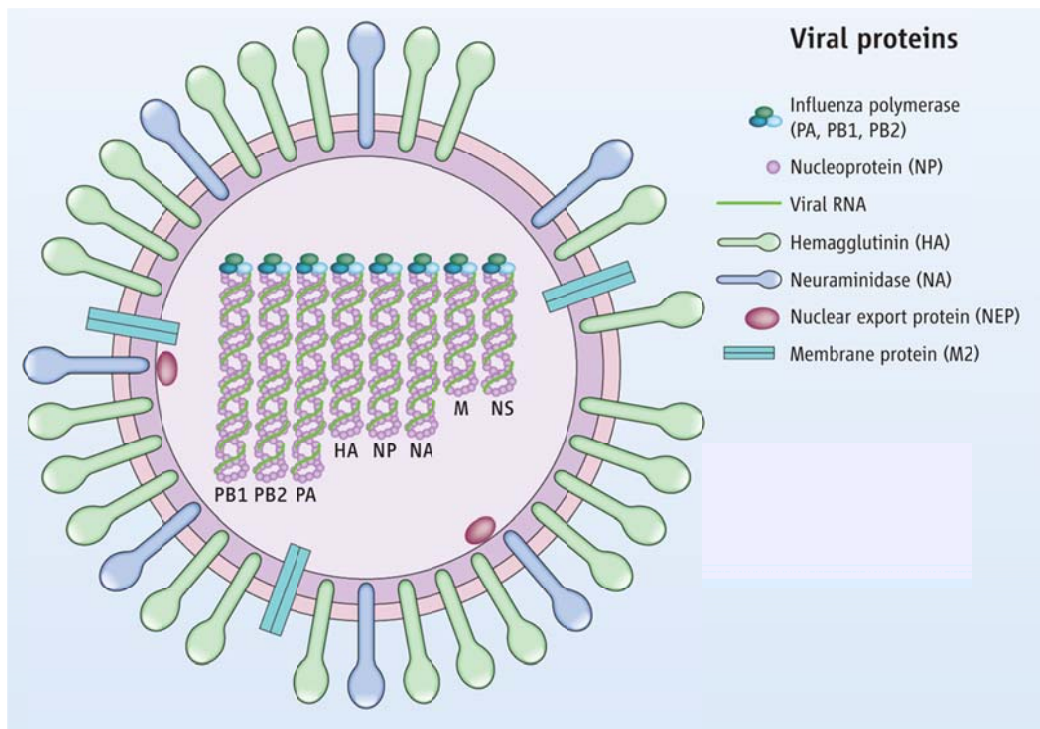


Figure 1.1: Structure of the avian influenza virus. Diagram obtained from DOI: 10.1126/science.1231588

The full complement, or ‘constellation’, of 8 gene segments is used to define influenza genotypes. To date, 18 different HA subtypes and 11 NA subtypes have been identified in aquatic waterfowl with the exception of H17(N10) and H18(N11) that have only been detected in bats (Tong *et al.*, 2012; Tong *et al.*, 2013). In addition to taxonomic classification into subtypes and genotypes, avian influenza viruses are designated as either highly pathogenic (HPAI) or low pathogenic (LPAI) based on their virulence phenotype in gallinaceous poultry (chickens).

Aquatic waterfowl are the natural reservoirs of 16 of the 18 known HA subtypes, and many different HA/NA combinations may be found in wild bird populations. Due to the segmented nature of the genome, the genetic diversity of influenza A viruses is continuously shaped by processes of reassortment (Lu *et al.*, 2014; CDC, 2016).

In most bird species, viral replication occurs mainly in the gastrointestinal tract, and to a lesser extent in the respiratory tract. Birds infected with LPAI generally have no apparent signs of illness (Munster *et al.*, 2007). Two of the HA subtypes, A(H5) and A(H7), have repeatedly undergone mutations to produce a polybasic cleavage site involved in protein activation, to generate highly pathogenic viral lineages. Indeed, the presence/absence of the polybasic cleavage site has such a well-established association with virulence that it is widely used as a molecular determinant in surveillance screening programmes. Infections with HPAI viruses in chickens typically result in fatal systemic infections, whereas the pathogenicity of HPAI viruses may be variable in ducks and other waterfowl (Taubenberger and Morens, 2017). The designation of HPAI versus LPAI refers exclusively to bird infections and has no bearing on whether human exposure to such viruses can cause symptomatic or severe disease (Taubenberger and Morens, 2017).

Internal proteins

The gene segments are numbered according to size from long to short. The polymerase complex (PB2, PB1, and PA) consists of polymerase enzymes encoded by segments 1, 2, and 3, respectively, that are responsible for transcription and replication of viral RNA (Mehle and Doudna, 2009). The PB2 protein plays a critical role in the initiation of transcription as it is responsible for binding the cap of host RNA (Ulmanen *et al.*, 1981; Blaas *et al.*, 1982) and is involved in replication (Gastaminza *et al.*, 2003). PB1 also initiates the process of transcription of RNA segments (Ulmanen *et al.*, 1981) and is responsible for elongating the growing RNA chain by addition of nucleotides (Braam *et al.*, 1983; Biswas and Nayak, 1994). Although no specific function has been described for the PA protein, mutations in PA affect both transcription (Fodor *et al.*, 2002; Fodor *et al.*, 2003) and replication (Huarte *et al.*, 2003). The PA subunit has the endonuclease activity responsible for cleaving the 5' cap from cellular mRNA (Li *et al.*, 2001; Dias *et al.*, 2009;

Yuan *et al.*, 2009). Furthermore, PA plays a role in stabilising the polymerase complex, cap binding activity as well as binding of the polymerase complex to the promoter region of vRNAs (Fodor *et al.*, 2002; Hara *et al.*, 2006). Additionally, the recently identified polypeptides of PB1-F2, PB1-N40, PA-X, PA-N155, and PA-N182 encoded within segments 2 and 3, respectively, have expanded our understanding of the virus and its ability to utilise the genome and these functional, accessory polypeptides (Sonnberg *et al.*, 2013). PB1-F2 protein plays a role in pro-apoptotic activity, while N40 protein, which is encoded by the same gene (PB1), interacts with the polymerase complex in the cellular environment but does not contribute to transcription function (Wise *et al.*, 2012). PA-X protein has been shown to modulate host response and viral virulence (Jagger *et al.*, 2012).

Additional proteins encoded by internal genes include NP (segment five), M (segment seven), and NS (segment eight); products of these genes play important roles in packaging of viral genomic RNA during replication, in intracellular transporting pathways and in the shipping mechanism through the channel components (Hutchinson and Fodor, 2013; Zheng and Tao, 2013). NP is involved in RNA synthesis and interaction with the viral RNA polymerase to allow initiation of RNA transcription to form cRNA and genomic vRNA (Newcomb *et al.*, 2009). Segment seven has been shown to code for two proteins, M1 and M2. M1, the major structural protein, is the dominant protein in determining virus morphology and also plays an important role in virus assembly and budding (Hale *et al.*, 2008). M2 is the ion channel that regulates the pH, and is responsible for virus uncoating, the step that follows virus entry into the host cell (Hale *et al.*, 2008). The final eighth segment of the influenza genome codes for NS1 and NS2. NS1 has a major role in inhibition of host immune response via limitation of interferon (IFN) production (Hale *et al.*, 2008). NS2 (also called nuclear export protein or NEP) plays a role in the export of

RNPs from the nucleus to the cytoplasm during viral replication, in addition, it also regulates virus transcription and replication processes (Wise *et al.*, 2012).

Surface glycoproteins

The fourth segment of the viral genome codes for HA, the most abundant glycoprotein on the surface of the influenza virus. The HA is involved in virus attachment to the cell receptors; and after being cleaved by host proteases, it facilitates fusion with the host cell membrane allowing entry of viral genetic material into the cell. HA is synthesised as a single polypeptide chain (HA0) with a length of approximately 560 amino acid residues, which is subsequently cleaved into two subunits, HA1 and HA2 (Wiley and Skehel, 1987; Palese and Shaw, 2007) (Figure 1.2). These subunits remain covalently linked to one another through disulphide bonds. In order for HA to undergo the conformational change necessary for fusion it must be cleaved (Choppin and Tamm, 1960). Cleavage of HA0 is essential for the molecule to mediate membrane fusion between the viral envelope and the host cell membrane (Skehel *et al.*, 1982; Palese and Shaw, 2007). Cleavage of HA0 to HA1 and HA2 is a necessity for virus infectivity and thus determines pathogenicity and spread of infection (Skehel *et al.*, 1982; Palese and Shaw, 2007). HA1 is responsible for binding of the virus to cellular sialic acid receptors, and the receptor-binding pocket is located close to the very tip of the molecule (Skehel and Wiley, 2000). HA1 also contains the major antigenic (immunogenic) epitopes of the molecule (Wiley *et al.*, 1981). The virus enters the cytoplasm through endocytosis followed by fusion of the host and viral membranes via conformational changes of the HA2 due to low pH in the endosome (Skehel *et al.*, 1982; Wharton, 1987; Wiley and Skehel, 1987). The HA cleavage site is the primary virulence determinant for AI virus, with the type and the number of basic amino acid at the cleavage sites (Capua and Alexander, 2004). HA is the primary viral antigen to

which the host's antibody response is directed and the only antigen inducing a neutralising antibody response.

Segment 6 of the influenza genome encodes the NA protein which encompasses approximately 17% of the surface glycoproteins. NA functions in promotion of the assembly and the release of the virus progenies from the cell receptor and is the target of a class of antiviral drugs knowns as neuraminidase inhibitors, which bind to the NA enzymatic site effectively blocking the ability of the NA to release progeny virus from the infected cell (Mair *et al.*, 2014).

Table 1.1: Avian influenza segments and their encoded polypeptides.

Segment	Length (nt)	Protein name	Encoded polypeptide	Length (aa)	Major functions	References
1	2341	Polymerase basic 2	PB2	759	Component of RNA polymerase; plays a role in RNA replication by mRNA cap recognition	(Nichol <i>et al.</i> , 1981; Ulmanen <i>et al.</i> , 1981; Blass <i>et al.</i> , 1982; Shi <i>et al.</i> , 1995; Bouvier and Palese, 2008; Guilligay <i>et al.</i> , 2008)
2	2341	Polymerase basic 1	PB1	757	Component of RNA polymerase; endonuclease activity; elongation	(Digard <i>et al.</i> , 1989; Poch <i>et al.</i> , 1989; Li <i>et al.</i> , 2001; Bouvier and Palese, 2008; Yuan <i>et al.</i> , 2009)
			PB1-F2	87	Component of RNA polymerase; cell apoptosis; virulence determinants	(Chen <i>et al.</i> , 2001; García-Sastre, 2006; Bouvier and Palese, 2008; Krumbholz <i>et al.</i> , 2011)
			N40	718	Polymerase complex interaction	(Bouvier and Palese, 2008)
3	2233	Polymerase acidic	PA	756	Component of RAN polymerase, protease; endonuclease activity	(Bouvier and Palese, 2008; Yuan <i>et al.</i> , 2009)
			PA-X	252	Modulates the host response and viral virulence	(Bouvier and Palese, 2008)
4	1778	Haemagglutinin	HA	566	Surface glycoprotein, receptor binding, fusion activity, major surface antigen, main target for neutralising antibodies	(Wilson and Cox, 1990; Webster <i>et al.</i> , 1992; Bouvier and Palese, 2008)
5	1565	Nucleoprotein	NP	498	RNA binding protein, RNA synthesis, nuclear import regulation	(Wilson and Cox, 1990; Webster <i>et al.</i> , 1992; Bouvier and Palese, 2008)
6	1413	Neuraminidase	NA	454	Minor surface glycoprotein for neutralising antibodies; sialic acid activity, cleavage of progeny virions from host cell receptors and virus release	(Wilson and Cox, 1990; Webster <i>et al.</i> , 1992; Bouvier and Palese, 2008)
7	1027	Matrix	M1	252	Major component of virion; RNA nuclear export regulation, viral assembly and budding	(Wilson and Cox, 1990; Webster <i>et al.</i> , 1992; Bouvier and Palese, 2008)
			M2	97	Ion channel for controlling pH during virus uncoating and HA synthesis (viral release)	(Wilson and Cox, 1990; Webster <i>et al.</i> , 1992; Bouvier and Palese, 2008)
			M42	99	Functionally replace M2 in M2-null viruses	(Wilson and Cox, 1990; Webster <i>et al.</i> , 1992; Bouvier and Palese, 2008)
8	890	Non-structural polypeptides	NS1	230	Multi-functional; inhibition interferon synthesis; induction apoptosis; mRNA maturation	(Wang <i>et al.</i> , 1999; Bouvier and Palese, 2008)
	395		NS2	121	NS2/NEP: nuclear export of vRNPs	(Bouvier and Palese, 2008; Robb <i>et al.</i> , 2009)

1.1.2. Molecular determinants of pathogenesis

The HA gene of influenza A viruses is the primary determinant of pathogenicity in avian and mammalian species (Bosch *et al.*, 1979), and because of the role of HA in binding to host cell receptors, it is critical for species specificity and host cell tropism. AI viruses preferentially bind to α -2,3 linked sialic acid receptors present in avian respiratory epithelial cells, while human influenza viruses preferentially bind to the predominant α -2,6 linkage receptor types present in human respiratory epithelial cells. Studies have demonstrated that humans also have small amounts of α -2,3 linkage receptor types, making them susceptible to certain avian influenza viruses, including HPAI A(H5N1) virus (Matrosovich *et al.*, 1999; Matrosovich *et al.*, 2004; Lorusso *et al.*, 2011). Swine have both receptor types in significant numbers so they are susceptible to infection with not only swine influenza viruses but also human and avian influenza viruses (Lorusso *et al.*, 2011).

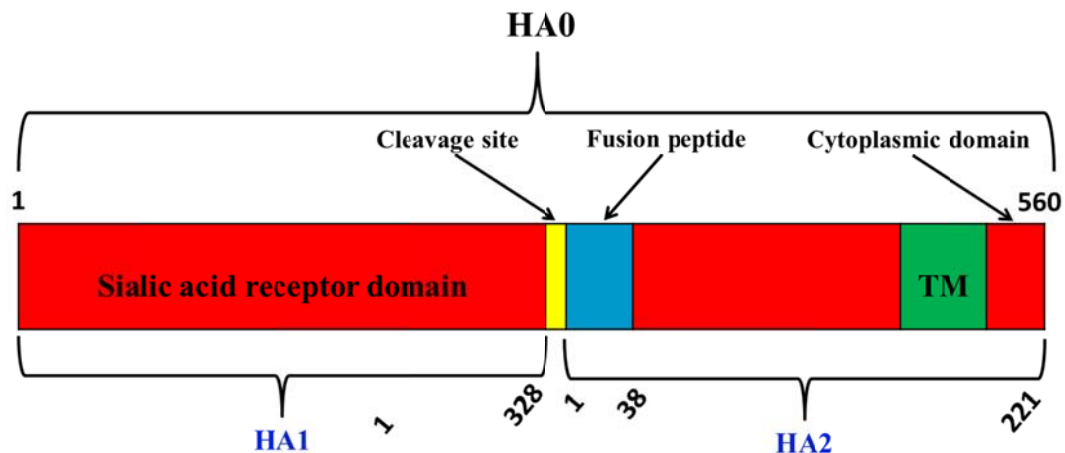


Figure 1.2: Schematic structure of HA of influenza A virus.

For LPAI viruses, the HA protein is cleaved by trypsin-like proteases located in the intestinal and respiratory tract, resulting in specific tissue tropism for these viruses, and containment of infection within these tissues. In contrast, HPAI viruses may be cleaved by trypsin-like proteases as well as ubiquitous furin-like proteases that are present throughout the cells of the visceral organs, cardiovascular system and nervous system, allowing for

systemic spread of these viruses (Perdue and Suarez, 2000). Alterations of the HA structure, particularly at the HA cleavage site, may confer a change in virulence. The substitution of non-basic with multiple basic amino acids and/or the insertion of basic amino acids in the cleavage site of an A(H5) or A(H7) virus can result in increased virulence and transformation of the virus from low pathogenicity to high pathogenicity for poultry (Senne *et al.*, 1996; Perdue and Suarez, 2000). A number of recent studies have demonstrated that a motif of multibasic amino acids around the HA cleavage site in HPAI A(H5N1) viruses also can act as a virulence factor in mammals. The substitution of even one amino acid can result in a significant increase or decrease in virulence (Schrauwen *et al.*, 2012; Suguitan *et al.*, 2012; Zhang *et al.*, 2012).

1.1.3. Antigenic drift and antigenic shift

Antigenic variation is a common strategy for an infectious pathogen to evade its host immunological response. The infectious pathogen can alter its surface protein(s) so that it will not be recognised by specific host antibodies. As a result, the pathogen can re-infect the same host. Antigenic variation in influenza A virus can occur through antigenic drift by accumulating mutations or through antigenic shift from exchanging surface proteins by two different strains. For human influenza A virus, antigenic drift typically leads to seasonal epidemics and antigenic shift to pandemics.

The term *antigenic drift* refers to genetic evolution of the HA gene in response to immune selection pressure in the host. Antigenic drift may result in virus escape from host immunity, and may be linked to changes in pathogenicity, protein temperature stability, or other relevant phenotypes that influence virulence and transmissibility. Drifted HA may not be recognised by immune responses to HA from earlier influenza viruses facilitating re-infection of hosts with viruses with the new HA, particularly for long-lived hosts such

as humans (Alexander and Brown, 2000). The phenomenon of antigenic drift of influenza viruses (both influenza A and B) is arguably the most well studied example of virus evolution known, and forms the rationale for the international network of reference laboratories that are devoted to annual evaluations of vaccine seed strain selection for human, seasonal influenza vaccines (Smith, 2003). The relationship between HI-reactive antibodies and immune correlates of protection has been extensively studied for both avian and human subtypes, and it is well established that antigenic ‘matching’ is a key factor for predicting the ability of a particular vaccine formulation to prevent severe clinical disease, block infection and viral replication, and is correlated with the level of virus shedding (Swayne *et al.*, 2000a; Lee *et al.*, 2004; Swayne *et al.*, 2015).

Antigenic shift is a result of reassortment and it occurs when two or more different influenza A viruses subtypes infect a single cell simultaneously. Because influenza A viruses are segmented, new viruses with a variety of segment constellations can be produced by reassortment (Holmes *et al.*, 2005; Nelson *et al.*, 2008). Reassortment may result in the emergence of new subtypes, which may be more pathogenic than the original parent viruses and may be associated with pandemics (Neumann *et al.*, 2009; Van-Tam and Sellwood, 2009). Pigs are thought to have an important role in reassortment because of their ability to become infected with different types of influenza A viruses (avian and human viruses), and thus they act as an intermediate host, or “mixing vessel”. The new reassortant strain may cause a pandemic or panzootic if the hosts (humans or birds) have little or no immunity against it (Smith, 2001; Van-Tam and Sellwood, 2009). Such a scenario happened recently in April 2009 when an H1N1 virus of presumed swine origin (H1N1pdm09) composed of genes from avian, porcine and human virus origin emerged and spread throughout the world from Mexico (Michaelis *et al.*, 2009). Other historical examples of known human influenza pandemics that involved antigenic shift events were

A(H2N2) in 1957 and A(H3N2) in 1968 (Viboud *et al.*, 2005; Salomon and Webster, 2009; Greninger *et al.*, 2010). In the last 4 years, detections of human infections with novel subtypes have raised concerns regarding new threats, including annual outbreaks of A(H7N9) and sporadic detection of A(H10N8), A(H6N1) and A(H5N6) viruses, all of which are circulating in Chinese poultry, and appear capable of occasionally infecting humans and other mammals, but have yet to develop human-to-human transmissibility (Bi *et al.*, 2015b; Su *et al.*, 2015).

1.2. History of HPAI A(H5) in human and poultry

1.2.1. Global history of HPAI A(H5)

HPAI A(H5N1) viruses were first detected in 1996 among geese from Guangdong province, China, and the first human cases were reported from Hong Kong in 1997. Among the first 18 recorded human cases, six cases were fatal (Steel *et al.*, 2009). In 2003, HPAI A(H5N1) re-appeared first in Vietnam and also in other parts of east and south-east Asia including Hong Kong, Japan, Korea and Thailand, with devastating economic consequences to the poultry industry and high mortality rates in human cases (approximately 60%) (Claas *et al.*, 1998). By 2005, the virus had spread to China, Indonesia and Malaysia as well as Africa, Europe, the Middle East and the Pacific.

HPAI A(H5N1) can be transmitted from wild birds to poultry by a faecal–oral route, oral–oral route, and also indirectly by respiratory droplets and aerosols (Sturm-Ramirez *et al.*, 2004; Jeong *et al.*, 2009). Most human cases, however, appear to have been exposed through unprotected direct or indirect contact with contaminated environments (CDC, 2013). To date, human cases of HPAI A(H5N1) have been identified in 16 countries (WHO, 2017), and the global total as of 14 February 2017 was 856 cases, of which 452 (53%) were fatal (WHO, 2017). Within Asia, HPAI A(H5N1) outbreaks have resulted in

human fatalities in Bangladesh, Cambodia, China, Indonesia, Lao People's Democratic Republic, Myanmar, Pakistan, Thailand and Vietnam, most often after direct contact exposure with infected poultry flocks (Kwon *et al.*, 2011; Van Kerkhove *et al.*, 2012).

In addition to A(H5N1) viruses, a number of other AI subtypes have been known to cause sporadic human infections. For example, humans have occasionally been shown to be infected with subtypes of A(H7N7) (Koopmans *et al.*, 2004), A(H9N2) (Butt *et al.*, 2005; Gao *et al.*, 2013; Huang *et al.*, 2015), A(H6N1) (Zhuanqiang *et al.*, 2014), A(H5N6) (Shen *et al.*, 2016) and A(H10N8) in China (Zhang, 2014). However, when infected with these subtypes, the associated symptoms tend to be mild flu-like-illness, conjunctivitis or gastrointestinal symptoms (Chmielewski and Swayne, 2011; Arzey, 2012; Pepin *et al.*, 2013) except for one fatal case during an outbreak of HPAI A(H7N7) in the Netherlands (Fouchier *et al.*, 2004; Koopmans *et al.*, 2004). Most notably, an LPAI virus of subtype A(H7N9) emerged in China in 2013 resulting in annual outbreaks with numerous human cases and fatalities. As of 23 February, 2017, the Chinese lineage A(H7N9) virus has caused 1,292 human infected cases and 435 deaths (FAO, 2017), thereby surpassing A(H5N1) in total case numbers. Although the majority of A(H7N9) virus isolates have been confirmed as LPAI and demonstrated no pathogenicity for poultry, at least 3 virus isolates from human clinical cases have shown evidence of mutations to HPAI forms (FAO, 2017) and were virulent for chickens in laboratory experimental infections (FAO, 2017). In the first months of 2017, increasing detections of the highly pathogenic form of A(H7N9) containing the HA polybasic cleavage site have been detected in chickens and environmental samples from Guangdong Province (FAO, 2017), thus raising alarms and heightened concern regarding risk potential. To date, neither the A(H5N1) nor A(H7N9) viruses have acquired the ability to cause sustained human-to-human transmission (Bui *et al.*, 2015; Tibayrenc, 2017).

Since the first emergence of HPAI A(H5N1) in southern China in 1996, the virus has spread to more than 60 countries (WAHIS, 2016), persisting in 2016 in 20 countries (OIE, 2016). The spread of HPAI A(H5N1) virus among poultry has been attributed to the movement of infected poultry and poultry products and in some cases, by wild migratory birds (Chen *et al.*, 2005; Magalhães *et al.*, 2010; Tian *et al.*, 2015). Reassortment with other influenza viruses has led to the replacement of most internal viral genes of the original A(H5N1) virus. However, the HA gene A(H5) has remained present in all isolates and was therefore used to develop a standardised ‘clade’ nomenclature, first adopted in 2008, based on the evolution and divergence of A(H5N1) viruses that evolved from the original HA gene of the 1996 A(H5N1) virus (WHO/OIE/FAO, 2008). In the initial years from 1996 to 2008, 10 distinct clades (0–9) had been generated and by 2012, 11 distinct actively circulating clades had been identified (WHO/OIE/FAO, 2015). Between 2009 and 2013, HPAI A(H5) viruses from the clade 2.3.4 showed an apparent geographical range expansion and were not only of the A(H5N1) subtype. Continuous live-poultry market surveillance in China identified novel clade 2.3.4 reassortant viruses of different A(H5N2), A(H5N5) and A(H5N8) subtypes, alongside A(H5N1) (Gu, 2011; Zhao *et al.*, 2012; Zhao *et al.*, 2013). All these viruses were part of an A(H5) monophyletic group of viruses that shared the A(H5) gene of an A(H5N1) clade 2.3.4 variant with NA genes from different viruses (Gu, 2013). Consequently, the nomenclature of A(H5) viruses that clustered in this divergent HA group was updated as a new clade 2.3.4.4, in addition to two other new clades (WHO/OIE/FAO, 2015). From January 2014 onward, viruses of clade 2.3.4.4 started spreading internationally. The first HPAI A(H5N8) virus outbreaks outside of China were reported in South Korea and Japan in spring 2014 (Hill *et al.*, 2015). In May 2014, a novel A(H5N6) clade 2.3.4.4 reassortant caused outbreaks in China and Lao PDR (Wong, 2015; Wu *et al.*, 2015) and thereafter from Myanmar and Vietnam. In November 2014, HPAI A(H5N8) viruses were reported from Germany, Italy, Netherlands, the

Russian Federation and United Kingdom in rapid succession. In the same autumn and winter 2014/2015, HPAI A(H5N2) were reported from outbreaks in British Colombia, Canada. This virus contained genes similar to those of the Eurasian clade 2.3.4.4 alongside genes from North American wild bird lineages (WAHIS, 2016). In Taiwan, novel [A(H5N2), A(H5N3)] reassortants also caused several outbreaks in 2014 (Lee *et al.*, 2016). In December 2014, two new HPAI A(H5N8) and A(H5N2) viruses were detected in wild birds in Washington USA, before being found in poultry. By February 2015, the HPAI A(H5N2) virus had triggered an epidemic in commercial poultry in the US, with nearly 43 million chickens and 7 million turkeys killed or culled across more than 20 different states (Association, 2016). All these HPAI A(H5N1), A(H5N2), A(H5N6) and A(H5N8) viruses found in Eurasia and North America shared an H5 gene segment belonging to clade 2.3.4.4 (Claes *et al.*, 2014; FAO, 2016). In summary, we can describe two periods and groups of viruses. From 2003 to 2010, the HPAI A(H5) viruses responsible for international spread and most outbreaks in poultry were of the N1 type, with continuous evolution of the H genes into different sub-lineages and gradual changes in its internal genes yielding clades and sub clades. From 2010 onward, HPAI A(H5) clade 2.3.4 viruses reassorted with several other AI viruses leading to generation of a diversity of A(H5) clade 2.3.4.4 viruses bearing NA other than N1 in Asia. These novel reassortant viruses then spread internationally in 2013, in some cases further reassorting with viruses from other geographic lineages to yield new viruses, all bearing an A(H5) clade 2.3.4.4 HA.

In early March 2017, the United States Department of Agriculture (USDA) National Veterinary Services Laboratories (NVSL) confirmed detection of a HPAI A(H7N9) virus in a chicken sample collected from Lincoln County, Tennessee. Sequencing of the genome confirmed that all eight gene segments are of a North American wild bird lineage that is distinct from the Chinese lineage currently causing human cases in China (FAO, 2017).

The frequency of detecting novel HPAI subtypes within domestic poultry and various avian species underscores the perpetual threat to human health worldwide (Morens *et al.*, 2013).

1.2.2. HPAI A(H5) in Vietnam

Although the first major epizootics of HPAI A(H5N1) occurred in 2003-2004, retrospective analysis of samples collected in LBMs suggest that HPAI viruses may have been circulating in healthy ducks and geese of northern Vietnam as early as 2001 (Nguyen *et al.*, 2005). The temporal distribution of HPAI A(H5) outbreaks reported between 2003 and 2016 in Vietnam is shown in Figure 1.3. Five distinct waves can be identified, with the first two occurring around the Tết (Vietnamese [lunar] new year) festival, the next two finishing about one month before Tết, and the last occurring in summer 2007 (May – June). The duration of epizootic waves varied between one and four months, and the duration decreased overtime, which may be indicative of the effectiveness of control measures. Apart from the fourth epidemic wave which was characterised by comparatively limited spatial spread in several southern provinces, the spatial and temporal pattern of HPAI A(H5N1) outbreaks shows that outbreaks simultaneously occurred in different provinces of Vietnam, concentrating in the areas of highly poultry density, i.e. the Red River Delta (RRD) in the north and the Mekong River Delta (MRD) in the south. In April 2014, the first outbreak of HPAI A(H5N6) occurred in the north of Vietnam, and the virus spread quickly and became dominant in the northern and central regions later in 2014. The occurrence of sporadic outbreaks of both HPAI A(H5N1) and A(H5N6) outbreaks between 2014 and the present time indicates persistent circulation of both subtypes.

Concurrent with poultry outbreaks, the HPAI A(H5N1) virus has caused a total of 127 human cases of disease in Vietnam, with 64 fatalities since December 2003 (WHO, 2017). Although close contact with infected poultry has been shown to be the main source of transmission of A(H5N1) to humans (Dinh *et al.*, 2006), the epidemiological linkage between poultry exposure and human cases is not always clear. Most human cases of HPAI A(H5N1) in Vietnam have occurred in rural areas, and are thought to be related to local marketing and poultry management practices. However, Vietnam has not reported any human cases of HPAI among supposed ‘high risk’ populations (i.e. veterinarians, poultry traders and slaughterers), and serological studies of occupational risk factors do not show high levels of exposure or asymptomatic past infection among these groups (MARD, 2014).

HPAI A(H5N1) IN HUMANS

2003: 03 human cases (03 fatal)
 2004: 29 human cases (20 fatal)
 2005: 61 human cases (19 fatal)
 2006: No human cases reported
 2007: 08 human cases (05 fatal)
 2008: 06 human cases (05 fatal)
 2009: 05 human cases (05 fatal)
 2010: 07 human cases (02 fatal)
 2011: No human cases reported
 2012: 04 human cases (02 fatal)
 2013: 02 human cases (01 fatal)
 2014: 02 human cases (02 fatal)
 2015: No human cases
 2016: No human cases
Total: 127 human cases (64 fatal)

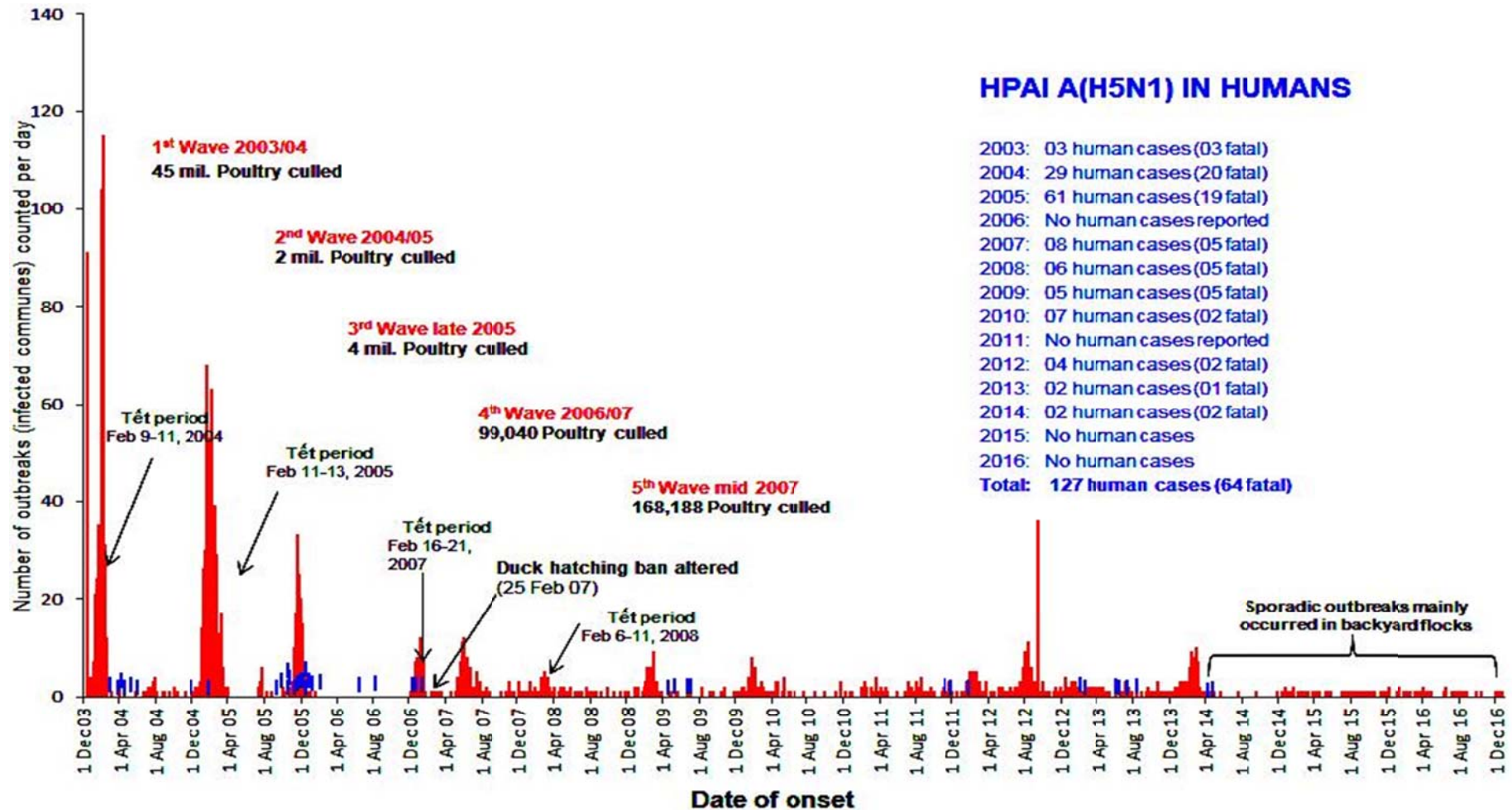


Figure 1.3: Temporal distribution of HPAI A(H5) outbreaks occurred between late 2003 and 2016.

1.3. HPAI control and prevention

1.3.1. Control and prevention strategies in other countries

Effective prevention and control of HPAI requires a transboundary response to contain and eliminate the disease at its source. Different approaches have been applied in various countries; however the key measures typically involve stamping out (depopulation), movement control, closure of live bird markets, enhancement of biosecurity, and vaccination. Early detection and stamping out have proven to be particularly effective in countries with relatively low poultry density (Sims, 2013; Spackman and Swayne, 2013; Swayne *et al.*, 2014). Vaccination was a key strategy for controlling the HPAI A(H5N2) outbreaks in Mexico in 1994-1995 and Central America in 2001- 2002. Stamping out has not been as successful in regions with high poultry density, especially if disease reporting and surveillance systems are not sufficiently robust to detect all cases where they first occur.

Biosecurity measures introduced to farms or in markets to reduce the likelihood of introduction of AI viruses can markedly reduce the risk of infection of poultry and provide a return on investment (Sims, 2013). A key constraint is that the measures must be cost-effective for the production system and producer (Fasina *et al.*, 2012). Most small village flocks in Asia are not confined, despite some advice to the contrary, because once the birds are confined it costs more to house and feed them (Abdelwhab *et al.*, 2010; Sultana *et al.*, 2011). The appeal of scavenging poultry to villagers is that they can be reared with virtually no financial input. This demonstrates that the measures proposed for small scale poultry producers must be practical and affordable, focusing in particular on behavioural change and simple, cost-effective measures. For some production systems, such as free-grazing ducks, only marginal improvements can be made to biosecurity, which means that

other ways to protect these poultry must be found such as better controls on movement and vaccination (Les Sims, 2016).

1.3.2. Control and prevention strategies in Vietnam

Since the first HPAI A(H5N1) epidemic in 2003-2004, the Vietnamese government has implemented aggressive control policies. The total number of birds destroyed during the first epidemic wave was approximately 45 million, accounting for 16.8% of the total poultry population, of which 30.4 million were chickens and 13.5 million were waterfowl (MARD, 2007; MARD, 2009). This massive culling reduced the impact of disease during the first epidemic, but did not eliminate the virus because the virus could be maintained in ducks without showing clinical signs, and periodic re-introductions continued to occur from China (Wan *et al.*, 2008).

Given the intensity and persistence of endemic HPAI transmission in Vietnam over the last decade, the government has focused on a variety of control and containment efforts, but no longer refers to ‘elimination’ as a feasible goal. A national ban on waterfowl hatching was introduced between February 2005 and February 2007, in recognition of the accumulating evidence that domestic waterfowl were responsible for silent spread of HPAI (Chen *et al.*, 2004). In March 2007, the ban was lifted, however movement controls for itinerant grazing (field running) ducks were introduced (Diep, 2011). Regarding vaccination, programmes have targeted chickens and ducks in high-risk areas, as defined by high poultry density, a history of prior outbreaks, or regions with large numbers of free grazing duck flocks. In these areas, culling in response to outbreaks is limited to affected flocks only (DAH, 2007; Diep, 2011). Prior to 2010, the number of poultry culled was approximately 30-300,000 animals per year, however the amount of culling has been reduced in recent years. The reasons for reduced culling rates are complex, and may be related to the overall reduced

size of outbreaks, as well as vaccine usage (since flocks are not culled if they can show proof of vaccination). New regulations aimed at changing practices in LBMs (e.g. segregation of poultry slaughter areas, restrictions on holding live birds overnight, cleaning and disinfection protocols) have been gradually introduced, especially since the advent of A(H7N9) emergence in China in 2013 (DAH, 2016c). In addition to control measures focusing on the poultry sector, nationwide education campaigns on risks associated with HPAI A(H5N1) virus have been implemented (Diep, 2011). Overall, the incidence of both poultry outbreaks and human cases has decreased over time, which may be interpreted as evidence for effective control policies (Figure 1.3).

1.4. HPAI surveillance

1.4.1. Surveillance programmes in other countries

Most poultry producing countries across the world have developed some forms of surveillance for HPAI, and the structure and implementation are guided by epidemiological considerations and available resources, most importantly the capacity of veterinary services. For countries that remain free of HPAI, the programmes tend to focus on surveillance at international borders and on wildlife. Results of national surveillance activities are shared through international organisations, such as the World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), and World Organization for Animal Health (OIE), that have jointly developed the Global Early Warning and Response System (GLEWS). GLEWS is linking network to improve communication among countries regarding HPAI and other emerging infectious disease threats, to promote coordinated responses and timely interventions.

1.4.2. Surveillance for HPAI in Vietnam

Establishing and maintaining a strong surveillance network have been the foundation of national HPAI control programmes in Vietnam, and many advances have been made within the last 14 years. The primary objective of HPAI surveillance has been to track genetic diversity of HPAI lineages, in order to provide early detection of mutations or reassortments that may alter risks for animal or human health, and to inform preparedness and control strategies. Both active and passive activities have been conducted routinely since 2004 (Figure 1.4). Passive surveillance has relied mainly on voluntary reporting of sudden death, die-offs and outbreaks by poultry owners and field veterinarians to local animal health authorities through community-based surveillance (Desvaux *et al.*, 2008). When an outbreak is reported, local veterinary authorities send experienced staff to carry out investigations using a national standardised outbreak investigation protocol (DAH, 2016a).

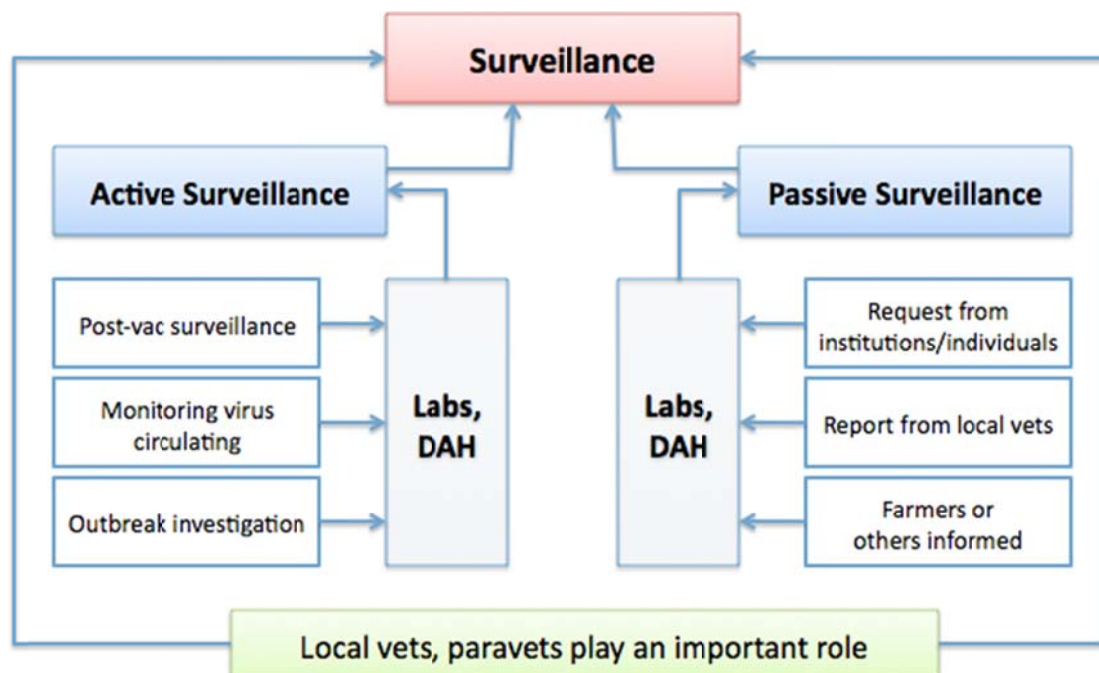


Figure 1.4: Structure of HPAI surveillance strategies in Vietnam.

Active surveillance comprises the routine periodic collection of samples from apparently healthy animals. These programmes were initially implemented only during the presumed ‘high risk’ periods of the year (i.e. during the annual Tét festival) and mainly at poultry raising households or poultry farms (Long, 2007; Taylor and Dung, 2007). Subsequently, the programmes were extended for year-round implementation and focused more intensively on LBMs. Annual meetings are held to review and revise the design of active surveillance programmes. In recent years, sampling has been also carried out at the non-traditional LBMs (poultry gathering areas), slaughterhouses and wild bird sanctuaries (Hoa *et al.*, 2010; Long *et al.*, 2013; Nishi *et al.*, 2013; Okamatsu *et al.*, 2013; Nguyen *et al.*, 2014; Chu *et al.*, 2017). The principle objective has been to monitor and track genetic diversity of currently circulating HPAI A(H5) viruses, through production of representative egg-grown virus isolates for use in molecular epidemiological studies (sequencing), phenotypic characterisations (e.g. antigenic characterisations and virulence testing), and for use in laboratory experiments and production of reagents. A secondary objective has been to collect epidemiological and demographic information for defining the main risk pathways of HPAI. Although many researchers wish to use surveillance data for further studies, for example for prevalence and incidence measures, network analysis, or mapping/modelling studies of transmission dynamics, to date the use of the surveillance system has been largely limited to sequencing and descriptive tracking of emerging clades.

For both active and passive surveillance activities, the NCVD and the seven Regional Animal Health Offices (RAHOs) coordinate sampling and carry out laboratory testing within their assigned geographical areas (Figure 1.5). Before 2004, capacity of these agencies for HPAI laboratory diagnosis was limited to conventional techniques such as virus isolation and serological testing (HA, HI). After the first wave of the HPAI epidemic,

molecular techniques such as Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), and the USDA-validated M gene and A(H5N1) HA RT-PCR (Spackman *et al.*, 2002; NVSL, 2008) were adopted by the Vietnamese Department of Animal Health (DAH) (MARD, 2005) and used to detect influenza M gene and subtype AI viruses in samples collected from both active surveillance and outbreak investigations.

Since 2006, real-time RT-PCR (rRT-PCR) has been used in active surveillance and outbreak confirmations for highly sensitive and rapid screening for influenza A virus positive samples. Due to the high volume load of surveillance activities, swab samples are typically pooled in the field, with 5 swabs from individual birds aggregated together.

Following screening for influenza A virus M gene, all positive and suspect-positive pools are further subtyped using subtype-specific HA and NA primer sets. A national laboratory and epidemiology network (EpiLabnet) was established in 2006 to enable sharing laboratory and epidemiological information, as well as technical training exchanges to support adoption and updating of standard operating procedures (SOPs). The national and regional laboratories take part in proficiency testing led by OIE reference laboratories (e.g. Australian Animal Health Laboratory), and have participated in OIE twinning programmes to enhance performance. Currently, apart from the NCVD and RAHO labs, there are an additional 20 provincial laboratories capable of HPAI diagnosis using both serological and molecular tests in Vietnam. All original swab samples that have tested positive are transferred to NCVD for virus isolation and further characterisations. Representative influenza virus isolates are selected and sent to the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, US for sequencing and phylogenetic analyses. Challenge viruses for use in vaccine efficacy evaluations are selected based on genetic diversity of contemporary clade variants and HA clusters/subclades.

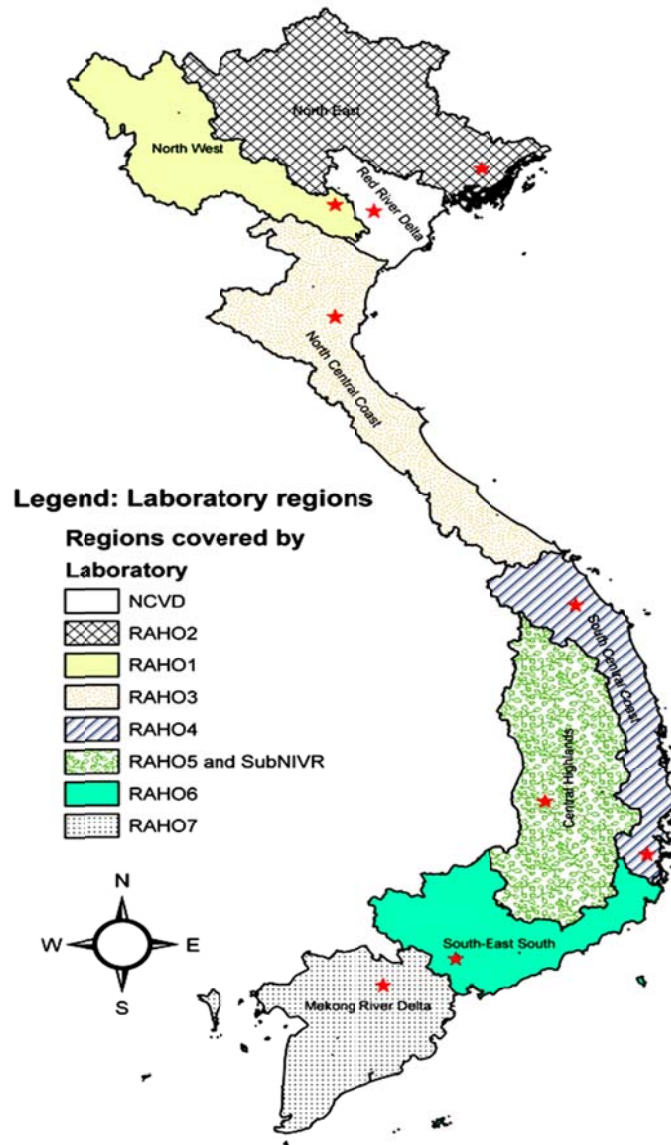


Figure 1.5: Map of Vietnam showing regional boundaries and the veterinary diagnostic laboratories servicing each region. Red stars indicate the location of each lab.

Surveillance programmes

1.4.2.1. Post-vaccination monitoring programme

Between late 2003 and 2004, during the peak of the first HPAI epizootic, little or no structured surveillance and investigation programmes were conducted. Following the introduction of mass poultry vaccination campaigns in 2005 and 2006, post-vaccination

monitoring was introduced to assess vaccine-induced immunity to HPAI using Haemagglutination Inhibition (HI) assays (Long, 2007; Long, 2008; Diep, 2011). The programme involved sampling large numbers of birds approximately one month after vaccination (annual collections comprised nearly 50,000 serum samples from 33–42 provinces). In addition to serological testing, swab samples were collected from unvaccinated chickens (called sentinels), as well as unvaccinated Muscovy ducks (at the time there was no vaccine available for this species) or any other unvaccinated poultry. The aim was to identify whether unvaccinated birds exhibited antibody responses to field virus. Results of these programmes demonstrated widespread circulation of HPAI A(H5N1), with prevalence of infection much higher than rates suggested through only passive reporting of outbreaks (Taylor and Dung, 2007).

1.4.2.2. Live bird market surveillance

Starting in 2011, the major focus of HPAI active surveillance shifted from on-farm sampling of diverse poultry species (sera and swabs) to exclusively sampling of ducks at LBMs (typically pooled oropharyngeal (OP) swabs, however individual OP sampling commenced in 2015). Sampling of chickens and environmental swabbing was introduced in 2014, in selected border provinces, with the goal of early detection of emergent Chinese lineage A(H7N9) viruses. To date, this second surveillance activity has screened over 40,000 pooled chicken oropharyngeal swabs and environmental samples, however, no A(H7N9) viruses have been identified in Vietnam by March 2017 (DAH, 2017). The results of active surveillance carried out between 2008 and 2015 indicated persistent and continuous asymptomatic circulation of HPAI A(H5) at prevalence levels ranging from 1.3% to 7.1% (Figure 1.7). The surveillance programme uses a stratified, multi-stage cluster sampling frame that has been periodically reviewed and revised over the years (Figure 1.6)

Output from the first and second rounds, conducted from September 2011 to February 2012 (six months) and from October 2012 to June 2013 (nine months) are the subject of **Chapter 2**.

In addition to monthly swab collections in LBMs of different sizes (both large district markets as well as informal smaller street markets), the programme attempts to obtain additional information about the origins of the sampled poultry (species, breed, age, poultry seller identification). To date, the vaccination status of birds sampled in LBM surveillance has not been documented. The shortcomings and limitations of ‘metadata’ collected in association with surveillance samples are discussed further in **Chapter 5**.

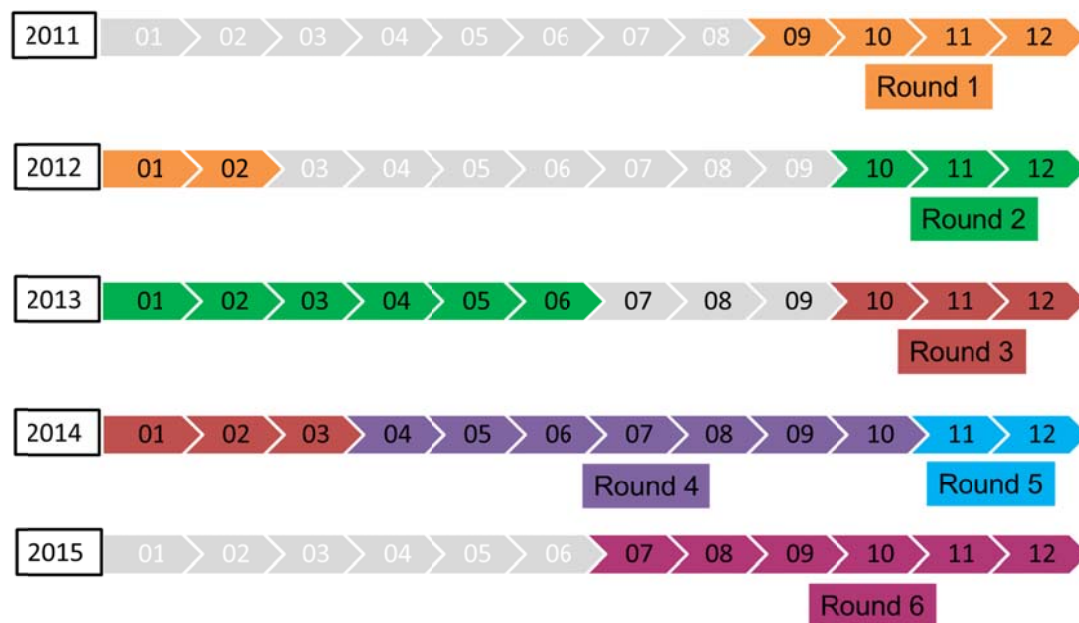


Figure 1.6: Time frame of surveillance activities from 2011 to 2015 in Vietnam.

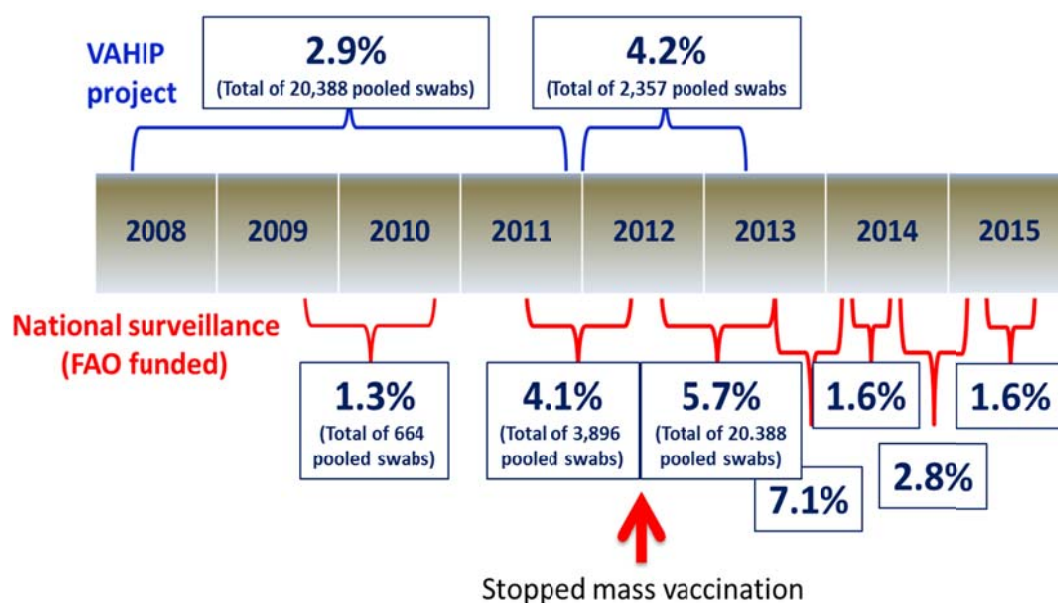


Figure 1.7: Schematic showing the average detection rates of HPAI A(H5N1) from ducks sampled at LBMs, 2008-2015.

1.5. Molecular epidemiology of HPAI

1.5.1. Evolutionary properties of influenza A

Rapid evolution is a crucial feature of influenza A viruses, and the rates of evolution are thought to vary among different reservoirs and hosts (Oxford *et al.*, 2003; Nelson *et al.*, 2014). Evolutionary rates for the gene segments encoding the two surface proteins, HA and NA, which are driven by positive selection and population immunity, are more rapid than sequence divergence of internal gene segments (Nobusawa *et al.*, 1991; Webster *et al.*, 1992).

The epidemiology of AI viruses within poultry reservoirs, as well as phylogenetic analyses, have suggested that avian viruses evolve at a slower evolutionary rate than viruses adapted to mammals (e.g. humans) (Swayne *et al.*, 2000b; Bouma *et al.*, 2009). Evolutionary rates can be used to estimate dates of emergence or origins of new lineages and depend on the

selective immune pressures and viral adaptation in the host species, as well as population size and host lifespan / turn-over rates (Suarez, 2000; Chen and Holmes, 2006).

While theoretically all combinations of HA and NA subtypes can be generated via reassortment, experimental studies of genetically engineered recombinant strains suggest that only some HA/NA combinations will yield replication-competent progeny viruses. Surveys of influenza A virus genetic diversity in various species have also clearly indicated that particular HA/NA subtypes and genome constellations have become adapted to different hosts with numerous factors that restrict the frequency and emergence of reassortant genome constellations.

The HA and NA genes of the HPAI A(H5N1) viruses in Southeast Asia are derived from the prototype A/goose/Guangdong/1/96 virus, whereas the genes encoding the six internal proteins (PB2, PB1, PA, NP, M, NS) are derived from LPAI A(H9N2) viruses (Gutiérrez *et al.*, 2009; Bui *et al.*, 2015). Since its first appearance in 1996 in Guangdong (Southern China), the progenitor HPAI A(H5N1) lineage diverged into 12 genotypes: GD, A, B, C, D, E, X(X0-X3), V, Y, W, Z(Z+) and G (Li *et al.*, 2004a; Smith *et al.*, 2006b). Shortly after the first emergence in Guangdong, the original genotypes (GD, A, C, D, E) disappeared, and were replaced by the next series of genotypes/subgenotypes V, W, X1, X2, X3, Y, Z, and Z+ (Macken *et al.*, 2006). The appearance of the Z genotype with strains of higher pathogenicity in Southeast Asian countries was considered antigenic drift (Li *et al.*, 2004b; Wu *et al.*, 2008). Genotype Z was characterised by deletions in NA (reducing the length of the ‘stalk’ region of the extracellular domain, which has been associated with adaptations from aquatic to gallinaceous avian species) as well as deletions in the NS segment. Viruses detected in Southern China (Guangdong, Guangxi, and Hunan provinces)

have substantially diversified to form further genotypes Z, V, W, and G. Genotype G appears to have been derived from reassortment between W and Z viruses (Li *et al.*, 2004b; Duan *et al.*, 2008).

Phylogenetic analyses of HPAI A(H5N1) HA genes resulted in the distinct classification of twelve clades (WHO/OIE/FAO, 2012). Between 2013 and 2014, an addition of eight clade designations were reported based on division of clade 1.1 (Mekong River Delta), 2.1.3.2 (Indonesia), 2.2.2 (India/Bangladesh), 2.2.1.1 (Egypt/Israel), and 2.3.2.1a, b and c (Asia) (WHO/OIE/FAO, 2015). More recently (from 2014), detection of HPAI A(H5N8) in East Asia and Europe, A(H5N8) and A(H5N2) in North America, and A(H5N6) in East and Southeast Asia, have prompted further reviews and updates to the A(H5) HA clade nomenclature, as each of these subtypes is associated with HAs from clade 2.3.4.4.

1.5.2. History and evolution of HPAI A(H5) in Vietnam

Clade distribution

Phylogenetic analyses of HA sequences of HPAI A(H5) viruses detected among poultry outbreaks in Vietnam between 2003 and 2014 identified at least 15 distinct HA clades [1, 1.1, 1.1.1, 1.1.2, 2.3.4, 2.3.4.1, 2.3.4.2, 2.3.4.3, 2.3.2.1a, b and c, 3, 5, 7.1, 7.2] (Wan *et al.*, 2008; Davis *et al.*, 2010; Creanga *et al.*, 2013) with timelines (Figure 1.8, Figure 1.9) as below:

- **2003-2005:** The HPAI A(H5N1) viruses were introduced into Vietnam for the first time and during this period, viruses of clade 1 were predominantly circulating in all provinces of the country (Nguyen *et al.*, 2008; Wan *et al.*, 2008).
- **2006:** Vietnam applied a nationwide poultry vaccination programme (Re-1, clade 0 vaccine), and there were no outbreaks reported that year.

- **2007-2009:** Clade 2.3.4 viruses of the Fujian sublineage (and its further subclades 2.3.4.1, 2.3.4.2, and 2.3.4.3) were dominant in the north of Vietnam and subsequently disappeared; no report of clade 2.3.4 was made after 2009. Viruses of clade 1 (and its subclade 1.1) were dominant in the Mekong Delta region in the south of Vietnam, and evolved into clades 1.1.1 and 1.1.2 (Nguyen *et al.*, 2008; Tung *et al.*, 2012). Re-1 (clade 0) and Re-5 (clade 2.3.4) vaccine imported from China were in use and a national project of testing NIBRG-14 master seed (reverse genetic virus strain A/Vietnam/1194/2004, clade 1, obtained from the National Institute for Biological Standards and Control [NIBSC, UK]) was launched for vaccine production (Davis *et al.*, 2010; Le *et al.*, 2011). In this period, a newly designated clade 7 was detected for the first time at ports of entry in Lang Son province at the Chinese border (Nguyen *et al.*, 2009b; Davis *et al.*, 2010).

- **2010:** The emergence of newly introduced clade 2.3.2 and further evolution of clade 2.3.2.1 into 2.3.2.1a and b was noted in the north and the continual evolution of clade 1.1 of the Cambodian lineage into 1.1.1 and 1.1.2 occurred in the south (Carrel *et al.*, 2012; Tung *et al.*, 2012; Creanga *et al.*, 2013; Bui *et al.*, 2014a). Re-1 and Re-5 vaccines were in use.

- **2011-2012:** The dynamic formation of 2.3.2.1a, b and particularly 2.3.2.1c clades was in progress in the whole country (Creanga *et al.*, 2013; Le and Nguyen, 2014); and because of antigenicity-matching failure for clade 2.3.2.1b, the Re-1/Re-5 vaccination was ceased in some provinces in the north. NIBRG-14 based vaccine (Navet Vifluvac) was successfully tested, indicating reliable efficacy against viruses of the current clades, particularly when used with immunomodulating adjuvants like β -glucan (Davis *et al.*, 2010; Le *et al.*, 2011; Li, 2011). Clade 1.1.1/1.1.2 viruses were dominant in the south where Re-1 vaccine was still in use (Carrel *et al.*, 2012; Gu, 2013).

- **2013-2014:** There was a wide circulation of clade 2.3.2.1a and c in the whole country and there were no reports of clade 2.3.2.1b. Concurrently, clades 1.1.1/1.1.2 were detected in both the Mekong Delta region and central Vietnam. Furthermore, the re-recognition of clade 7.1/7.2 evolving from clade 7 was reported. Due to decline in protection and the wide spread of clade 2.3.2.1c, Re-6 (clade 2.3.2.1b) vaccine was imported from China for use in 2014 (Gu, 2013).

Tracing back the geographic origin of A(H5N1) viruses in Vietnam, it appears that all viruses of the pre-existing clades (2.3.4, 2.3.2, 1.1, 7 before 2010) and the currently circulating clades (2.3.2.1a, b, c, 1.1.1, 1.1.2, 7.1, 7.2) were derived from progenitor viruses circulating in the southern provinces of China during 2003-2014 (Wan *et al.*, 2008; Creanga *et al.*, 2013; Lee *et al.*, 2015) (Figure 1.9). The viruses found in southern Vietnam evolved from clade 1, resulting in subclades 1.1.1 and 1.1.2, similar to that of the Cambodian clade 1.1 (Sonnberg *et al.*, 2013; WHO/OIE/FAO, 2014) (Figure 1.9).

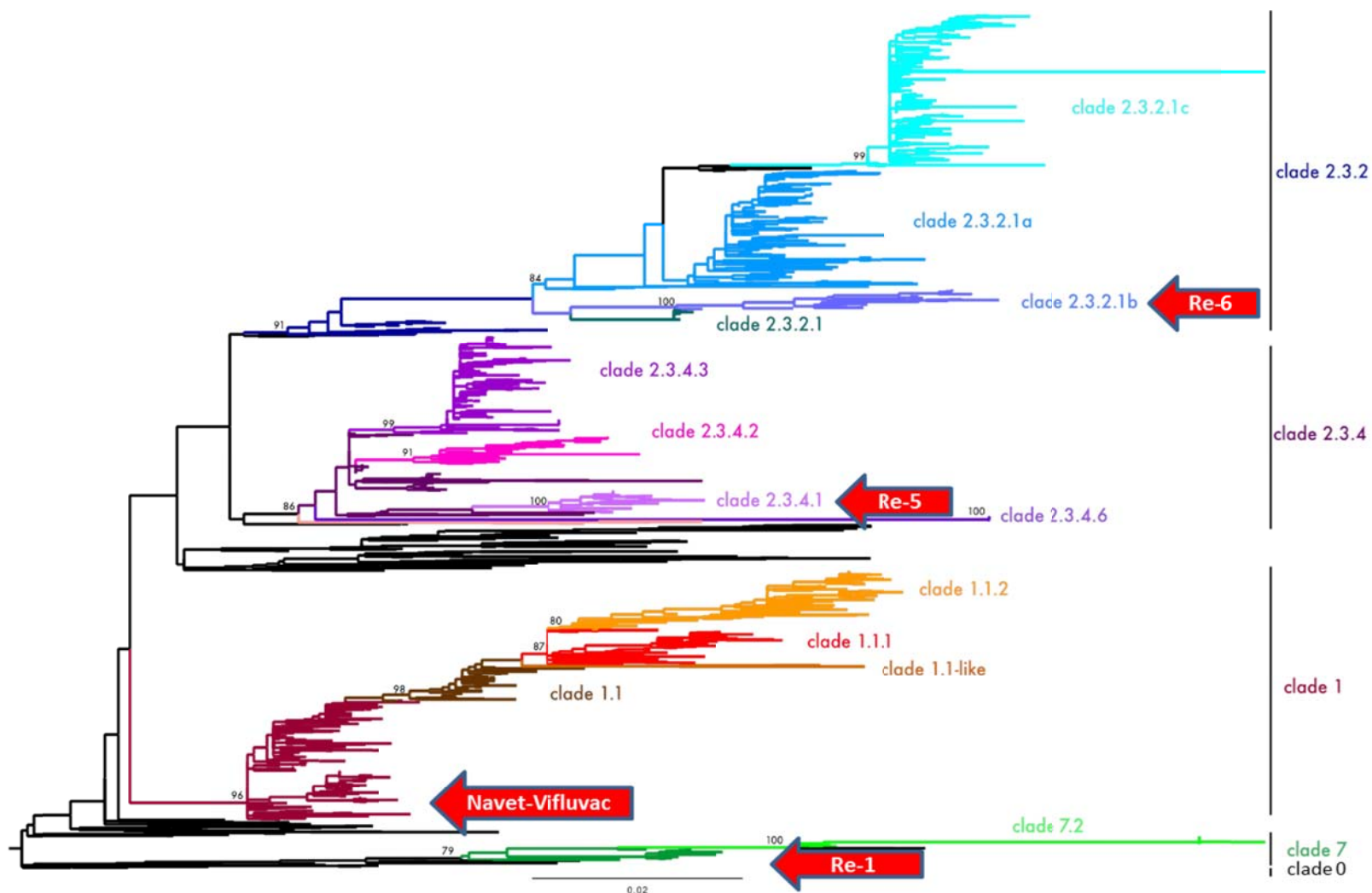


Figure 1.8: Evolution of HPAI A(H5) viruses in Vietnam between 2003-2014.

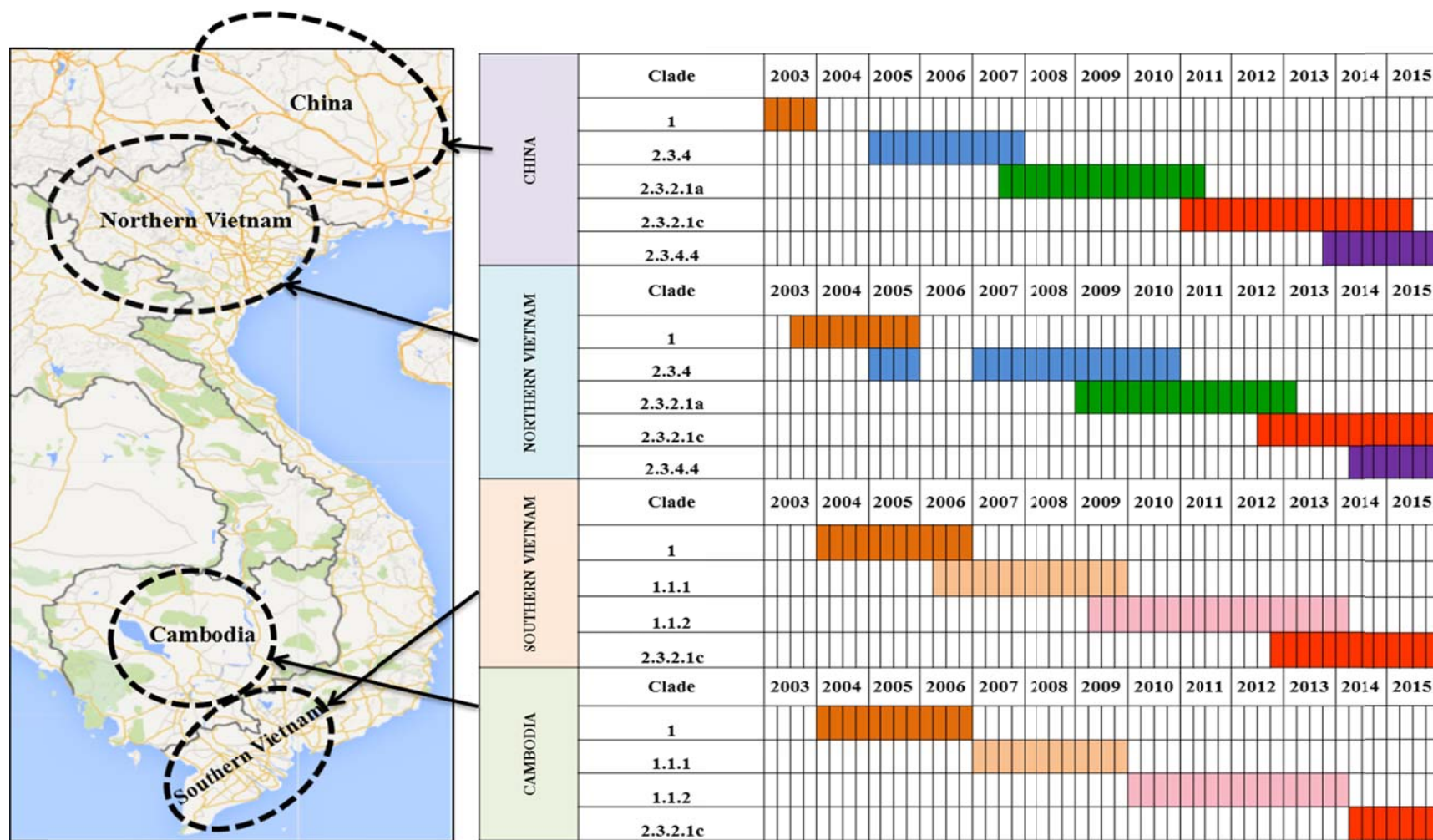


Figure 1.9: Emergence timelines and geographic distribution of HA clades of A(H5) in Vietnam, 2003-2015.

Genomic distribution in Vietnam

In Vietnam, genotypes Z and G have been dominant since 2005 (Nguyen *et al.*, 2008; Wan *et al.*, 2008; Tung *et al.*, 2012). The A(H5N1) viruses detected in Vietnam during 2004-2006 belonged to the Guangdong sublineage of genotype Z. In 2007-2008, the additional genotype G emerged; and after 2010, both Z and G genotypes coexisted (Wan *et al.*, 2008; Creanga *et al.*, 2013). Two subgenotypes of Z and G have been found to exist in Vietnam, subgenotype N (North) predominant in the North, and subgenotype S (South) in the South between 2007 and 2008 (Smith *et al.*, 2006c; Nguyen *et al.*, 2008). Between 2007 and 2010, circulating viruses were of mixed sublineages of Guangdong-like, Fujian-like and Qinghai-like (Nguyen *et al.*, 2008; Creanga *et al.*, 2013).

Molecular determinants of pathogenicity in A(H5) HA

Amino acid analysis of the A(H5) HA of the emerging 2.3.2.1 clades detected during 2010-2013 in Vietnam revealed that the polybasic cleavage site (amino acids 338-345, H5 numbering) was similar to the A(H5) HAs belonging to viruses of clade 2.3.2.1 and its subgroups (a, b, c) reported in China, Bangladesh, Indonesia, Japan, Laos, Mongolia, Nepal, Russia and South Korea (Sonnberg *et al.*, 2013; Xu *et al.*, 2013; Gerloff *et al.*, 2014); and other 2.3.2 subclades, but different from the cleavage site of clade 2.3.4 and clade 1 viruses (and 1.1) (Tung *et al.*, 2012; Creanga *et al.*, 2013; Sonnberg *et al.*, 2013).

Amino acids located at the HA receptor binding site (QSG, position 238-240) in all Vietnamese A(H5) clades sequenced to date have retained a binding specificity for avian type α -2,3 receptors (Nguyen *et al.*, 2009b; Mair *et al.*, 2014). The prototype vaccine strains representing clade 2.3.2 and 2.3.2.1c (A/Hubei/1/2010 and A/barn-swallow/Hong Kong/1161/2010, respectively) (WHO, 2014a; WHO/OIE/FAO, 2014), share close identity

to the viruses within clade 2.3.2.1 at most of the major sites, except antigenic site 2. Whether amino acid substitutions at these antigenic sites have contributed to antigenic-mismatching is still a question to be clarified. Glycosylation at amino acid 170 in HA1 (position 170-172) is completely lost by changing from NST (as seen in clade 1 and 1.1 strains) and NNT (clade 2.3.4) to amino acids DNA in all viruses of clades 2.3.2 origin including 2.3.2.1a, b, c of Bangladesh, China, Indonesia, Vietnam and other countries (Nguyen *et al.*, 2009b; Dung *et al.*, 2011; Creanga *et al.*, 2013).

1.6. Antigenic characterisation of HPAI A(H5) viruses in relation to vaccination programmes

1.6.1. Antigenic characterisation of HPAI A(H5) viruses

The WHO Global Influenza Surveillance and Response System (GISRS) is responsible for global virological surveillance of influenza in humans (WHO, 2014b), and has the following objectives: 1) Surveillance and monitoring of seasonal and emerging influenza viruses; 2) Recommendations in areas including laboratory diagnostics, vaccines and antiviral susceptibility; 3) Risk assessment for seasonal and pandemic influenza; and 4) Detection and response to influenza outbreaks.

Since 1952, the WHO has coordinated monitoring of the antigenic properties of influenza viruses in humans and, more recently, in animals (WHO, 2014b). The GISRS is currently comprised six WHO collaborating centres (CCs) four essential regulatory laboratories (ERLs), 143 national influenza centres (NICs) in 113 WHO member states and 13 WHO A(H5) reference laboratories (WHO, 2014b). The responsibilities of the NICs include collecting samples from patients with symptoms of influenza-like illness or acute respiratory disease and reporting on influenza activity. The CCs receive clinical samples or

virus isolates from NICs and carry out detailed characterisation, which includes assessment of antiviral drug susceptibility, genetic sequencing, phylogenetic analysis, serology, vaccine efficacy, and antigenic analysis.

The WHO Global Influenza Programme supports a Vaccine Composition Meeting held twice annually to select human seasonal influenza viruses for the northern and southern hemisphere vaccine formulations. This process incorporates data from the GISRS and makes recommendations to vaccine manufacturers regarding which viruses to incorporate into the next season's human seasonal influenza vaccines. National laboratories are an integral part of the process because they supply the viruses to the CCs. The CCs are responsible for virus isolations, sequencing, and antigenic characterisations of thousands of influenza A viruses each year. This formal, internationally recognised process informs the selection of candidate vaccine viruses (CVVs).

Although the efficacy of human seasonal influenza vaccines are regularly evaluated by a variety of public health programmes and surveillance indicators, the conduct of human clinical vaccine trials is extremely expensive, and thus the GISRS uses a great many surrogate indicators to evaluate the protective efficacy of human vaccination (WHO, 2014c). In contrast, although the evaluation of veterinary vaccines does not benefit from a coordinated international effort such as GISRS, it is far more feasible to test vaccine performance within the targeted host. CVVs for use in human pre-pandemic A(H5) vaccines are prepared each year by WHO CCs and ERLs, using data collected through country-specific animal influenza surveillance activities. Experts choose CVVs based on sequence data, HI analyses, and other phenotypic analyses to indicate which currently circulating AI viruses may pose the greatest threat to human health. Wildtype HPAI A(H5)

and A(H7) viruses are transformed into CVVs by genetic engineering to remove the polybasic cleavage site in the HA protein, and are tested for safety to confirm attenuation in chickens and ferrets (Li *et al.*, 1999). Using this approach, CVVs have been developed against >30 A(H5) viruses (WHO, 2016b), and are updated each year to reflect which A(H5) clade variants are deemed predominant. Because these CVVs are attenuated, they offer a potential source of seed virus for use in poultry vaccines, and these CVVs have sometimes been transferred to veterinary vaccine manufacturers for this purpose. For instance, the Navet Vifluvac vaccine, which was the first domestically produced poultry A(H5) vaccine in Vietnam, was based on the clade 1 pre-pandemic CVV selected in 2004. Typically, the HA and NA of choice are genetically engineered into the backbone of high growth non-pathogenic virus, A/Puerto Rico/8/34 (PR8), in essentially the same process that is used for generating pre-pandemic A(H5) CVVs. In this way, Harbin has produced the following vaccine series: A/goose/Guangdong/1/96 (Re-1), A/bar-headed goose/Qinghai/3/2005 (Re-3), A/chicken/Shanxi/2/2006 (Re-4), A/duck/Anhui/1/ 2006 (Re-5), A/duck/Guangdong/S1322/2006 (Re-6), and (A/chicken/Liaoning/S4092/2011) (Re-7).

1.6.2. Antigenic characterisation and use of antigenic cartography

Rapid detection of antigenic variants and accurate quantification of divergence is essential for selection of a vaccine strain and for development of timely vaccination programmes. Antigenic characterisation of influenza viruses is performed using immunological assays, such as HI, microneutralisation (MN) and enzyme-labelled immunosorbent (ELISA) assays.

Analysis of these immunological data is complicated for the following reasons: (i) serological assays are often considered ‘crude,’ and difficult to standardise both within and between laboratories, hence there is significant ‘noise’ present in datasets; (ii) it is common that there are empty or missing entries in immunological datasets requiring use of cross-testing (i.e. incomplete results for a given panel of antigens or antisera); and (iii) the data distribution in these datasets is unique, and unobserved values are not distributed randomly in the tables, especially for datasets that contain viruses with large isolation time intervals.

Antigenic characterisation based on HI assays has long been a routine procedure for influenza vaccine strain selection. The HI assay measures how a test influenza antigen and a reference antiserum (antibody) react. The antibody is usually diluted 5 fold first and then diluted in powers of 2. Thus, the titre from HI assay will be 5×2^n , $n \geq 0$. The larger the n is, the more closely the testing antigens match the reference antigens, for which the reference antisera are generated. Usually an HI titre < 20 is considered as a low level of reactivity between antigen and antibody. In many cases, HI experiments are used to measure the antigenic distance between two test antigens through their immunological reactions to the same reference antiserum. For instance, consider an example where one test antigen is a high reactor for a given reference antiserum (e.g. with a titre of 320) while another test antigen is a low reactor (e.g. with a titre of 40). The antigenic distance could be approximately 3 units, which is $\log_2(320/40)$. In reality, the antigenic distances are usually measured by a set of reference antisera, thus the calculation is more complicated.

In a typical influenza HI assay, generally less than 15 reference antisera are used but the number of test antigens can be more than 100. Interpretations of HI results are complicated because: (1) they only show the indirect relationship between antigens and antisera, since

each value reflects a reaction from antigen, red blood cells (RBCs), and antibody. Many variables from RBCs and antibody will influence the HI titres; (2) it is not possible to perform two-way HI analysis for all antigen and antisera reactions. Thus, the resulting HI table is generally incomplete, and the percentage of missing data could be up to 95% depending on availability of antisera.

By applying the metric multidimensional scaling (MDS) method to reduce the shape space into less than three dimensions, Lapedes and Farber (Lapedes and Farber, 2001) were able to show a linear correlation between the logarithmic values of HI titres and the spatial distances between influenza antigens. Based on this method, Smith et al. (Smith et al., 2004) constructed antigenic cartography to visualise the distance among influenza antigens and sera from HI tables by further developing the metric MDS method. This method is useful in both influenza antigenic variant identification and influenza vaccine strain selection (Cai et al., 2010).

Antigenic cartography has been shown to effectively detect influenza antigenic drift events; therefore, cartography is useful in antigenic characterisation and vaccine strain selection (Ducatez et al., 2011; Smith et al., 2004; Wan et al., 2011). Antigenic cartography has become a standard tool in influenza vaccine strain selection by the WHO influenza reference laboratories (Barnett et al., 2012).

1.6.3. Poultry A(H5) vaccine use in Vietnam

Vaccine recommendations

Vaccination is used to help control AI virus and limit production losses to poultry in areas where the virus is endemic. Currently, two types of vaccines are available for the control of

AI in Vietnam: adjuvanted inactivated vaccines based on whole virions and adjuvanted inactivated vaccines based on recombinant engineered vaccines. The former can be produced from master seed viruses isolated from the field, whilst the latter is generated by reverse genetic technology. Although vectored vaccines are available and licensed in some countries, 95.5% of the AI virus vaccine used for poultry, by dose, is oil emulsion, inactivated whole virus vaccine (Swayne *et al.*, 2011). Despite the disadvantage that this type of vaccine must be applied to each bird individually, inactivated vaccines are safe, effective, and relatively inexpensive, and therefore will remain highly utilised for AI virus in poultry, particularly in areas where labour costs are low. Individual inoculation does have the advantage that it can ensure high coverage within the vaccinated population. AI viruses require adjuvants to elicit a sufficient immune response. Vaccine adjuvants are chemical substances, microbial components or proteins that enhance the immune response to inactivated vaccines. An ideal adjuvant should be stable and environmentally safe, should not cause an inflammatory reaction at the injection site and must be cost effective. Adjuvants have been shown to increase the breadth of antibody responses to influenza in humans, and have been used in human seasonal vaccines and trial pandemic vaccines (Osterhaus *et al.*, 2011; Lorenzo and Fenton, 2013). However, the specific adjuvants and dosing/formulations are different between humans and animals. A recent study on adjuvants in poultry showed that some adjuvants (namely Montanide ISA 70VG and 71VG) provided modest improvements to protection compared to other adjuvants and non-adjuvanted vaccine, however, the correlation between antibody responses and different adjuvants was not significant (Lone *et al.*, 2017).

Table 1.2 provides a summary of a number of poultry vaccines used for the control of HPAI A(H5) viruses in Vietnam since the first major epizootics. The majority of poultry

vaccines used to date in Vietnam have been reassortant, inactivated oil-in-water A(H5N1) vaccines produced by Harbin, although some live vectored vaccines were used in the early years of mass vaccination campaigns. The vaccine most widely used from 2005-2010 during the period of biannual mass vaccination campaigns was based on the clade 0 A/goose/Guangdong/1/1996 (Re-1) virus (Qiao et al., 2006) with about 300 million vaccine doses (Table 1.2). Re-1 was used subcutaneously for both chickens (at 2 week-age) and ducks, however, ducks required two doses (at 2 week-age and 4 week-age) and showed poorer responses in terms of seroconversion and protection. Production of Re-1 was discontinued by most manufacturers in China in mid-2008 (Chen, 2009), however, Re-1 continued to be used in Vietnam until stocks were depleted in 2009. In addition to Re-1, some of the early campaigns used the live fowlpox vectored Trovac vaccine based on A/turkey/Ireland/1378 [A(H5N1)] with about 40-55 million vaccine doses and was used once intranasally for one day old chicks. This vaccine contained live recombinant fowlpox (recFP), expressing the haemagglutinin gene of an AI A(H5) subtype isolate. Another vaccine used in the early campaigns was a recombinant inactivated Intervet vaccine based on A/chicken/Mexico/232/1994 [A(H5N2)] recommended for day-old chicks and was used subcutaneously with about 9,5 million vaccine doses (Table 1.2). In 2008, use of Trovac vaccine was discontinued, because it showed poor efficacy under laboratory conditions, and evaluations of performance in the field were difficult and controversial (Lee et al., 2004; Escorcia et al., 2008; Pfeiffer et al., 2010). In addition to government subsidised vaccines in response to outbreaks, the Vietnamese commercial poultry sector has widely used A(H5N1) vaccines for many years, however obtaining data on the extent of coverage and details on vaccine use is problematic (Table 1.2).

Starting in 2011, following the emergence of the clade 2.3.2.1a and b variants, the Vietnamese government began recommending different poultry vaccine formulations for use in different regions of the country (DAH, 2011). The following inactivated oil-emulsion adjuvanted vaccines have all been used to some extent in various provinces since mid-2012: Re-5 (reverse engineered reassortant with the clade 2.3.4 A/Anhui/1/2005 HA and NA), Re-6 (reverse-engineered with clade 2.3.2.1b A/duck/Guangdong/S1322/2010 HA and NA), and the Vietnamese produced Navet ViFluvac vaccine (NIBRG-14 reverse-engineered with clade 1.1 A/Vietnam/1194/2004 HA and NA). These vaccines are used subcutaneously for both chickens (one shot at 2 week-age) and ducks (two shots at 2 and 4 week-age).

Although it is fast to make an updated formulation of poultry vaccines in other countries, the problem is that Vietnam manufacturers do not have those capacities, such as: 1) manufacturers in Vietnam do not currently have capacity to generate new candidate vaccine strains, because they are not yet competent in basic reverse genetics; 2) since the Vietnamese government is dependent on strain updates performed in China, they must wait for a new licensed product to become available from Harbin Veterinary Institute; and 3) even after a new vaccine strain is licensed in China, the regulatory process to allow importation into Vietnam requires extensive paperwork and more laboratory testing, which causes further delays. As an example, a clade 1 vaccine strain was used from 2003 to 2012, despite significant antigenic evolution of field strains during this period. The first domestically produced vaccine in Vietnam was the result of a national project, and used the NIBRG-14 master seed [reverse genetic virus strain A/Vietnam/1194/2004, A(H5N1), clade 1] (Davis et al., 2010; Le et al., 2011). It was not approved for use until 2012, by which time it was already outdated (Davis et al., 2010; Le et al., 2011; Li, 2011). Clade

2.3.4 viruses of the Fujian sublineage (and its further subclades 2.3.4.1, 2.3.4.2, and 2.3.4.3) were first detected in China in 2005, but vaccines containing variants with that clade were not available until 2008 (a three-year delay), when Re-5 [A/duck/Anhui/1/2006, A(H5N1), clade 2.3.4] began to be imported. Similarly, 2.3.2.1a, b and particularly 2.3.2.1c clades first emerged during the period from 2010 to 2014, and a new vaccine containing clade 2.3.2.1b (Re-6, A/duck/Guangdong/S1322/2006) vaccine was approved for use in the mainland China by 2012, but was not imported from China until 2014. Since late 2013, the clade 2.3.4.4 viruses began to spread in the region but a new vaccine with HA from this clade (Re-8 [(A/chicken/Guizhou/4/2013, A(H5N1), clade 2.3.4.4] has only just now become available in 2017. Thus, regardless of theoretical rapidity for producing updated vaccine formulations, the complexity of the manufacturing, regulatory, and political situation caused considerable delays. This means that the number of doses needed to implement and sustain an AI vaccination programme need to be evaluated carefully. Vaccine banks for emergency vaccination should be set up and maintained, and the vaccines need to be routinely evaluated based on continuous surveillance of the antigenic characteristics of the circulating viruses.

Determination of vaccine use in Vietnam

Vaccine efficacy studies are routinely conducted each year at NCVD in chickens and/or ducks, using selected representative challenge field viruses. Data generated from these annual experiments are used to formulate recommendations for local authorities and poultry owners. Selected vaccines are proposed that have the best antigenic match to circulating field viruses and offer an optimal immune response. However, challenge experiments are costly and can only be conducted for a limited number of challenge viruses. The experiments are carried out 2-3 times per year at NCVD within their BSL 2+

animal containment facility, using relatively small sample sizes (10 birds per challenge group). The challenge experiments typically comprise a 3×3 experimental design, i.e. testing of 3 candidate vaccines against challenge with 3 viruses. The endpoints measured are survival rate, time to death, and viremia at days 3, 5, 10, 14, and post-mortem. The results are used internally but have not yet contributed to peer-reviewed scientific publications. The methodologies explored in this thesis are motivated in part to help improve capacity for more systematic evaluations of the protective efficacy of candidate poultry vaccines, by establishing and validating surrogate measures of response to vaccination.

Table 1.2: HPAI vaccines licensed and used in Vietnam.

Product name	H5 HA Clade	Manufacturer's purported seed strain	Licensed in Vietnam	Recombinant backbone	Usage history (M=million)	Reference for efficacy data
Trovac AIV (Merial)	0	A/turkey/Ireland/1378 A(H5N1)	2005	Fowlpox vector w/ HA and NA of H5N1	~40-55 M doses/year in 2005-2006; discontinued use in 2007 due to poor performance	(Bublot <i>et al.</i> , 2010)
Bioflu (Merial)	1	A/chicken/Italy/22A/98 A(H5N9)	2006	Inactivated, whole virus H5N9	~7 M doses used for Muscovy ducks in 2007; discontinued use in 2008	(Bublot <i>et al.</i> , 2007)
Nobilis Influenza (Intervet)	North Am. lineage	A/chicken/Mexico/232/94/CPA A(H5N2)	2005	Inactivated whole virus	~9.5 M doses used in 2009, for chickens only	(Ellis <i>et al.</i> , 2004; Swayne, 2006; Swayne <i>et al.</i> , 2015)
(Chinese company)	Eurasian lineage	A/turkey/England/N28/73 A(H5N2)	2006	Inactivated, for chickens only	Never used in federally funded programmes, only in commercial operations	(Swayne <i>et al.</i> , 2015)[5]
Re-1 (Harbin)	0	A/goose/Guangdong/1/1996 A(H5N1)	2005	Inactivated, Rg in PR8	~300 M doses used/year throughout country from 2005-2010; 110 M doses in 2011-2012*; manufacturing ceased in 2011 but use continued until stocks depleted	(Tian <i>et al.</i> , 2010; Swayne <i>et al.</i> , 2015)
Re-5 (Harbin)	2.3.4	A/duck/Anhui/1/2006 A(H5N1)	2011	Inactivated, Rg in PR8	In current use, 2013-present**	(Chen, 2009)
Re-6 (Harbin)	2.3.2.1b	A/duck/Guangdong/S1322/2010 A(H5N1)	2012	Inactivated, oil-in-water emulsion, Rg in PR8	In current use, 2013-present**	(Cha <i>et al.</i> , 2013; Swayne <i>et al.</i> , 2015)
Navet-Vifluvac	1	NIBRG-14; A/duck/VN/1194/2004 A(H5N1)	2013	Inactivated, oil-in-water emulsion, Rg in PR8	In current use, 2013-present**	(Dau <i>et al.</i> , 2013)
Vectormune (Ceva)***	2.2	unknown [Clade 2.2 Egyptian A(H5)]	pending	live, HVT vector strain FC126	Registered; licensure pending field trials in 2016; applied at hatchery	(De Vriese <i>et al.</i> , 2010; Rauw <i>et al.</i> , 2011)
NDV-H5 (Boehringer Mannheim)	NA	synthetic HA, designed to contain composite epitopes	pending	inactivated, NDV vector with HA recombinant subunit	Registered; licensure pending field trials in 2016; applied at hatchery	
HVT-H5 (Pirbright)	2.3.2.1c and 1.1	A/duck/Vietnam/NCVD-1544/2012 A(H5N1)	pending	live, HVT vector	In development thru ZELS project	(Iqbal, 2012)

*: in 13 southern provinces only; **: Total estimated usage for 2014 of Re-5, Re-6, and Navetco vaccine was ~185 million dose; ***: Licensed and stockpiled for emergency use in USA.

Similar to other countries with recognised endemic transmission of A(H5) viruses (e.g. China, Egypt, and Indonesia), poultry vaccination has been considered a key component of HPAI control programmes in Vietnam (Sims, 2007; Capua and Alexander, 2008; Swayne, 2012b; Kapczynski *et al.*, 2015). Here, government commitment to preventive routine vaccination has varied over the years, however, expenses directly related to poultry vaccination still constitute the largest component of budget expenditures within the national control plan. Formal evaluations of vaccine intervention strategies were conducted by FAO in 2010 (Hinrichs *et al.*, 2011), and various meta-studies have incorporated analysis of Vietnamese datasets (Swayne, 2012b), as well as academic investigations using modelling approaches (Tran *et al.*, 2016). Despite these efforts, it remains challenging to systematically assess poultry vaccine interventions, largely due to the dearth of data on population coverage rates, post-vaccination immunity, and limitations of the passive reporting system for documenting outbreaks.

During the first major epizootic of HPAI in 2003, Vietnam reported 2,388 poultry outbreaks. The number of reported outbreaks had dropped to 45 in 2011. From 2012 to the present, the number of reported sporadic outbreaks was relatively stable, ranging from 39 to 167 per year (Figure 1.3). The significant drop in outbreak activity between 2003 and 2011 was attributed to a combination of measures, including increased biosecurity, depopulation and vaccine implementation (FAO, 2011). When used properly, poultry vaccination is widely accepted as an effective measure for protecting poultry against clinical signs and death, and reducing virus shedding. However, in recent years (since 2014), the co-circulation of both HPAI A(H5N1) and A(H5N6) may have comprised vaccine efficacy.

In countries that have used the same vaccines for an extended period of time, there is some evidence to support vaccine-driven evolution of field viruses, based upon the appearance of new variants capable of evading vaccine-induced immunity (Lee et al., 2004; Smith et al., 2004; Escorcia et al., 2008). Improper vaccination and inadequate coverage of poultry populations (Swayne et al., 2011) make control of virus spread difficult, which may contribute to the emergence of antigenic variants. ‘Improper vaccination’ may be defined as giving a low dose by either injecting less volume than required, or using of a vaccine with a low antigen load, use of expired vaccine, or use of vaccine which has lost potency due to improper storage. Additional ways in way vaccination could be improper or suboptimal include using a vaccine which is a poor antigenic match to the field virus; vaccinating at the wrong age (for example vaccinating at hatch when there are still maternal antibodies present); vaccinating too few birds within the flock; for ducks, 2 doses are required, so failure to complete both doses will compromise protective effect). Modelling studies (Gandon *et al.*, 2001) and molecular epidemiological analysis of evolutionary rates (Cattoli *et al.*, 2011) have been used to explore questions relating to the long term evolutionary impact of poultry A(H5) vaccines. These research questions remain highly relevant to the situation in Vietnam, and merit future prospective analyses using the resources of the current active and passive surveillance systems.

Table 1.3: Vaccine efficacy and shedding data from selected A(H5) challenge experiments conducted at NCVD, 2011-2015.

HA clade	A(H5) challenged virus	GMT of homologous antibody titre prior to challenge#			Percent mortality			Mean death time			Virus shedding in dead birds (survived birds)		
		Re-1	Re-5	Re-6	Re-1	Re-5	Re-6	Re-1	Re-5	Re-6	Re-1	Re-5	Re-6
1.1	A/Chicken/Vietnam/NCVD-778/2011	7	6	3	0	0	50	-	-	2.6	NA (+)	+++ (+)	+++ (+)
	A/Chicken/Vietnam/NCVD-1192/2012	8	7	3	0	10	50	-	3.0	2.4	+++ (+)	+++ (+)	+++ (+)
	A/Chicken/Vietnam/NCVD-2750/2013	ND	7	6		10	10		3.0	3.0	+++ (+)	+++ (+)	+++ (+)
	A//Vietnam/NCVD-14A47/2014	ND	7	4		10	70		3.0	2.4	+++ (+)	+++ (+)	+++ (+)
2.3.2.1a	A/Chicken/Vietnam/NCVD-675/2011	7	5	7	0	0	0	-	-	-	NA (+)	+++ (+)	+++ (+)
	A/Duck/Vietnam/NCVD-1207/2012	7	5	7	0	30	0	-	2.5	-	+++ (+)	+++ (+)	+++ (-)
	A/Chicken/Vietnam/NCVD-2749/2013	7	4	7	0	30	0	-	2.5	-	+++ (+)	+++ (+)	+++ (+)
2.3.2.1b	A/Duck/Vietnam/NCVD-672/2011	3	3	8	50	90	0	2.6	2.9	-	+++ (+)	+++ (+)	+++ (+)
	A/Duck/Vietnam/NCVD-1163/2012	2	3	8	50	70	0	2.6	2.4	-	+++ (+)	+++ (+)	+++ (-)
	A/Chicken/Vietnam/NCVD-2693/2013	ND	3	8			0			-	+++ (+)	+++ (+)	+++ (+)
2.3.2.1c	A/Duck/Vietnam/NCVD-1648/2012	5	5	8	0	10	0	-	2.0	-	+++ (+)	+++ (+)	+++ (+)
	A/Duck/Vietnam/NCVD-2745/2013	ND	7	7		10	0		2.0	-	+++ (+)	+++ (-)	+++ (+)
	A//Vietnam/NCVD-14A86/2014	ND	7	6		10	10		2.0	2.0	+++ (+)	+++ (+)	+++ (-)
	A/Duck/Vietnam/NCVD-15A7/2015	ND	8	5		60	10		5.5	4.0	+++ (+)	+++ (-)	+++ (+)
2.3.4.4 (H5N6)	A/Chicken/Vietnam/NCVD-A324/2014	ND	7	5		0	60		-	2.7	+++ (+)	+++ (+)	+++ (+)
	A/Duck/Vietnam/NCVD-15A17/2015	ND	7	5		30	60		7.0	6.7	+++ (+)	+++ (+)	+++ (+)
	A/Duck/Vietnam/NCVD-15A52/2015	ND	7	5		30	30		4.7	7.3	+++ (+)	+++ (+)	+++ (+)

Homologous titre was determined by HI of each homologous antigen to antisera collected 3-weeks post-vaccination; ND: Not determined;

+, Ct >33; ++, 29<Ct<33, +++, Ct<29

1.7. Aims and structure of the thesis

The overall objective of my thesis was to evaluate the genetic and antigenic evolution of influenza A(H5) viruses in poultry of Vietnam. Specifically, I aimed to:

- provide a comprehensive analysis of molecular epidemiology of contemporary HPAI A(H5) viruses collected through the national surveillance system in poultry Vietnam;
- measure antigenic diversity of these A(H5) viruses using haemagglutination inhibition testing with antigenic cartography and compare the use of both ferret and chicken antisera to accomplish this;
- compare evolutionary rates and antigenic distance among A(H5) clades variants within the context of poultry vaccine selection.

Collectively, my results may contribute to the establishment and validation of standard operating procedures for generating chicken antisera against A(H5) viruses circulating in Vietnam, and the assessment of the current active and passive poultry surveillance systems to detect virus and characterise both genetically and antigenically.

I present and discuss my data in three result chapters in the format (with slight modifications) of peer-reviewed publications (**Chapters 2 and 3**) and draft manuscript about to be submitted for peer review (**Chapter 4**).

In **Chapter 2**, I present the results from national active surveillance in live bird markets (LBM) between 2011 and 2013, and address the strengths and limitations of the system for assessing the risk of poultry outbreaks and various intervention strategies. In **Chapter 3**, I have compiled a comprehensive analysis of whole genome sequences of Vietnamese HPAI

A(H5) viruses from 2012 to 2015, and describe the results from initial assessments of antigenic relationships using ferret reference antisera. In **Chapter 4**, I describe methods for chicken reference antisera production, and in-depth investigations of the antigenic relationships among A(H5N1) viruses comparing the use of both ferret and chicken reference antisera. Finally, I conclude my thesis with a general discussion in **Chapter 5**, where I reflect on the major findings and diverse methodologies used throughout the thesis, and their contribution to the global advances of influenza research.

Surveillance and molecular epidemiology of avian influenza A(H5N1) viruses in live bird markets of Vietnam, 2011-2013

Abstract

Active surveillance for AI viruses in poultry sold at LBMs was conducted in 44 of 63 provinces throughout Vietnam over two periods from September 2011 to February 2012 and October 2012 to June 2013. The study objectives were to assess the prevalence of AI type A, A(H5) and A(H5N1) subtype viruses and characterise the geographical and temporal distribution of A(H5N1) virus genetic variants across the country. Monthly sampling was conducted in 394 LBMs located in 372 communes. A total of 9,790 oropharyngeal swabs from poultry were screened for influenza A virus by rRT-PCR. Virus isolation was attempted on all positive samples in embryonated chicken eggs and the HA1 region of each A(H5) virus isolate was sequenced. Market prevalence of A(H5) subtype virus was 32.2% (127/394) over the cumulative 15 months of surveillance. Phylogenetic analyses indicated that clade 1.1 viruses persisted in the south, whereas three genetically distinct subgroups of clade 2.3.2.1 were found simultaneously in northern, central and southern Vietnam. Clade 2.3.2.1c viruses first appeared in July 2012 and spread rapidly to the centre and south of Vietnam in late 2012, where they were predominant among clade 2.3.2.1 viruses and were detected in both active LBM surveillance and poultry outbreaks. Given the overlapping geographic distribution of clade variants and the antigenic divergence previously described for these clades, AI poultry vaccines used in Vietnam during this time period may have required bivalent formulations containing representatives of both clade 1.1 and clade 2.3.2.1 viruses.

Diep T. Nguyen, Juliet E. Bryant, C. Todd Davis, Leo Loth, Long V. Nguyen, Ken Inui, Long T. Pham, Tung Nguyen, Yunho Jang, Thanh L. To, Tho D. Nguyen, Diep T. Hoang, Hoa T. Do, Trang T. Nguyen, Scott Newman, Jennifer Siembieda, and Dong V. Pham. *Avian Disease*. 2014 Dec; 58(4):599-608.

2.1. Introduction

HPAI A(H5N1) viruses continue to pose a significant economic disease burden to the poultry sector of Vietnam, as well as to human public health (DAH, 2013a; WHO, 2013). Although reported HPAI A(H5N1) outbreaks have been mainly confined to the MRD and the RRD, an increasing number of outbreaks have been reported from coastal provinces located along major north-south trade routes and in mountainous regions (DAH, 2013a). At the end of 2013, several provinces previously free of disease (Kon Tum, Lao Cai and Phu Yen), reported their first confirmed outbreaks of HPAI. Until 2013, the geographic distribution of clade variants across Vietnam suggested non-overlapping transmission zones; virus descendants from clade 2 lineages were detected exclusively in the north, with frequent lineage replacement likely due to introduction of new viruses from bordering countries, whereas clade 1 lineages circulated exclusively in the MRD (Wan *et al.*, 2008) and neighbouring Cambodia. In addition, clade 7 viruses were occasionally detected in poultry illegally brought from China to LBMs located in the north of Vietnam (Nguyen *et al.*, 2009a; Davis *et al.*, 2010).

The development of an efficient national program of active surveillance to monitor silent circulation of AI viruses in poultry at LBMs represents an enormous achievement in tracking and monitoring risk and spread of HPAI A(H5N1) in Vietnam. To date, this program has been funded by the US Agency for International Development (USAID), and implemented by the FAO and the regional network of veterinary laboratories administered by the DAH. Previously, assessment of the prevalence of HPAI A(H5N1) based on poultry outbreak data alone was problematic due to the potential bias and unreliability of reporting systems. Passive detection of poultry outbreaks did not allow adequate assessment of asymptomatic “silent” infections, which likely play a role in the ecology of endemic

transmission cycles. Surveys conducted in 2009 and 2010 established the feasibility and utility of routine sampling in LBMs (Minh *et al.*, 2012) and recommendations were subsequently made to establish a nation-wide program for active market-based surveillance to complement passive reporting of poultry outbreaks.

In late 2011, the national LBM active surveillance program was initiated with the goal of providing data for risk assessment and evidenced-based allocation of resources for disease control measures. Here we present results of the first 15 months of the LBM surveillance program and discuss the relevance of the findings for poultry outbreak risk assessment and poultry vaccination strategies.

2.2. Methodologies

Sample collection

The national surveillance program for AI was developed by the DAH using a stratified, multi-stage cluster sampling design (DAH, 2011). The first phase of the program was conducted in 30 provinces over a period of six months from September 2011 to February 2012. The second phase was conducted from October 2012 to June 2013 (nine months) and included an additional 14 provinces for a total of 44 (out of 63 provinces). Criteria used to select provinces included historical HPAI outbreak data, previous active surveillance results, census data on poultry density, and consideration of cross-border trade in poultry. Within each province, the sub-Department of Animal Health (sDAH) was requested to identify one small-scale LBM in each of 3-4 districts. Small-scale LBMs are markets that receive birds from the district and/or province and therefore capture/represent the local circulation of HPAI A(H5N1). Markets were required to have at least six duck vendors to simplify sample collection. In addition, large-scale LBM were selected for inclusion from

the 20 cities in Vietnam with the largest human population. Large-scale LBM are markets that receive birds from outside the province and therefore capture/represent the national and/or regional circulation of HPAI A(H5N1), i.e. large catchment areas. The first surveillance period involved 272 LBMs located in 263 communes of 127 districts belonging to 30 provinces; the second period involved 184 LBMs located in 169 communes of 144 districts of 44 provinces.

On each monthly sampling visit, during the first surveillance period, staff from local District Veterinary Services (DVS) sampled a total of 20 ducks randomly. Sample collections were increased during the second period of surveillance to 30 ducks per LBM per month. The sample size was calculated based on logistics and feasibility, and it was estimated to detect at least one positive A(H5) sample in a given LBM if the prevalence of A(H5) was 10% with 95% confidence interval (CI) and an infinite population. Ducks (broiler or layer ducks) were primarily targeted for sampling, but spent hens were also sampled if an insufficient number of ducks ($n = 20$) were available in the market at the time of sampling. DVS staff collected one OP swab per bird and then pooled 5 individual OP swabs into a single tube with 2 mL viral transport medium (PBS pH 7.2 supplemented with antibiotics, gentamicin, and buffering agents). Samples were kept in a cold box with wet ice and transported to the designated RAHOs or the NCVD within 48 hours of sampling. Samples were stored in -80 °C freezers until further processing.

DAH provided a standardised collection form for sample tracking and to obtain information about spatial data (names and location of LBMs, communes, districts, provinces), sampling date, dates of sample submission to the assigned laboratories, and information about species, breed, age, and origin of poultry. However, data on poultry

breeds, ages, origin of poultry, and poultry seller identifier were not always recorded completely for all samples.

Influenza A typing and subtyping

Pooled swab samples were extracted manually using the Qiagen Viral RNA (Qiagen, Hilden, Germany) kits and then screened by rRT-PCR to detect the influenza A virus Matrix (M) gene (MARD, 2005). All M gene positive samples were subsequently tested using A(H5) HA primer/probe pairs (Appendix 1). All A(H5) positive samples were then tested for the N1 neuraminidase. Each RAHO used the same primer/probe sets designed and validated internally at NCVD (the national reference laboratory for avian influenza virus in Vietnam), and positive controls, virus transport media (VTM) and sampling consumables were obtained through the national laboratory network. Primer/probe sets for the A(H5) HA remained unchanged over the surveillance period and are listed in Appendix 1. The threshold cycle used to determine a positive sample was $Ct \leq 35$ for M gene, A(H5) and N1 rRT-PCR assays. The use of this cut-off for rRT-PCR positivity has been developed in consultation with the resident FAO laboratory expert, for use in standardising reporting systems within the LabEpiNet. The Ct-values of influenza A virus infected birds in the surveillance studies typically range from 15 to 40, and a direct relationship between viral RNA (i.e. Ct-values) and the ability to isolate virus in eggs has been observed over the years. In general, samples with Ct-values less than 25 yield the highest rate of isolation, and virus isolation is not attempted if $Ct > 35$ due to low expected yield and resource limitations. For practical reasons it is desirable to set a cut-off Ct value for positivity that can be applied through the national laboratory network, despite the fact that each laboratory may have slightly variable sensitivity thresholds in practice, and sensitivity thresholds may vary when primers are updated. Limit of detection experiments will also be

performed in the future to establish cut-off values that reflect sensitivity of the rRT-PCR assay with positive control vRNA. The rRT-PCR screening results were reported to the DAH Epidemiology Division and original specimen material and RNA extractions of all positive samples were subsequently transferred to NCVD for further analysis.

Prevalence of influenza type A, A(H5) and A(H5N1) virus infection was estimated from the pooled samples at the market, commune, district and province levels. The prevalence was defined as the number of positive pooled samples divided by the total number of pooled tested at each level. A commune or district or province was considered positive if it had at least one positive pool for influenza type A, A(H5) and A(H5N1) virus. Standard errors were calculated using the epiR package (Stevenson, 2013) implemented within R (Team, 2013). Chi-square tests were performed for all comparisons, the formula as follows:

$$\chi_c^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

The subscript “c” are the degrees of freedom. “O” is the observed value and E is the expected value. 95% confidence intervals were performed for all proportions. Maps were generated using a commercial ArcGIS 9.3. The Epidemiology Division of the DAH provided data on reported HPAI outbreaks collected at the individual commune level since September 2011. A poultry outbreak of HPAI in Vietnam was defined based on the presence of characteristic clinical signs among farm flocks and positive rRT-PCR laboratory confirmation (DAH, 2012; Long et al., 2013).

Virus isolation

Virus isolation was attempted at NCVD on all pooled swab samples that were positive with influenza type A and/or A(H5) virus based on the rRT-PCR screening results from the

RAHOs. Samples were inoculated into the allantoic cavity of 9-day old embryonated chicken eggs (2 eggs per pool), and harvested after 72 hours. Eggs were provided by a commercial hatchery that was certified as influenza virus free since 2003. Allantoic fluids were clarified by centrifugation at 10,000 rpm for 10 minutes at 4 °C, and presence of virus was determined by HA assay as previously described using 1:2 dilutions and chicken erythrocytes (OIE, 2009). Positive samples were titrated by HA serial dilution, RNA extractions were performed, followed by confirmatory M gene rRT-PCR testing. For isolates with a Ct value of less than 25, partial haemagglutinin (primarily the HA1 region) sequencing of a 900bp region was performed. Bidirectional Sanger sequencing reactions were completed at Macrogen, Inc. (Seoul, Republic of Korea). Sequence editing was performed in DNA baser 3.11.

Phylogenetic analysis

Data used for analyses comprised the A(H5) HA sequences from Vietnamese poultry isolates as well as additional publicly available sequences identified by BLAST analysis of the GISAID database (<http://platform.gisaid.org>). The full dataset was aligned using the MUSCLE algorithm. Neighbour-joining methods with the Kimura 2-parameter substitution model were implemented in MEGA5 (www.megasoftware.net) and bootstrap analysis were conducted with 1000 replications. Trees were either rooted to A/goose/Guangdong/1/1996 or were mid-point rooted. HA clades were defined using the criteria described by WHO/OIE/FAO A(H5N1) Evolution Working Groups (WHO, 2008; WHO/OIE/FAO, 2012; WHO, 2014a) and gene lineage nomenclature previously used for analysis of A(H5N1) virus genotypes from Vietnam (Tung *et al.*, 2012).

2.3. Results

2.3.1. Prevalence of influenza type A and A(H5) viruses

A total of 9,790 pooled OP swabs were collected and processed by regional laboratories, of which 2,162 were influenza virus A positive (22.1%, 95% CI 21.3 – 22.9) (Table 2.1).

Among LBMs, overall prevalence of influenza type A virus was 75.4% (95% CI 70.8 - 79.6) for the entire surveillance period (Table 2.2). Influenza A viruses were detected in all 44 provinces surveyed (Table 2.1). At the commune level, the prevalence of influenza type A virus was relatively high, 68.1% (95% CI 62.1 - 73.6) for the first surveillance period (from September 2011 to February 2012) and 86.9% (95% CI 80.9 - 91.7) for the second surveillance period (from October 2012 to June 2013).

Overall, A(H5) viral RNA was detected in 531 of 9,790 OP swab pools (5.4%, 95% CI 4.9-5.8) (Table 2.1) and in 127/394 of LBMs (32.2%, 127/394) (Table 2.2). The proportion of A(H5) positive LBMs was 17.3% (95% CI 12.9 – 22.3) during the first surveillance period (6 months duration), and increased to 49.5% (95% CI 42.0 – 56.9) during the second period (9 months duration). The neuraminidase N1 subtype could not be confirmed in all A(H5) positive samples; A(H5N1) virus was detected in only 387 of 9,790 OP swab pools (3.9%, 95% CI 3.6 – 4.4), and in 104 of 394 LBMs (26.4%, 95% CI 22.1-31.0) (Table 2.2). These values corresponded to a LBM A(H5N1) prevalence of 13.2% during the first surveillance period, and 41.3% in the second period.

Examining LBM prevalence versus outbreaks on the commune level, the data indicated that 17.5% (46/263) of communes were positive for A(H5), and of these, 39.1% (18/46) reported HPAI poultry outbreaks during the first 6 months of surveillance (Table 2.2). For

the second round of 9 months of surveillance, 51.5% (87/169) of surveyed communes were A(H5) positive, and 13.8% (12/87) reported HPAI poultry outbreaks.

The geographic distribution of market prevalence for influenza A and A(H5) virus for the two surveillance periods is shown in Figure 2.1. The highest detection rates of influenza A virus were found in markets in central coastal provinces such as Da Nang (95.2% positive, CI 83.8 – 99.4) and Ba Ria Vung Tau (77.1%, CI 62.7 – 98.0), however these provinces did not consistently rank highest in terms of A(H5) detections. Provinces where A(H5) was detected in more than 10% of all influenza type A positive pools were Quang Ninh, Da Nang, Binh Dinh, Khanh Hoa, Dak Lak, Ba Ria Vung Tau, Tay Ninh and Ca Mau.

Table 2.1: Summary of molecular diagnostic screening results for influenza A (M gene), A(H5) and A(H5N1) at the individual pool level, stratified by surveillance period and species origin

	Influenza A			A(H5)			A(H5N1)		
	No. tested	No. positive	% positive (95% CI)	No. tested	No. positive	% positive (95% CI)	No. tested	No. positive	% positive (95% CI)
Period									
Sept 2011 - Feb 2012	3876	730	18.8 (17.6-20.1)	730	105	2.7 (2.22-3.27)	105	82	2.12 (1.7-2.6)
Oct 2012 - June 2013	5914	1432	24.2 (23.1-25.3)	1432	426	7.2 (6.6-7.9)	426	305	5.2 (4.6-5.7)
Species									
Duck	9732	2159	22.2 (21.4-23.0)	9732	516	5.3 (4.9-5.8)	516	386	3.9 (3.6-4.4)
Muscovy Duck	35	2	5.7 (0.7-19.2)	35	1	2.9 (0.1-17.9)	1	1	2.9 (0.1-17.9)
Chicken	23	1	4.3 (0.1-21.9)	23	0	0.0 (0.0-21.1)	0	0	0.0 (0.0-21.1)
Total	9790	2162	22.1 (21.3-22.9)	2162	531	5.4 (4.9-5.8)	5312	387	3.9 (3.6-4.4)

¹ Confidence interval = CI

² Species not certain for 15 samples due to labelling error.

Table 2.2: Prevalence of influenza A, A(H5) and A(H5N1) viruses for period 1 (September 2011-February 2012) and period 2 (October 2012-June 2013) at the live bird market, commune, district and provincial levels.

June 2013) at the live bird market, commune, district and provincial levels.									
Level	Total units	Influenza A		A(H5)		A(H5N1)		Poultry outbreaks	
		No. positive	% positive (95% CI)	No. positive	% positive (95% CI)	No. positive	% positive (95% CI)	Reported HPAI outbreaks	% Outbreak (95% CI)
Period 1: September 2011 to February 2012									
Province	30	29	96.7 (82.8-99.9)	17	56.6 (37.4-74.5)	15	50.0 (31.3-68.7)	11	40.0 (22.6-59.4)
District	127	108	85.0 (77.6-90.7)	33	25.9 (18.6-34.5)	26	20.5 (13.8-28.5)	15	16.5 (10.5-24.2)
Commune	263	179	68.0 (62.1-73.6)	46	17.5 (13.1-22.6)	36	13.7 (9.8-18.4)	18	9.1 (5.9-13.3)
Market	272	187	68.7 (62.8-74.2)	47	17.3 (12.9-22.3)	36	13.2 (9.4-17.8)	n/a	n/a
Period 2: From October 2012 - June 2013									
Province	44	44	100.0 (88.2-100.0)	35	79.5 (64.7-90.2)	31	70.4 (54.8-83.2)	9	20.4 (9.8-35.3)
District	144	135	93.7 (88.5-97.1)	81	56.2 (47.7-64.5)	68	47.2 (38.8-55.7)	12	8.3 (4.4-14.1)
Commune	169	147	86.9 (80.9-91.6)	87	51.5 (43.7-59.2)	73	43.2 (35.6-51.0)	12	7.10 (3.7-12.1)
Market	184	152	82.6 (76.3-87.8)	91	49.5 (42.0-56.9)	76	41.3 (34.1-48.8)	n/a	n/a
Total for entire surveillance period									
Province	44	44	100.0 (88.2-100.0)	36	81.8 (67.3-91.8)	32	72.7 (57.2-85.0)	12	27.3 (14.9-42.8)
District	214	195	91.1 (86.5-94.6)	102	47.6 (40.8-54.6)	84	39.2 (32.7-46.1)	21	9.8 (6.2-14.6)
Commune	372	286	76.9 (72.3-81.1)	124	33.3 (28.6-38.4)	102	27.4 (22.9-32.3)	24	6.4 (4.2-9.4)
Market	394	297	75.4 (70.8-79.6)	127	32.2 (27.6-37.1)	104	26.4 (22.1-31.0)	n/a	n/a

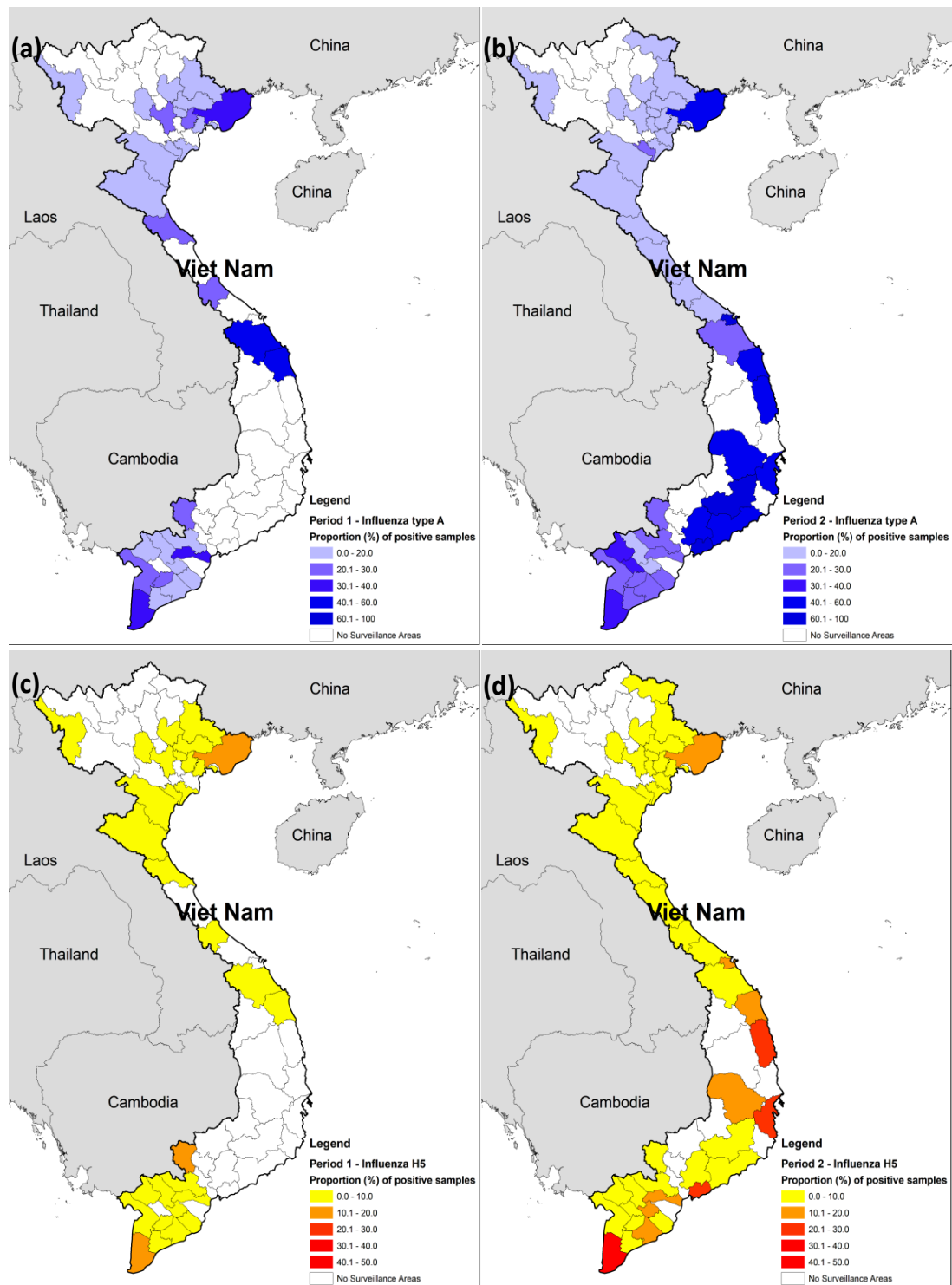


Figure 2.1: Spatial distribution of influenza A viruses detected in LBMs. Figure 2.1a and Figure 2.1c for Influenza A and A(H5) from period 1 (September 2011–February 2012), respectively; Figure 2.1b and Figure 2.1d for Influenza A and A(H5) from period 2 (October 2012–June 2013).

The temporal distribution of A(H5N1) virus detected from LBMs in relation to the numbers of reported HPAI poultry outbreaks (number of infected communes) is shown in Figure 2.2. Note this analysis was performed using A(H5N1) positives [not all A(H5)], because 100% of all suspected HPAI outbreaks were confirmed A(H5N1) positive. From LBM, A(H5N1) was consistently detected during all sampling months, with the highest detection levels observed during February-March 2013. In contrast, the period with the highest number of reported HPAI outbreaks coincided with the hiatus of surveillance activities from February to November 2012. This included a notable peak in outbreaks during September 2012. Despite the apparent increase in A(H5) detection in markets during the nine months of surveillance from October 2012 to June 2013, HPAI outbreaks during this period remained constant.

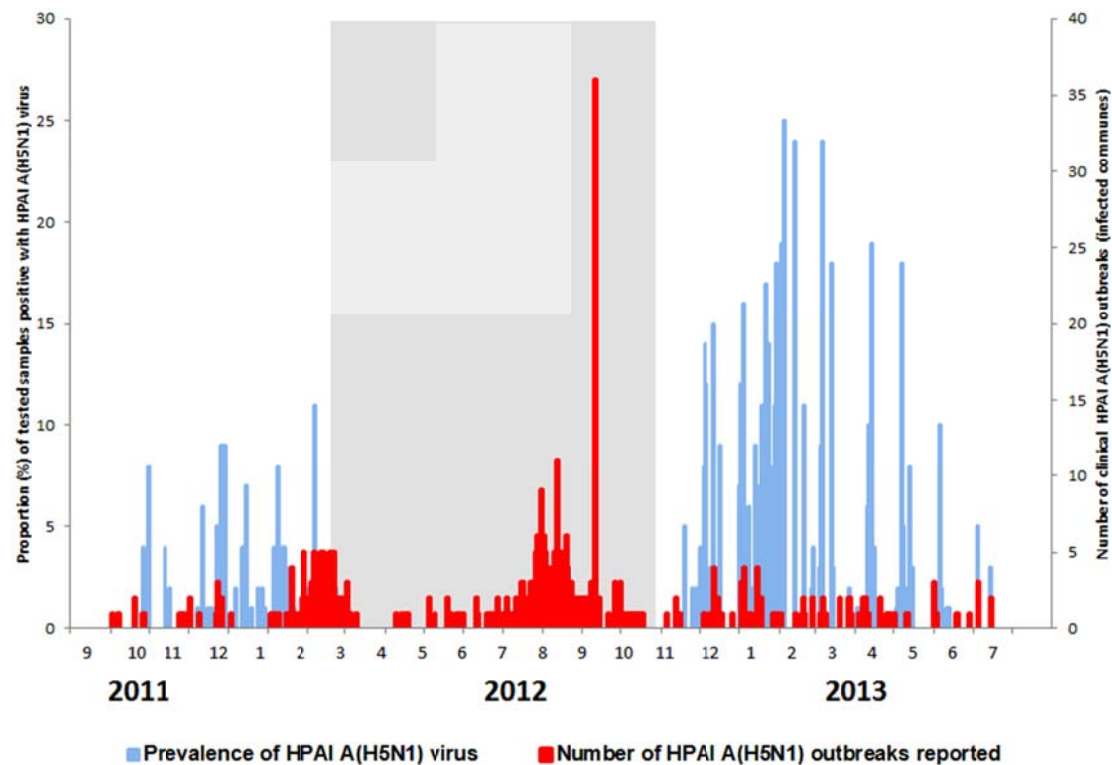


Figure 2.2: Temporal distribution of A(H5N1) virus detected from LBMs surveillance and A(H5N1) poultry outbreaks from September 2011 to June 2013. Shaded area indicates lack of surveillance activities, from February to November 2012.

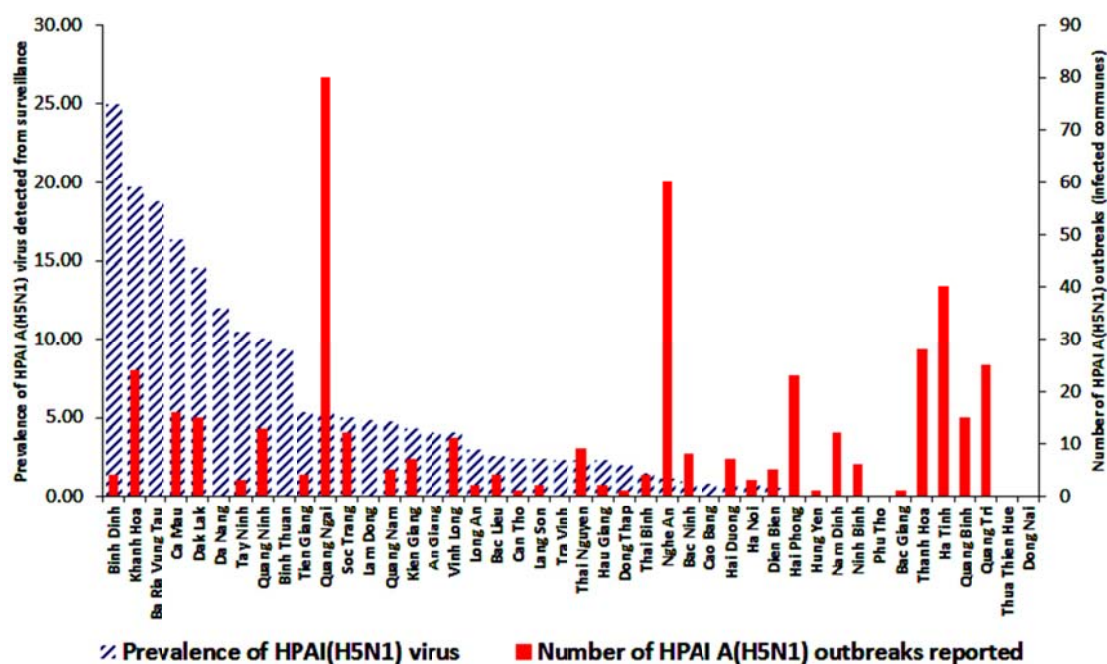


Figure 2.3: LBMs prevalence of A(H5N1) virus estimated at the provincial level (hatched bars), in descending order. Number of A(H5N1) poultry outbreaks reported per surveillance province (red bars).

Market prevalence of A(H5N1) virus detection by province, ranked from highest to lowest, and the corresponding numbers of reported HPAI outbreaks are shown in Figure 2.3. The data reveals poor correlation between viruses detected in markets and reported outbreaks. Provinces with the highest number of reported HPAI outbreaks (Quang Ngai, Nghe An, Ha Tinh, Thanh Hoa, Quang Tri) did not have the highest frequency of A(H5) detected in markets, and other provinces with relatively high detection rates of A(H5) in markets (e.g., Ba Ria Vung Tau, Da Nang and Binh Thuan) did not report any HPAI outbreaks.

2.3.2. Identification of clade variants

Of the 2,162 influenza A virus positive OP pools, a total of 1,669 samples (with Ct < 35, sufficient volume, complete metadata and proper sample tracking and storage quality) were tested for virus isolation in embryonated chicken eggs. Two hundred forty-three (243) virus isolates were generated (243/1,669, 14.6%), with 231 M gene confirmations. Two

hundred eight (208) of these yielded an HA amplicon of the correct size and 194 A(H5) HA sequences were generated (ranging in length from approximately 900-1200 nucleotides of the HA gene). Phylogenetic analyses of the partial HA gene sequences identified the presence of viruses from six previously designated clades: clade 2.3.2.1a (44 isolates), clade 2.3.2.1b (3 isolates), clade 2.3.2.1c (90 isolates), clade 1.1.1 (12 isolates), clade 1.1.2 (32 isolates) and clade 7.2 (13 isolates). Clade 7.2 viruses were found exclusively in 13 samples from spent hens collected from Hanoi markets in February 2013. Based on phylogenetic divergence from progenitor viruses (marked by an increase of 0.02 nucleotide substitutions per site), distinct sub-clades of clade 1.1 emerged in 2012 (1.1.1) and in 2013 (1.1.2) (Sorn et al., 2013). Figure 2.4 shows the A(H5) HA phylogeny of a representative subset of the novel LBM sequences ($n = 63$ of 193) with additional sequences from global datasets ($n = 36$).

Numerous HA1 protein amino acid differences were identified in these viruses relative to viruses used in Vietnam as antigen for poultry vaccines. Clade 1.1 viruses had 8 conserved amino acid substitutions relative to A/Vietnam/1194/2004 with four identified in predicted antigenic sites (Figure 2.3). Clade 2.3.2.1b viruses, which belong to the same clade as the vaccine virus, A/duck/Guangdong/S1322/2010 (Re-6), had very few conserved changes ($n = 3$) relative to the vaccine strain. In contrast, both clade 2.3.2.1a and 2.3.2.1c viruses had 17 conserved changes in the HA1 region, five of which were found in putative antigenic sites. The clade 7.2 viruses detected during 2013 had more than 40 amino acid differences relative to A/chicken/Shanxi/2/2006 indicating substantial genetic divergence from this vaccine strain (Figure 2.4).

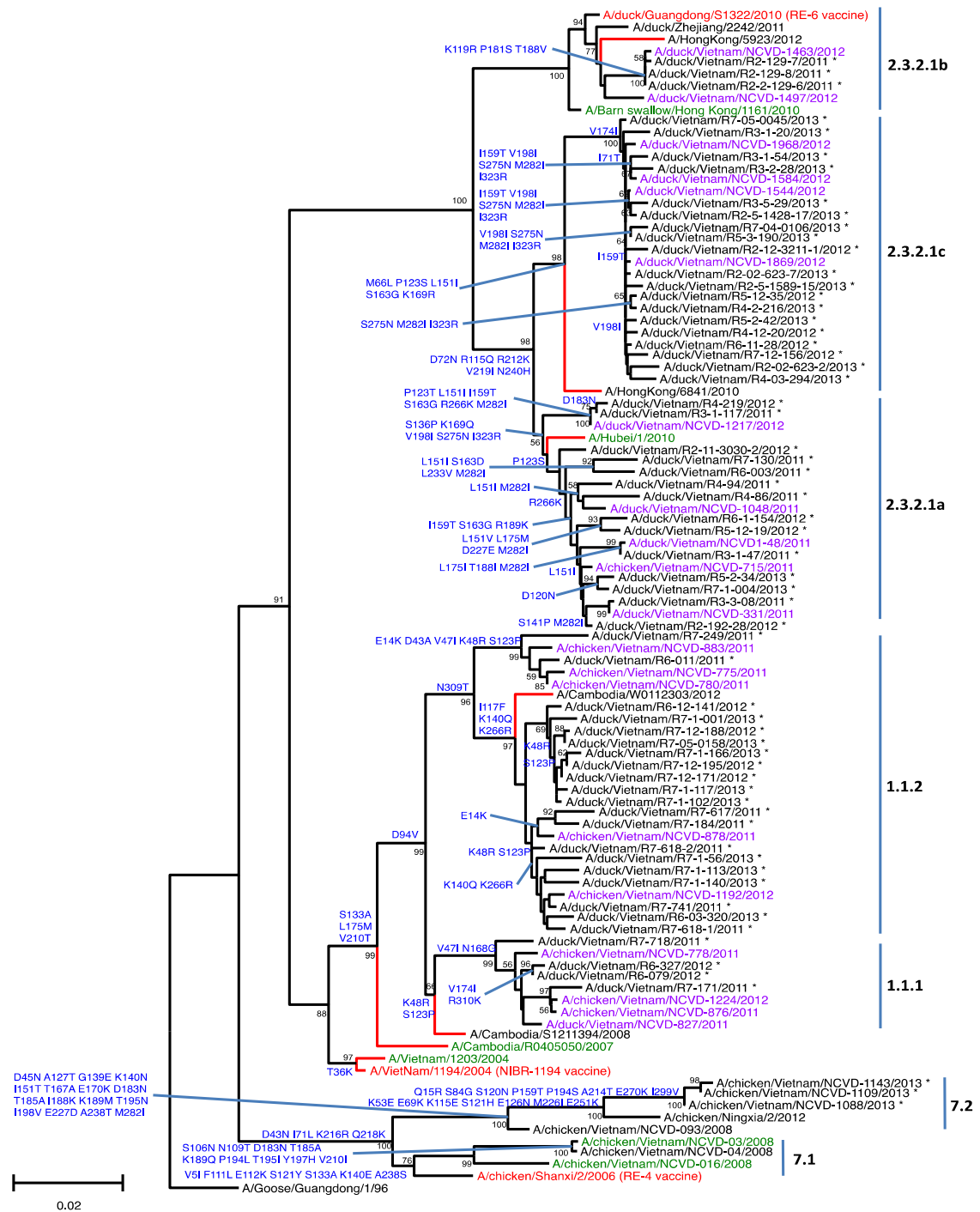


Figure 2.4: Phylogeny of A(H5N1) Haemagglutinin detected in Vietnam from September 2011 to June 2013. LBM viruses are coloured in black with * and outbreak viruses are coloured pink. The poultry vaccine viruses are coloured red. The nearest reassortant WHO candidate vaccine viruses (CVV) for each clade are coloured green. Amino acid differences at branch nodes indicate shared HA1 substitutions relative to the poultry reference strains. Branches on the tree with HA sequences from human cases are coloured red. The tree was constructed by neighbour-joining analysis using MEGA 5.3 with 1000 bootstrap replications. The scale bar represents nucleotide substitutions per site.

2.3.3. Geographic distribution of clade variants

The geographic distribution of A(H5) HA clade variants detected in markets across the country from September 2011 to June 2013 is shown in Figure 2.5. With the exception of a few rare detections of clade 2.3.2.1b and clade 7.2 viruses, the four predominant clades detected over the entire surveillance period were clades 2.3.2.1a, 2.3.2.1c, 1.1.1 and 1.1.2. Importantly, clade 2.3.2.1a was the first clade 2 virus detected in southern provinces; the first detections were in markets in Dong Thap in September 2011, and subsequently in Tay Ninh markets in January 2012. One year later (January 2013, in the week before ‘Tết’ holiday), the first poultry outbreaks caused by clade 2.3.2.1a viruses in the south were reported from Tay Ninh. This clade has continued to circulate in the south, as evidenced by positive detections in Long An, in the last month of surveillance reported here (July 2013). In the north, clade 2.3.2.1a was continuously detected throughout 2011-2013, from both markets and poultry outbreaks in four of the surveyed northern provinces (Hai Duong, Quang Ninh, Thanh Hoa and Thai Nguyen).

The clade 2.3.2.1c viruses were first reported in Vietnam from poultry outbreaks in mid-2012 (Creanga *et al.*, 2013), during the hiatus in market surveillance between the first and second rounds of sampling. Subsequently, starting in November and December 2012, clade 2.3.2.1c became the most frequently detected A(H5) variant, both from markets and poultry outbreaks. The province with the most frequent and consistent detection of clade 2.3.2.1c was Dak Lak, in the central highlands. Clade 1.1 virus was the predominant variant of A(H5) detected in markets and outbreaks from the south until late 2012, when the appearance of clade 2 viruses (2.3.2.1a and 2.3.2.1c) began to increase in frequency. Clade 1.1 viruses have only occasionally been detected from markets in central provinces (Ha Tinh, Quang Nam and Thanh Hoa) in late 2011 and, notably, were also detected in

northern outbreaks (Ha Tinh and Quang Ninh) in late 2011 and early 2012. Clade 7.2 was detected in spent hens in Hanoi during February 2013 (presumably smuggled from southern China), however this clade has never been detected in either market ducks or poultry outbreaks in the country. Finally, clade 2.3.2.1b viruses were detected only 3 times from market samples, in Thai Nguyen during the first months of surveillance (in November, 2011). Since that time, clade 2.3.2.1b viruses have not been detected in Vietnam, although WHO reports indicate they were reported in China in 2013.

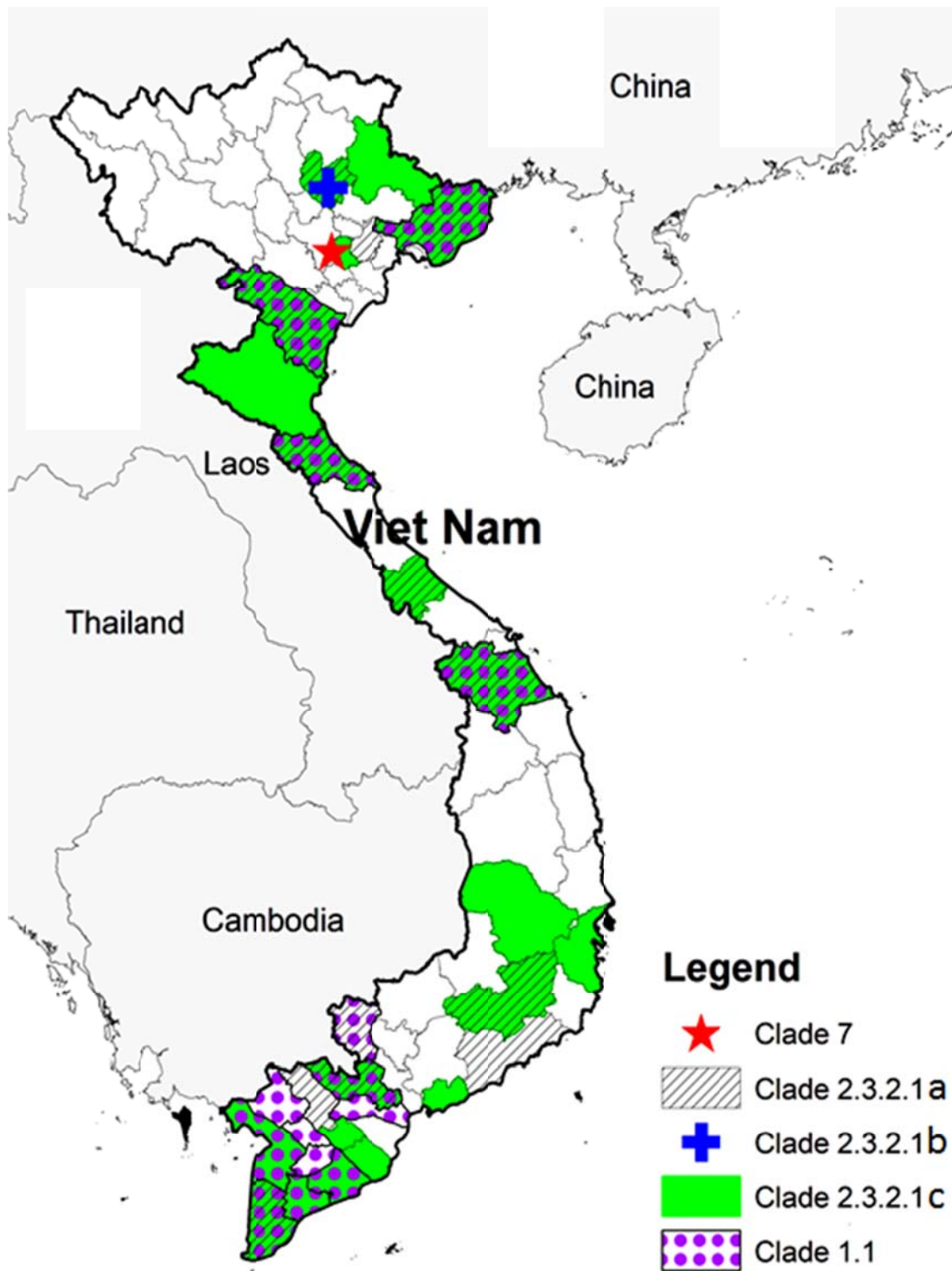


Figure 2.5: Geographic distribution of A(H5N1) virus clades detected from LBMs, September 2011- June 2013.

2.4. Discussion

Epizootics of HPAI in Vietnam continue to pose extraordinary challenges for disease control and intervention, requiring a massive coordination effort by the national infrastructure for veterinary services. The ongoing AI LBM surveillance programme described here complements the outbreak response activities by providing systematic monitoring at the commune, district and provincial levels. As such, the prevalence and geographic distribution of influenza A virus and A(H5N1) subtype variants that circulate silently within the domestic duck population of the country can be measured and assessed. The data is consistent with previous observations that the two major river deltas (Mekong and Red River) are important foci of endemic transmission, however a number of valuable new observations have arisen. Firstly, higher overall prevalence of both influenza A and A(H5) viruses was detected from markets of the central provinces in comparison to the two deltas. Secondly, the incursion of clade 2 viruses (both clade 2.3.2.1a and 2.3.2.1c) into southern Vietnam, as well as the occasional northward spread of clade 1.1 into central coastal provinces as far north as Ha Tinh, indicates that the transmission ecology of northern and southern Vietnam forms a continuum and are more highly linked than previously thought. Indeed, from 2003 to 2010, the pattern of clade circulation in northern Vietnam was characterised by periodic incursion and lineage replacement of new strains (primarily clade 2.3.4 variants) from southern China, whereas in the Mekong Delta, virus circulation was limited to persistent endemic transmission of clade 1 viruses. Clade 2.3.2.1 A(H5N1) viruses were likely re-introduced into Vietnam via poultry trade or wild bird migration as this genetic group spread widely in many Asian and some European countries (e.g., Bangladesh, Bulgaria, China, Korea, Japan, Mongolia, Romania, Russia) (Gilbert *et al.*, 2012; Islam *et al.*, 2012; Marinova-Petkova *et al.*, 2012; Nagarajan *et al.*, 2012; Sakoda *et al.*, 2012; Uchida *et al.*, 2012; Choi *et al.*, 2013; Fan *et al.*, 2015; Naguib *et al.*, 2015;

Jiang *et al.*, 2017). The rapid dispersal of clade 2.3.2.1 variants (2.3.2.1a and 2.3.2.1c) to southern latitudes during 2012-2013 may reflect increased intensity of poultry trade along the north-south axis, the leakiness of transport barriers for infected or non-vaccinated flocks, and/or particularly high transmissibility of clade 2.3.2.1 variants in poultry.

The co-circulation of both clade 2.3.2.1a and 2.3.2.1c variants together with clade 1.1 has important implications for strategic planning of poultry vaccination programs. These phylogenetically divergent A(H5) lineages are not cross-reactive antigenically (Dau *et al.*, 2013; Xu *et al.*, 2013), and experimental challenge studies indicate that none of the currently commercially available A(H5N1) poultry vaccines provide adequate protection against both the clade 2.3.2.1 and clade 1.1 viruses (Cha *et al.*, 2013; Dau *et al.*, 2013).

Starting in 2011, following the emergence of the clade 2.3.2.1a and b variants, the Vietnamese government requested different vaccine formulations for use in different regions of the country (DAH, 2011): the inactivated oil-emulsion adjuvant Re-5 vaccine (a reverse genetic (rg) engineered reassortant with the clade 2.3.4 A/Anhui/1/2005 HA and NA), Re-6 vaccine (rg with clade 2.3.2.1b A/duck/Guangdong/S1322/2010 HA and NA), and the Vietnamese Navet Vifluvac vaccine (NIBR-1194 rg virus composed of clade 1.1 A/Vietnam/1194/2004 HA and NA). All have been used to some extent in various provinces since mid-2012. However, given that these variants are now co-circulating within the same regions, and given the poorly immunogenic cross-reactivity profiles, it would seem that bivalent or multivalent vaccine formulations will be required to achieve adequate vaccine efficacy. In addition, HA1 sequence data from viruses collected during this market surveillance revealed substantial genetic drift among viruses in each of these clades compared to these vaccine strains. Together with the identification of many of these substitutions in previously described antigenic sites, these findings suggest the likelihood

of significant antigenic drift between circulating strains and vaccine viruses. This is supported by lack of effective protection from currently in-use vaccines demonstrated in recent field studies (L. V Nguyen et al., 2013). The relationship between antigenic matching of field variants and vaccines and its influence on vaccine efficacy are exceedingly complex, and may be complicated by different immunologic responses in avian species or breeds (e.g., commercial vs village chickens, Peking ducks, Muscovy ducks, geese, and spent hens). Further research targeting development of appropriately cross-protective and highly efficacious A(H5N1) poultry vaccines is urgently needed to facilitate the continued use of vaccination as part of integrated programs of HPAI control.

Another key observation of the market surveillance program was the lack of correlation between observed market prevalence and provincial reporting of HPAI poultry outbreaks. This lack of correlation may be explained by several factors. Firstly, the outbreak data by definition reflects notifications of clinically apparent disease in farm flocks, including all domestic poultry species that are known to vary in their pathogenic response to infection, whereas the market data likely reflects silent circulation in domestic ducks. Secondly, the number of HPAI poultry outbreaks officially reported to DAH likely does not comprise all outbreaks; this is because the reliability of disease reporting varies across provinces, and among districts within provinces, depending on the infrastructure and local capacity of veterinary services at the communal level. Previous surveillance studies in the Mekong river delta have documented that poultry flocks often test positive with A(H5N1) virus for prolonged periods without any apparent outbreaks, even when these flocks comprise a mix of field running ducks and in-contact chickens (L. V Nguyen et al., 2013). Despite the lower pathogenicity of A(H5N1) in ducks relative to chickens, more than 70% of the reported A(H5N1) poultry outbreaks between 2012 and 2013 occurred in duck populations,

and only 30% in chickens (DAH, 2013a). Finally, ongoing A(H5N1) vaccination practice in certain provinces likely influences both the observed prevalence in market ducks, as well as the occurrence of HPAI outbreaks. Although the market data certainly provides an extraordinary resource for initial assessments of transmission intensity, there is a clear need for more focused research to parameterise the principle factors that drive transmission dynamics, in order to use the market data for evidenced-based risk assessments.

Although the surveillance output provides initial evidence of increasing prevalence and geographical expansion of HPAI transmission zones, a robust and systematic analysis of these trends is complicated due to inherent limitations of the system, the lack of continuity in the surveillance system over time, and possible ascertainment bias due to continued evolution of the surveillance infrastructure. For instance, implementation of the two rounds of surveillance were not comparable (e.g., modifications to the selection of provinces and districts for inclusion), and individual provinces clearly made improvements over time in the efficiency of sample collection, sample tracking, and transport logistics, as evidenced by variations in PCR detection rates, and rates of virus isolation success from samples originating from different RAHOs (data not shown). These sources of variability suggest that the program still suffers from technical difficulties, with the need for improved quality control procedures and standardisation among the participating laboratories. Additional technical issues regarding the relative sensitivities of the molecular screening assays used for influenza A matrix gene, HA A(H5), and NA N1 also complicate the analysis of trends and interpretation of prevalence data; indeed, we postulate that reduced sensitivity of the N1 rRT-PCR assay in comparison to the other two assays may explain the inability to confirm N1 subtype in 144 of the 531 A(H5) positive pools (27%). This interpretation is further supported by the fact that to date, all HPAI outbreak samples have been confirmed

A(H5N1), with no evidence of NA reassortment events among samples from clinically diseased animals. Vigilant monitoring of HA and NA sequence evolution is required to adapt the molecular diagnostic tools used for routine screening, and these efforts are supported by HA sequencing all egg-grown isolates. Finally, the roster of provinces and exact markets for inclusion within the surveillance program will necessarily require periodic updates, as the epizootic situation and the poultry trade sector continues to evolve; for instance, in the first five months of 2014, outbreaks have now been reported from 3 additional provinces (Kon Tum, Phu Yen, and Lao Cai) that were previously free of disease but had not yet participated in the program.

In summary, the market surveillance data confirms the widespread circulation of influenza A and A(H5) within domestic duck populations of Vietnam, and provides evidence for co-circulation of clades 2.3.2.1a, 2.3.2.1c and clade 1.1 subgroups throughout the Mekong Delta provinces, and as far north as Ha Tinh province. As the market data continues to accumulate, it will no doubt provide a substantial resource to complement the notification system for reporting of HPAI poultry outbreaks, and a rich source of new influenza virus isolates for further molecular epidemiological investigations. Whole genome sequencing and in-depth phylodynamic and ecological analysis of the existing datasets are ongoing, as well as analysis of viral diversity among other circulating influenza A subtypes.

Genetic diversity, reassortment and emergence of new subtypes and genotypes of highly pathogenic avian influenza A(H5) viruses collected in Vietnamese poultry from 2012 to 2015

Abstract

Whole genome sequences of representative highly pathogenic avian influenza A(H5) viruses from Vietnam were generated, comprising samples from poultry outbreaks and active market surveillance collected from January 2012 to August 2015. Six HA gene clades were characterised. Clade 1.1.2 was predominant in southern Mekong provinces throughout 2012 and 2013, but gradually disappeared and was not detected after April 2014. Clade 2.3.2.1c viruses spread rapidly during 2012 and were detected in the south and centre of the country. A number of clade 1.1.2 and 2.3.2.1c inter-clade reassortant viruses were detected with different combinations of internal genes derived from 2.3.2.1a and 2.3.2.1b viruses indicating extensive co-circulation. Although reassortment generated genetic diversity at the genotype level, there was relatively little genetic drift within the individual gene segments suggesting genetic stasis over recent years. Antigenically, clade 1.1.2, 2.3.2.1a, 2.3.2.1b, 2.3.2.1c viruses remained related to earlier viruses and WHO recommended pre-pandemic vaccine strains representing these clades. Clade 7.2 viruses, although only detected in small numbers, were the exception as indicated by introduction of a genetically and antigenically diverse strain in 2013. Clade 2.3.4.4 viruses [A(H5N1) and A(H5N6)] were likely introduced in April 2014 and appeared to gain dominance across northern and central regions. Antigenic analyses of clade 2.3.4.4 viruses compared to existing clade 2.3.4 CVVs indicated the need for an updated vaccine virus. A/Sichuan/26221/2014 [A(H5N6)], was developed and ferret antisera generated against this virus was demonstrated to inhibit some but not all clade 2.3.4.4 viruses suggesting consideration of alternative clade 2.3.4.4 CVVs.

Importance

HPAI A(H5) viruses have circulated continuously in Vietnam since 2003 resulting in hundreds of poultry outbreaks and sporadic human infections. Despite significant reduction in the number of

human infections in recent years, poultry outbreaks continue to occur and the virus continues to diversify. Vaccination of poultry has been used as a means to control spread and impact of the virus but due to the diversity and changing distribution of antigenically distinct viruses, the utility of vaccines in the face of mismatched circulating strains remains questionable. This study assesses the putative amino acid changes in viruses leading to antigenic variability, underscoring the complexity of vaccine selection for both veterinary and public health purposes. Given the overlapping geographic distribution of multiple, antigenically distinct clades of HPAI A(H5) viruses in Vietnam, the vaccine efficacy of bivalent poultry vaccine formulations should be tested in the future.

Diep T. Nguyen, Yunho Jang, Tho D. Nguyen, Joyce Jones, Samuel S. Shepard, Hua Yang, Nancy Gerloff, Thomas Fabrizio, Long V. Nguyen, Ken Inui, Genyan Yang, Adrian Creanga, Li Wang, Duong T. Mai, Sharmi Thor, James Stevens, Thanh L. To, David E. Wentworth, Tung Nguyen, Dong V. Pham, Juliet E. Bryant, C. Todd Davis. Shifting clade distribution, reassortment and emergence of new subtypes of highly pathogenic avian influenza A (H5) viruses collected in Vietnamese poultry from 2012 to 2015 (2016). **Journal of Virology**: 21 December 2016, doi:10.1128/JVI.01708-16.

3.1. Introduction

Measures to control the 2003 epizootics of HPAI A(H5N1) in Vietnam included stamping-out of infected and neighbouring flocks and restriction of poultry movements. Vaccination of poultry also became a major component of the disease control strategy since September 2005. Despite these efforts to control HPAI A(H5N1) in Vietnam, eradication has not been achieved likely due in part to the multiple introductions and spread of viruses with distinct genetic and antigenic properties from neighbouring countries. Phylogenetic analyses of whole genome sequences of A(H5N1) viruses isolated from poultry outbreaks in Vietnam have identified a total of 48 genotypes and circulation of at least 15 distinct HA clades [1, 1.1, 1.1.1, 1.1.2, 2.3.4, 2.3.4.1, 2.3.4.2, 2.3.4.3, 2.3.2.1a, b and c, 3, 5, 7.1, 7.2] (Wan *et al.*, 2008; Davis *et al.*, 2010; Tung *et al.*, 2012; Creanga *et al.*, 2013; Dau *et al.*, 2013). Until recently, the geographic distribution of A(H5) virus clades across Vietnam suggested non-overlapping transmission zones; virus descendants from clade 2.3.4 lineages were detected exclusively in the north, with frequent lineage replacement via introduction of new viruses from bordering countries, whereas clade 1 lineages circulated exclusively in the Mekong River Delta and neighbouring Cambodia (Wan *et al.*, 2008). In addition, clade 7 virus was occasionally detected in illegally imported poultry from China and then transported to LBMs located in northern Vietnam (Nguyen *et al.*, 2009b; Davis *et al.*, 2010; Nguyen *et al.*, 2014).

Emergence of clade 2.3.2.1 and three clusters within this group [i.e., A/Hubei/1/2010-like (Hubei-like, 2.3.2.1a), A/barn swallow/Hong Kong/1161/2010-like (BS-like, 2.3.2.1b) and A/Hong Kong/6841/2010-like (HK-like, 2.3.2.1c)] were reported in Vietnam in 2009 and continued to cause outbreaks through 2012 (Tung *et al.*, 2012). Significant antigenic diversity between these viruses suggested possible vaccine ‘breakthrough’. Evidence of

suboptimal protection of poultry vaccinated with Re-1, which has HA and NA genes derived from A/goose/Guangdong/1/1996 (clade 0), Navet Vifluvac, which has HA and NA genes derived from A/Vietnam/1194/2004 (clade 1), and Re-5, which has HA and NA genes derived from A/duck/Anhui/1/2006 [clade 2.3.4 A(H5N1) virus], led to a temporary suspension of the national avian influenza vaccination program in mid-2012 (Cha *et al.*, 2013). In addition, during 2012 and 2013 clade 2.3.2.1 viruses were detected in an expanding range, which accompanied the disappearance of the previously dominant clade 2.3.4 viruses in northern and central Vietnam (Creanga *et al.*, 2013). Subsequently, the Re-6 vaccine, which has HA and NA genes derived from A/duck/Guangdong/S1322/2010 (clade 2.3.2.1b) was produced in China and approved for use in Vietnam (DAH, 2012). Currently, both Re-5 and Re-6 vaccines are used in poultry (DAH, 2016b).

Outbreaks of HPAI A(H5) virus with high mortality have been sporadically reported in poultry throughout Vietnam. Despite ongoing detection in birds, the number of human case reports has declined over the last several years with no cases detected in 2015 (WHO, 2017). Importantly, the lack of recent human cases of A(H5) viruses suggests that control measures to prevent zoonotic infections are effective. However, both passive surveillance in response to A(H5) poultry outbreaks and active surveillance through monthly sampling in LBMs continue to detect circulation of the virus throughout the country (DAH, 2015).

These surveillance platforms provide essential components for monitoring viral diversity and guiding effective application of disease control interventions. Here we report on the epidemiological situation in Vietnamese poultry and molecular evolution of HPAI A(H5) in Vietnam from 2012 through 2015. Full genome sequence analysis of samples collected across the country, together with antigenic characterisation, demonstrate a shifting distribution and introduction of virus clades and antigenic variants with an increasing

number of reassortant viruses resulting from co-circulation of multiple clades and genotypes. Molecular clock analysis was performed to estimate the timing of these events and to assess variability among the evolutionary rates of the viruses detected. HI assays using panels of ferret antisera raised against previously circulating strains and pre-pandemic candidate vaccine viruses were performed to address the question of antigenic match between newly detected viruses and available vaccines.

3.2. Methodologies

3.2.1. Viruses used in this study

Viruses used in this study included HPAI A(H5) isolates obtained from poultry outbreaks and active surveillance in LBMs derived from national programs implemented by the NCVD and RAHOs between September 2012 and August 2015. Original samples from poultry outbreaks comprised tissue homogenates from poultry carcasses (typically brain or lung specimens from deceased birds). Market samples were pooled OP duck swabs (typically five swabs per pool). Spatial analyses of sample collection locations were conducted in ArcGIS 9.3 (ESRI Inc, 2009) using the Vietnamese national spatial database (Figure 3.1; Appendix 14) (NARENCA, 2012). Screening for detection of influenza A(H5) by rRT-PCR and virus isolation in embryonated eggs was performed as previously described (Nguyen *et al.*, 2014). Representative samples were shipped to the US CDC for virus isolation and/or passaging and further characterisation. The majority of whole genome sequences (WGS) were generated at US CDC by Sanger sequencing (Appendix 2). A subset of 2015 outbreak samples were deep sequenced using Illumina MiSeq in Vietnam with protocols adopted from St Jude Children's Research Hospital (Zaraket *et al.*, 2015). Sequences were submitted to the GenBank sequence database (accession numbers in Appendix 15). Handling of infectious materials at the US CDC was performed in

compliance with biosafety level 3 containment, including enhancements required by the U.S. Department of Agriculture and the Select Agents program (<http://www.cdc.gov/od/ohs/biosfty/bmb15/bmb15toc.htm>).

3.2.2. Molecular analyses

Analysis of A(H5) clade distribution was based on all HA sequences generated during the study period ($n = 214$). HA sequences were aligned via MUSCLE (Edgar, 2004) and trimmed to the mature A(H5) HA coding region using BioEdit v7.0 (Hall, 1999). A maximum likelihood tree was constructed using MEGA 5.05. The tree was rooted to A/goose/Guangdong/1/1996 (Figure 3.2). Amino acid differences on each branch reflect differences compared to the nearest Vietnamese poultry vaccine and underlined mutations indicate previously recognized residues associated with antigenic epitopes, changes in receptor affinity, mammalian adaptation, and increased virulence or transmissibility as identified from automated search tools within the Rainbow software package (CDC, 2014; Rith *et al.*, 2014). Branches with viruses sequenced from human cases are coloured red. Bootstraps greater than 50 generated from 1,000 replicates are shown at branch nodes. The scale bar represents nucleotide substitutions per site. For genotyping, we compiled a comprehensive dataset of all previously available WGS from Vietnam and combined these with the novel WGS generated here. Alignments for each gene segment were created. Additional non-Vietnamese A(H5) sequences were included as outgroups (Cambodian clade 1.1.2; Bangladesh clade 2.3.2.1a; China clade 2.3.4.4 and 7.2; A(H5N8) clade 2.3.4.4 from the US). Neighbour-joining trees for each gene with the Kimura 2-parameter substitution model were implemented in MEGA5 with 1000 bootstrap replications (data not shown). Trees were either rooted to A/goose/Guangdong/1/1996 or were mid-point rooted. HA gene lineages were defined using criteria from the WHO/OIE/FAO A(H5)

Evolution Working Group (WHO/OIE/FAO, 2008; WHO/OIE/FAO, 2012; WHO/OIE/FAO, 2014; WHO/OIE/FAO, 2015) and genotype assignments followed conventions previously established for Vietnamese A(H5) virus characterisation (Figure 3.2) (Wan *et al.*, 2008; Tung *et al.*, 2012; Creanga *et al.*, 2013). Codon complete amino acid alignments of the NA and internal proteins were also used to identify amino acid residues conferring previously described viral phenotypes (Table 3.3) (CDC, 2014).

3.2.3. Evolutionary rates and time of most recent common ancestor

For time to most recent common ancestor (tMRCA) analysis, we prepared a reduced dataset of unique WGS ($n = 140$) that remained representative of geographic regions, dates of collection, and phylogenetic clusters. Dates of collection (day/month/year) were transformed into decimal values. For viruses without precise collection dates, the middle of the collection year was used. Markov chain Monte Carlo (MCMC) runs were performed in BEAST v1.8.1 (Shapiro *et al.*, 2006; Drummond and Rambaut, 2007), with convergence evaluated in Tracer v1.5 after removal of 10% burn-in (Rambaut, 2009). Each analysis was repeated with the lognormal relaxed and exponential relaxed clock models and mean substitution rates with a normal prior distribution until the effective sample size (ESS) of each prior was at least 200. The model with best fit was the SRD06 codon partition model using the general time-reversible (GTR) substitution model for each partition with unlinked frequencies. We used the lognormal relaxed clock (uncorrelated) for all genes except for PB2 (exponential). The tree priors used were coalescent Bayesian Skyline with 10 groups and piecewise constant skyline model with a randomly generated tree as a start. Each MCMC run had a chain length of 50 million and was sampled every 5000 generations. For each tMRCA, a probability interval (Bayesian confidence interval) was estimated as the highest posterior density (HPD 95%) that represented an interval in the

domain of a posterior probability distribution. Maximum clade credibility (MCC) trees were generated in Tree Annotator 1.7.1 (Drummond and Rambaut, 2007) with 10% burn-in removed, posterior probabilities on 0.5, and median heights. Trees were visualised in FigTree version 1.3.1 and posterior probabilities are shown on or above nodes (Rambaut, 2009). Bayesian skyline plots estimating changes in effective population size through time were constructed in Tracer v1.5 for each HA clade and virus gene segment.

3.2.4. Haemagglutination inhibition assay

Antigenic characterisation of HPAI A(H5N1) and A(H5N6) viruses isolated from poultry in Vietnam was performed using the HI assay with polyclonal ferret immune sera produced against representative circulating strains and vaccine candidates. A panel of antiserum raised against representative Vietnamese A(H5N1) viruses and WHO pre-pandemic A(H5N1) vaccine candidates was used to test the antigenic relatedness of circulating viruses collected during this study (WHO, 2014a). HI assays were performed separately for clade 1.1.2, 2.3.2.1c, 7.2, and 2.3.4.4 viruses as previously described using turkey erythrocytes (Balish *et al.*, 2010).

3.2.5. Antigenic cartography construction

The HI results of each dataset (by clades) were formatted for processing and were imported to ACMACS to generate x/y coordinate output files (<https://acmacs-web.antigenic-cartography.org/>). Metadata associated with all reference and test antigens were prepared in Excel spreadsheets with an antigen table (test, antigen ID, strain name, clade, and abbreviation) and antisera table (test, serum ID, serum strain, serum clade, abbreviation, serum type). Antigenic maps were generated from ACMACS output files using Tableau version 9.1 by importing x/y coordinate output files and linking each coordinate with

reference or test antigen metadata (Figure 3.5). HA1 substitutions identified at putative antigenic sites between the consensus sequence of each clade and nearest poultry vaccine virus were displayed on models of the HA trimeric structure using Pymol version 7.2 (Figure 3.4).

3.3. Results

3.3.1. Sample collection, identification and sequencing

Between September 2012 and August 2015, over 19,000 samples collected from 55 provinces were analysed at NCVD and RAHOs; 666 samples were collected from poultry outbreaks, whereas 18,405 were collected through market-based surveillance (Table 3.1). A total of 1,448 A(H5) positive specimens were detected by rRT-PCR (259 A(H5) outbreak samples, 17.89%; 1,189 A(H5) market samples, 82.11% (Table 3.1). Virus isolation in embryonated chicken eggs yielded 214 isolates from 45 provinces (Appendix 14, Appendix 15). Amongst species, 59.8% (128 isolates) were from ducks, Muscovy ducks and geese, 35.5% (76 isolates) were from chickens, and 4.7% were from other species (6 quail, 2 pheasant, 1 swiftlet, and 1 tiger from a zoological collection). Of these, 90.6% (194 isolates) were A(H5N1) and 9.4% (20 isolates) were A(H5N6). One hundred nineteen full length HA and NA sequences and 95 partial HA1 sequences were generated from the 214 isolates. One hundred six WGSs were generated from isolates selected for sequencing based on representative collection locations and dates (Appendix 15). Additional partial genomes were obtained from 11 viruses. The complete dataset for analysis comprised the 106 novel Vietnamese A(H5) WGS from 2012-2015, plus 169 WGSs available from 2010-2012 described in previous studies (Creanga et al., 2013), for a total of 275 WGSs.

Table 3.1: Passive and active sample collection and A(H5) detection in Vietnam from September 2012 to August 2015

	Sept-Dec, 2012		2013		2014		Jan-Aug, 2015		Total	
	Market-based surveillance ^a	Outbreak ^b	Market-based surveillance	Outbreak	Market-based surveillance	Outbreak	Market-based surveillance	Outbreak	Market-based surveillance	Outbreak
Total samples	2,546	109	4,904	123	8,926	311	2,029	123	18,405	666
Total A(H5) positive samples	125	56	336	83	655	84	73	36	1,189	259
% A(H5) positive	4.91%	51.38%	6.85%	67.48%	7.34%	27.01%	3.60%	29.27%	6.46%	38.89%
Number of provinces	21	15	44	20	30	33	14	13	50	46

a Active surveillance through monthly sampling in live bird markets

b Passive surveillance in response to a poultry outbreak

3.3.2. HPAI A(H5) virus identification

The viruses identified belonged to six different clades including 1.1.2, 2.3.2.1a, 2.3.2.1b, 2.3.2.1c, 2.3.4.4 and 7.2 (Figure 3.2 and Appendix 6). With the exception of a few rare detections of clade 2.3.2.1a, 2.3.2.1b and clade 7.2 viruses, the predominant A(H5N1) clades detected throughout this period were clades 1.1.2 and 2.3.2.1c. Twenty-three clade 1.1.2 viruses were identified from October 2012 to April 2014, the majority of which ($n = 21$) were detected in the southern provinces of the Mekong Delta. The two remaining clade 1.1.2 viruses were found in the north (Bac Ninh) and central region (Khanh Hoa). Average pairwise nucleotide divergence within clade 1.1.2 viruses was 2.1%. The last detection of 1.1.2 viruses was in April 2014. Clade 2.3.2.1 HA viruses were classified into three subclades (2.3.2.1a, 2.3.2.1b and 2.3.2.1c). Clade 2.3.2.1a viruses previously circulated widely throughout Vietnam and Southeast Asia between 2010 and 2012. However, they were gradually replaced by clade 2.3.2.1c starting in July 2012 (Creanga *et al.*, 2013). Here we report detection of four clade 2.3.2.1a viruses in early 2013, but none after August 2013. Clade 2.3.2.1b never achieved a wider circulation beyond Hong Kong SAR and China, and the last detection was in August 2013. In contrast, clade 2.3.2.1c viruses were found in 16 provinces in the north, 12 provinces in the center and 9 provinces in the south of Vietnam. Although clade 2.3.2.1c were identified in 37 provinces, they were highly related to each other, forming a distinct monophyletic group within the larger multi-country 2.3.2.1c lineage. To date, clade 7.2 viruses ($n = 3$) have only been found in north Vietnam (Hanoi province) and were detected in poultry likely to have been smuggled across northern Vietnam borders. In February 2014, an emerging subclade of 2.3.4 A(H5N1), termed 2.3.4.4, was detected in central Vietnam (Quang Ngai and Kon Tum), followed shortly thereafter by detection of the same clade 2.3.4.4 HA gene but with an N6 NA in April 2014. A(H5N6) detections were then reported in 11 provinces in northern ($n = 6$) and central ($n =$

5) regions and were detected throughout the rest of the study period. Phylogenetic analysis indicated the A(H5N6) viruses originated through reassortment with Vietnamese A(H5N1) (Figure 3.2; Appendix 6). Through August 2015, two diverse clusters of Vietnamese A(H5N6) were identified. The first cluster included 2015 viruses ($n = 6$) that were closely related to the Jiangxi-like virus in China (A/duck/Jiangxi/NCDZT1126/2014-like), and the second group ($n = 14$) included viruses more closely related to Sichuan-like viruses (A/Sichuan/26221/2014-like).

The nearest reassortant WHO CVVs for each clade are coloured red. Amino acid differences at branch nodes indicate shared HA1 substitutions relative to the reference strains. Mutations to the right of each strain name indicate amino acid changes found only in that individual virus. Mutations that are underlined indicate previously recognised molecular markers. Branches on the tree with HA sequences from human cases are coloured red. Bootstraps greater than 50 generated from 1,000 replicates are shown at branch nodes. The scale bar represents nucleotide substitutions per site.

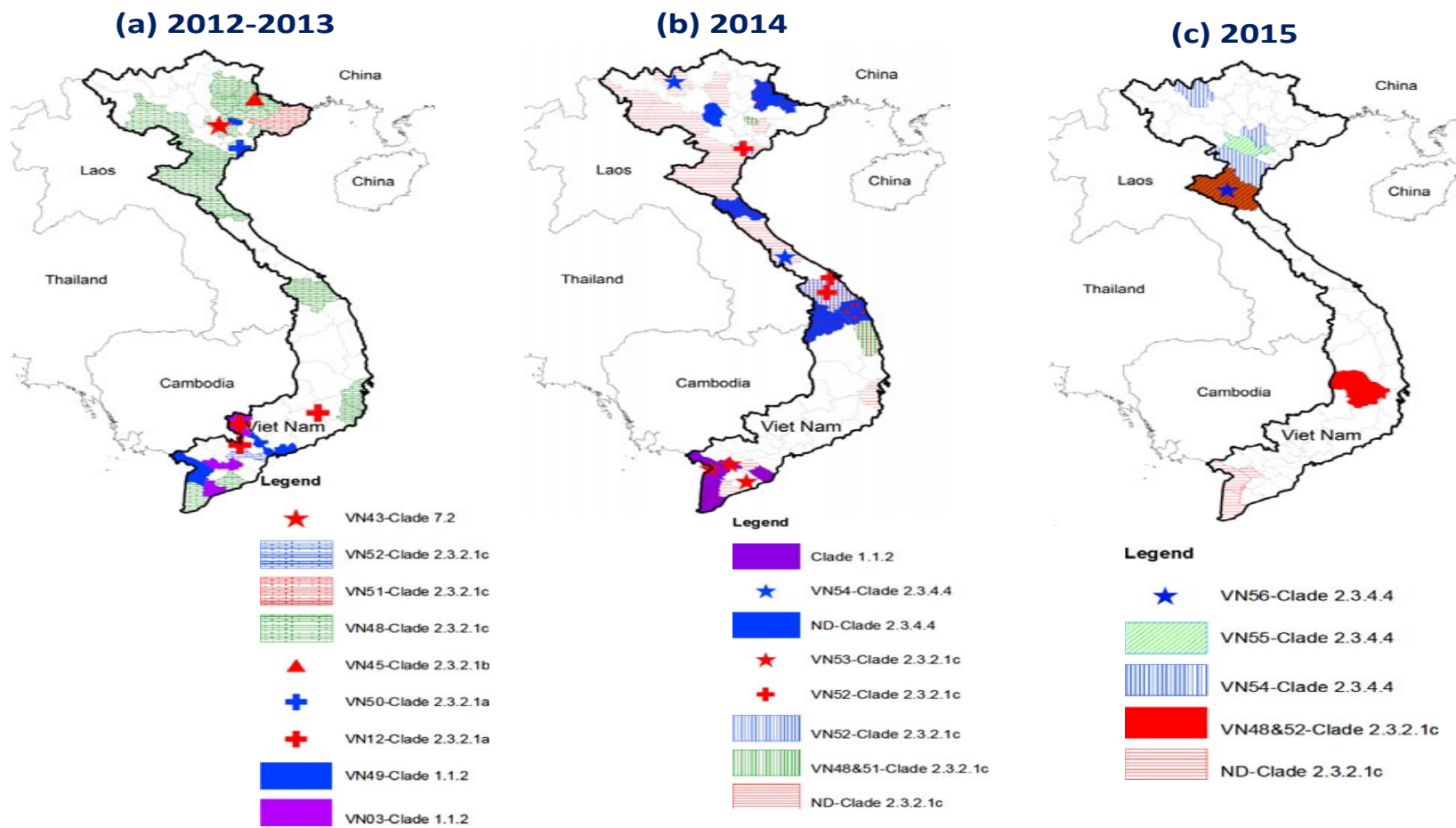


Figure 3.1: Spatial distribution of A(H5) virus clades and genotypes in Vietnam.

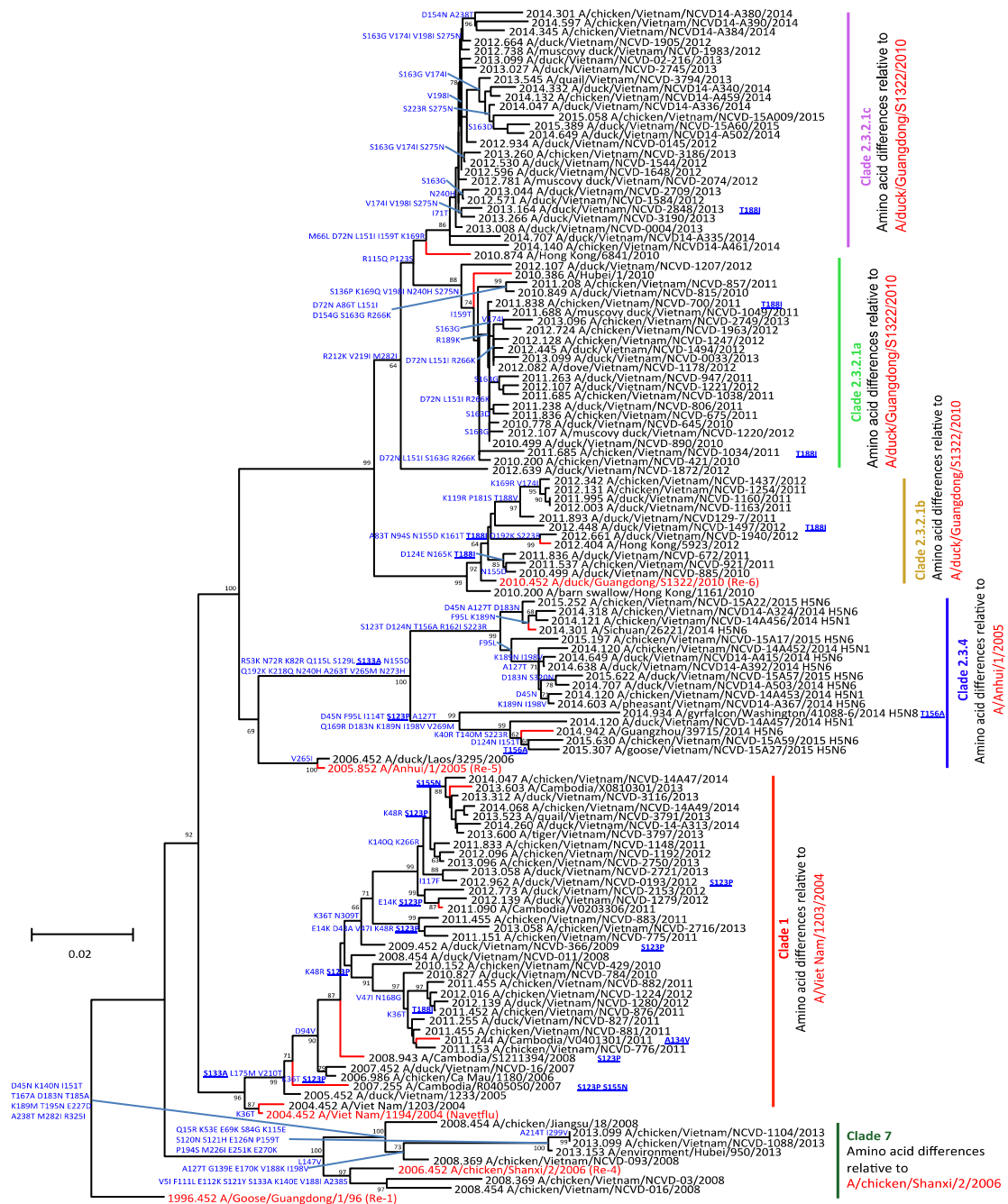


Figure 3.2: Maximum likelihood tree of the HA1 genes of HPAI A(H5) viruses in Vietnam. The nearest reassortant WHO candidate vaccine viruses (CVV) for each clade are coloured red. Amino acid differences at branch nodes indicate shared HA1 substitutions relative to the reference strains. Mutations to the right of each strain name indicate amino acid changes found only in that individual virus. Mutations with underline indicate previously recognised molecular markers. Branches on the tree with HA sequences from human cases are coloured red. Bootstraps greater than 50 generated from 1,000 replicates are shown at branch nodes. The scale bar represents nucleotide substitutions per site.

3.3.3. Reassortment of HPAI A(H5) internal genes

Phylogenetic analyses of the NA and internal genes (PB2, PB1, PA, NP, M, and NS) revealed numerous instances of reassortment among the 106 WGSs (Figure 3.3 and Appendix 6 - Appendix 13). Similar to the H5 HA gene phylogeny, multiple lineages of N1 NA and internal genes co-circulated in Vietnam. Eleven (of 19) clade 1.1.2 viruses were typical VN3 genotype, three out of four clade 2.3.2.1a viruses were genotype VN12, one clade 2.3.2.1b virus was genotype VN45, 42 (of 66) clade 2.3.2.1c viruses were genotype VN48, three clade 7.2 were genotype VN43.

Each gene segment for the A(H5) subtype viruses in Vietnam formed gene constellations related to the HA clades: clade 1.1.2, clade 2.3.2.1 a-c, clade 7.2 and clade 2.3.4.4 A(H5N6). Five previously described genotypes were identified (VN3, VN12, VN43, VN45, VN48) (Creanga, Thi, et al., 2013; T. Nguyen et al., 2012; Wan et al., 2008) and eight newly identified genotypes were identified [VN49 ($n = 8$), VN50 ($n = 1$), VN51 ($n = 2$), VN52 ($n = 15$), VN53 ($n = 7$), VN54 ($n = 9$), VN55 ($n = 3$) and VN56 ($n = 1$)]. NA and internal gene sequences of clade 1.1.2, clade 2.3.2.1a and clade 2.3.2.1b viruses were closely related with the corresponding genes found in viruses isolated before September 2012 from Vietnam, China and Cambodia (Wan *et al.*, 2008; Tung *et al.*, 2012; Creanga *et al.*, 2013).

NA and internal genes of the majority of clade 1.1.2 viruses shared a common ancestor defined by the progenitor strain A/duck/Cambodia/202W6M1/2013. However, the six internal gene sequences found in eight 2013 clade 1.1.2 viruses were related to those found in clade 2.3.2.1a viruses indicating reassortment and generation of genotype VN49. Clade 2.3.2.1c viruses also revealed evidence of reassortment and whole genome analysis

revealed 3 new genotypes in this clade with internal gene sequences previously found in the clade 2.3.2.1a or clade 2.3.2.1b viruses (Figure 3.3).

Analyses indicated a clade 2.3.2.1a reassortant in 2012 with HA, PB2 and PA from clade 2.3.2.1a and PB1, NP, NA, MP and NS from clade 2.3.2.1b (VN50). Further genetic reassortment was evident among clade 2.3.4.4 A(H5N6) viruses. The NA gene of all thirteen A(H5N6) viruses reported here originated most likely from related strains of A(H6N6) viruses circulating in the Fujian and Guangdong Provinces of China (Figure 3.3; Appendix 11). The internal genes of 9 viruses were related to clade 2.3.2.1b viruses, similar to strains found in domestic ducks from south-central and eastern China (Appendix 7 - Appendix 13) (VN54). Three of the A(H5N6) viruses had 5 internal genes related to clade 2.3.2.1b viruses, but the PB2 was distinct and related to A/duck/Yamagata/061004/2014 [A(H6N6)] (termed VN55). Similarly, one A(H5N6) virus (A/chicken/Vietnam/NCVD-15A22/2015) had the same five internal genes from clade 2.3.2.1b, but PB2 more closely related to A/chicken/Jiangxi/10784/2014 [A(H7N7)] (VN56).

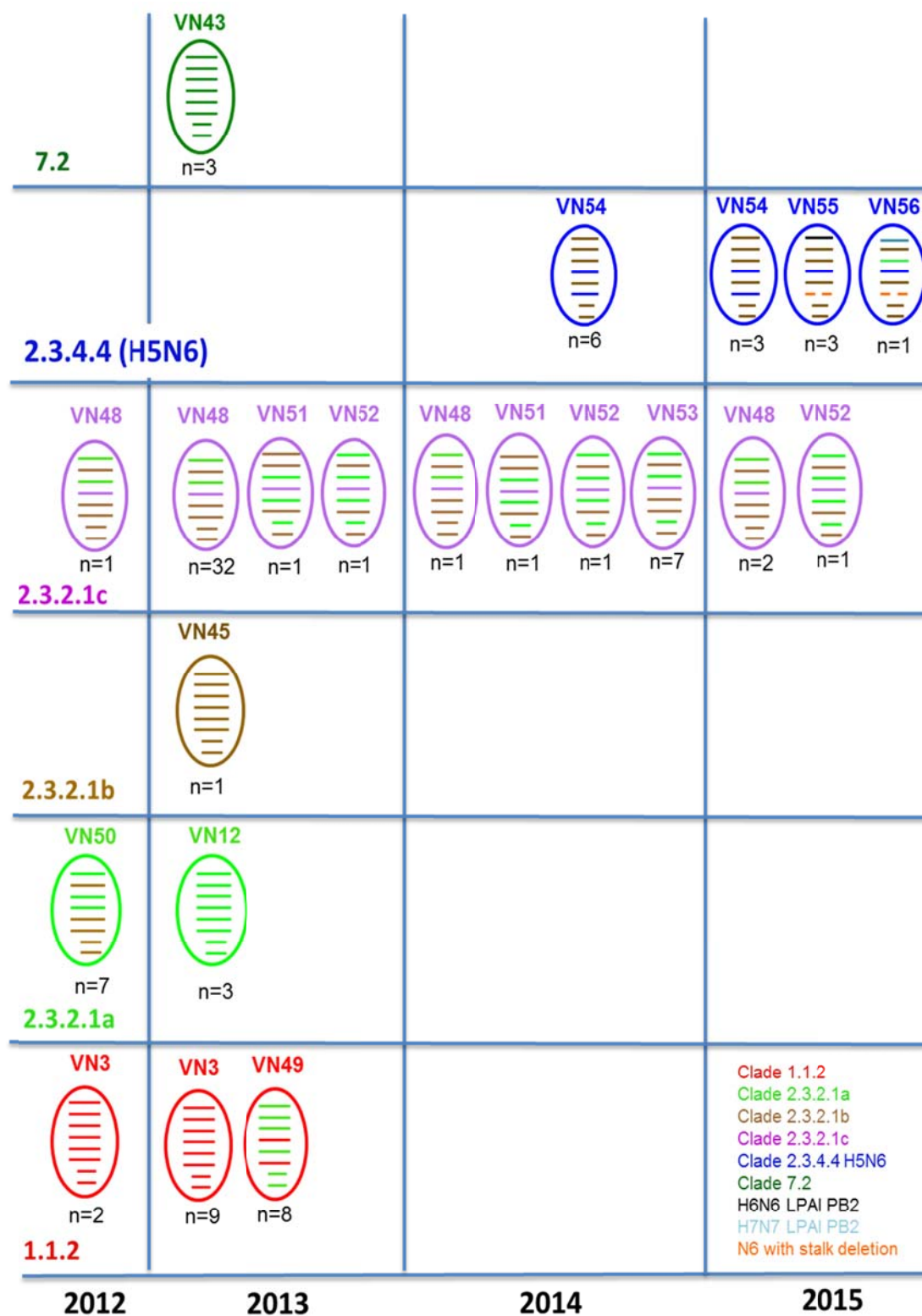


Figure 3.3: Schematic diagram of genome constellations of A(H5) viruses in Vietnam, 2012-2015.

3.3.4. Geo-temporal distribution of clades and genotypes in Vietnam

The geographic distribution of A(H5) HA clades and genotypes detected between September 2012 and August 2015 reveals the expansion of clade 2.3.2.1c (genotypes VN48 and VN52) throughout the country (Figure 3.1). Clade 2.3.2.1c VN53 viruses were only detected in the south, while VN51 viruses were only found in the north. Only VN50 virus was detected in a northern province (Nam Dinh) in 2012, however the older original clade 2.3.2.1a VN12 circulated in all three regions during 2013. Clade 2.3.2.1b (VN45) and clade 7.2 (VN43) were identified from the north early in early 2013 but did not circulate persistently. Clade 1.1.2 (VN3 and VN49) were found in southern provinces, with the last reported detection in April 2014 (Figure 3.1). Clade 2.3.4.4 A(H5N6) viruses (VN54, VN55 and VN56) were detected in the central and northern provinces from 2014 to August 2015, and appeared to be gaining dominance towards the end of 2015 (data not shown).

3.3.5. Substitution rates and divergence times

To further distinguish evolutionary features of the major clades detected and discern potential differences in selective pressures acting on each genomic segment, we estimated nucleotide substitution rates by Bayesian analysis of each gene segment (Table 3.2, Appendix 6 - Appendix 13). Clade 2.3.2.1c HA genes exhibited a higher mean substitution rate ($6.87\text{E-}3$) than clade 1.1.2 or clade 2.3.4.4, however the overlapping range of 95% HPD intervals indicated these differences were likely not significant and could also be due to a larger population sampled. Analysis of substitution rates by segment ranged from $2.63\text{E-}3$ to $5.61\text{E-}3$ substitutions/site/year; the mean rate for HA was highest among the gene segments, followed by NA and NS. However, these overlapping HPD intervals

suggested the differences were not statistically significant. Substitution rates of viral polymerase genes (PB2, PB1 and PA) fell in the range (3.83E-3 to 4.55E-3) and the MP gene had the lowest rate (mean 2.63E-3), which may be expected due to the highly conserved nature of this gene.

Table 3.2: Estimation of evolutionary rate of Vietnamese A(H5) viruses, 2012-2015.

Stratification	Uncorrelated relaxed clock model	Mean substitution rate (x 10 ⁻³)	95% HPD ^a interval (x 10 ⁻³)
Clade			
Clade 1.1.2	Lognormal	5.69E-3	4.72E-3 to 6.69E-3
Clade 2.3.2.1c	Lognormal	6.87E-3	5.39E-3 to 8.40E-3
Clade 2.3.4.4	Lognormal	5.98E-3	4.90E-3 to 7.16E-3
Gene			
PB2	Exponential*	4.55E-03	3.54E-3 to 5.78E-3
PB1	Lognormal	4.18E-03	3.61E-3 to 4.76E-3
PA	Lognormal	3.83E-03	3.27E-3 to 4.37E-3
HA	Lognormal	5.61E-03	4.98E-3 to 6.25E-3
NP	Lognormal	3.39E-03	2.70E-3 to 4.12E-3
NA	Lognormal	4.83E-03	4.25E-3 to 5.42E-3
MP	Lognormal	2.63E-03	2.10E-3 to 3.13E-3
NS	Lognormal	4.80E-03	3.085E-3 to 6.66E3

^a HPD, Highest posterior density

* Exponential was used due to the low effective sample size with the lognormal model.

Bayesian estimates of the divergence times of clades 1.1.2, 2.3.2.1c and 2.3.4.4 through calculation of times to the most recent common ancestor (tMRCA) indicated the HA gene for clade 1.1.2 viruses diverged in October 2011 from closely related viruses from Cambodia (Appendix 3 - Appendix 5). Skyline plot analysis of the virus population size revealed an increase in the genetic diversity of clade 1.1.2 that occurred from the first introduction of the clade in 2011 before stabilising again towards the end of 2012 (Appendix 3). The estimated tMRCA for the clade 2.3.2.1c HA gene indicated that most viruses diverged in June 2013 from a closely related viruses from Khanh Hoa province (central region; see Appendix 4). From mid-2012/early 2013 the population size of this

clade expanded as the virus spread and caused outbreaks in new regions across the country (Appendix 4). All Vietnam viruses of subtype A(H5N6) appear to have diverged in July 2012 from Chinese ancestors. The first cluster, including the most recent viruses collected in 2015, were estimated to have emerged in Vietnam during September 2014 from a closely related Jiangxi-like virus (A/duck/Jiangxi/NCDZT1126/2014). The second and larger group of A(H5N6) viruses was estimated to have diverged from ancestral strains around July 2012 and emerged in Vietnam in February 2014. This cluster was related to 2013/2014 Chinese viruses (e.g., A/Sichuan/26221/2014) (Appendix 5). Although the larger genetic lineage of A(H5N6) viruses had a stable population size from 2011 through 2013, the emergence of these clusters in Vietnam and the coinciding expansion and spread of the clade 2.3.4.4 HA genes in Europe and North America during 2014 resulted in an increased population size (Appendix 5). Overall, Bayesian skyline plot analysis of the NA and internal genes of all viruses included in this study showed a relative expansion of the A(H5) virus population size during 2012/2013 (Appendix 3 - Appendix 13), which appears to have resulted primarily from spread of clade 2.3.2.1c in the country and the numerous genotypes and internal gene lineages that were identified at this time (Figure 3.3).

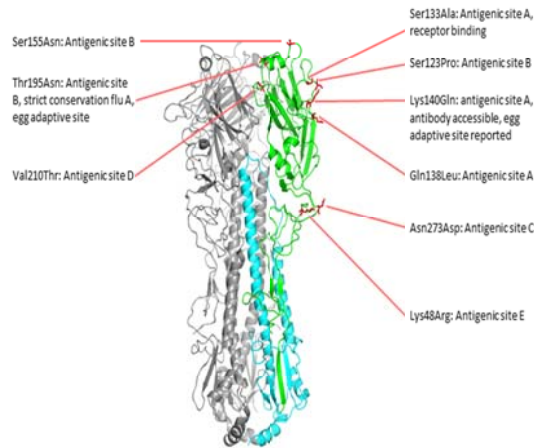
3.3.6. Molecular markers identified in A(H5) viruses

Molecular markers and putative antigenic sites of the HA1 protein

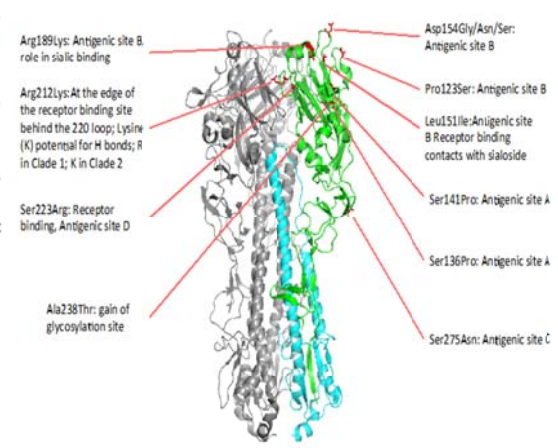
The multibasic cleavage motif was present in all A(H5) HA protein sequences, however there were consistently conserved differences across clades. The motif PQREERRKKR↓G was identified in 19/21 sequences from clade 1.1.2; PQRERRRKR↓G was found in clade 2.3.2.1c ($n = 73/77$) and clade 2.3.2.1a ($n = 4/4$); clade 2.3.2.1b had PQIERRRRKR↓G; clade 2.3.4.4 had PLREKRRKR↓G; and clade 7.2 had PQIEGRRRKR↓G. Three viruses had additional mutations in comparison to their respective clades: two clade 1.1.2 viruses

had PQREGRRKKR↓G and PQRKERRKKR↓G, respectively, and one clade 2.3.2.1c virus had PQKERRRRKR↓G. All viruses showed replication in MDCK cells in the absence of trypsin. Conserved amino acid mutations in the HA1 protein of each clade with respect to the closest A(H5) CVV are shown in Figure 3.2, Figure 3.4 and Appendix 16 –Appendix 21. Clade 1.1.2 viruses averaged 13 amino acid substitutions relative to A/Vietnam/1203/2004, 9 changes relative to A/Cambodia/R0405050/2007, and 12 changes compared to A/Vietnam/1194/2004 (Navet Viflucac; clade 1 Vietnamese poultry vaccine) (Figure 3.5a, Appendix 16). Clade 2.3.2.1a viruses had an average of 6 amino acid substitutions relative to A/Hubei/1/2010, of which 5 occurred in predicted antigenic sites, and 19 amino acid substitutions relative to the poultry vaccine Re-6 (A/duck/Guangdong/S1322/2010) (Appendix 17). Clade 2.3.2.1b viruses, which belong to the same clade as the vaccine virus, A/duck/Guangdong/S1322/2010 (Re-6), had 10 conserved amino acid substitutions in HA1 relative to the WHO CVV A/barn swallow/Hong Kong/1151/2010 or A/duck/Guangdong/S1322/2010 (Re-6), with 5 occurring in epitope regions (Appendix 18). Similarly, clade 2.3.2.1c viruses possessed an average of 9 coding changes relative to A/Hubei/1/2010, and 17 amino acid mutations relative to Re-6, of which 17 were identified in putative epitope regions (Figure 3.5b, Appendix 19). The greatest number of changes in relation to the nearest WHO CVV or available poultry vaccine was identified in clade 2.3.4.4 A(H5N6) viruses, with an average 27 and 40 mutations relative to Re-5 and Re-6, respectively (Figure 3.5c, Appendix 20). Finally, clade 7.2 HA1 sequences had an average of 34 amino acid substitutions compared to A/chicken/Shanxi/2/2006 (Re-4) vaccine strain (Figure 3.5d, Appendix 21).

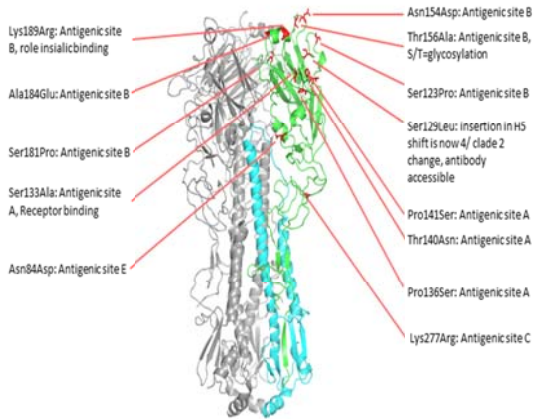
(A) Clade 1.1.2



(B) Clade 2.3.2.1c



(C) Clade 2.3.4.4



(D) Clade 7.2

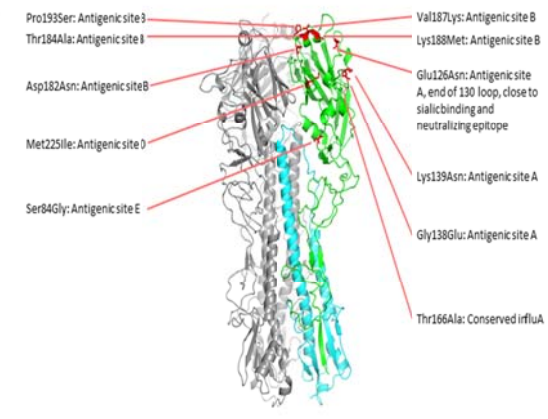


Figure 3.4: HA1 substitutions identified at putative antigenic sites on the HA trimeric structure. Amino acid differences between the consensus sequence of each clade and nearest poultry vaccine virus are displayed for clade 1.1.2 versus A/Vietnam/1203/2004 (Figure 3.4a); Clade 2.3.2.1c sequence differences to A/duck/Guangdong/S1322/2010 (Re-6) (Figure 3.4b); Clade 2.3.4.4 sequence differences to A/Anhui/1/2005 (Re-5) (Figure 3.4c); Clade 7.2 sequence differences to A/chicken/Shanxi/2/2006 (Re-4) (Figure 3.4d).

All 119 HA sequences had glycosylation sites at positions 286. The majority had additional putative glycosylation sites at positions 10 ($n = 99$), 11 ($n = 100$), 23 ($n = 101$), 165 ($n = 101$), 484 ($n = 108$) and 543 ($n = 108$). Analysis of the glycosylation motif at position 154 showed that all clade 1.1.2 and 7.2 sequences retained the motif, whereas the glycosylation was ablated in clade 2.3.4.4 sequences and clade 2.3.2.1a-c sequences, except for one recent single clade 2.3.2.1c sequence (A/chicken/Vietnam/NCVD14-A380/2014).

Molecular markers within NA and internal genes

Substitutions in NA proteins associated with altered susceptibility to neuraminidase inhibitors (NAIs) were identified in numerous clade 1.1.2 but not from other clades (Table 3.3). A V129A substitution (or residue 149 N2 numbering) associated with reduced susceptibility to zanamivir (Naughtin *et al.*, 2011; Creanga *et al.*, 2013), was found in 17 clade 1.1.2 viruses collected in 2012 ($n = 2$) and 2013 ($n = 15$). Another marker of reduced susceptibility to oseltamivir associated with an I203V mutation (or residue 222 in N2 numbering) was found in a single clade 1.1.2 virus. Mutation of R430Q, associated with reduced susceptibility to NAIs, was found in a single clade 1.1.2 virus (A/chicken/Vietnam/NCVD-2750/2013).

Table 3.3: Molecular markers identified in A(H5) viruses in Vietnam.

Gene	Mutation/Phenotypic consequences	Mutation	Clade 1	Clade 2.3.2.1c	Clade 2.3.4.4 A(H5N6)	Clade 7.2
HA	Antigenic site C	D43A	A (4.8%)			
	Antigenic site E	K48R	R (95.2%)			
	Antigenic site E	S84G				G (100%)
	Antigenic site B; Increased α 2-6 binding	S123P	P (90.5%)	S (85.7%)	P (30.7%)	
	Antigenic site A	E126N				N (100%)
	Antigenic site A; Increased α 2-6 binding	S133A	A (100%)		100%	
	Antigenic site A	Q138L	L (19.0%)			
	Antigenic site A	G138E				E (100%)
	Antigenic site A	K140N/Q	Q (90.5%); N (4.8%)			
	Antigenic site A	S141P		P (3.9%)	P (7.7%)	
	Antigenic site B; receptor binding	L151I		I (87.0%)	T (31.0%)	
	Antigenic site B	D154N/G		N (10.4%); G (2.6%)		
	Antigenic site B	S155N	N (47.6%)			
	Antigenic site B; predicted loss of glycosylation; Increased α 2-6 binding, increased transmission in mammalian models	T156A			A (100%)	
	Antigenic site B	S181P			P (23.08%)	
	Antigenic site B	T182A				A (100%)
	Antigenic site B	D183N			N(61.53%)	
	Antigenic site B	A184E			E (23.08%)	
	Antigenic site B	V184K				K (100%)
	Antigenic site B	K187M				M (100%)
	Antigenic site B	P188S				S (100%)
	Antigenic site B; Increased virus binding to α 2-6	R189K		K (3.9%)		
	Antigenic site B	Q192K			100%	
	Antigenic site B	P193S				S (100%)
	Antigenic site B	T195N	N (9.5%)			
	Antigenic site D	V210T	T (100%)			
	Edge of the receptor binding site	R212K		K (87.0%)		
	Increased virus binding to α 2-6; Antigenic site D	S223R		R (6.5%)	R (100%)	
	Antigenic site D	M225I				I (100%)
	Antigenic site C	N273D	D (4.8%)			
	Antigenic site C	S275N		N (79.2%)		
Highly pathogenic cleavage peptides			PQRE/KE/GRRK KR ↓ G	PQR/KERRR KR ↓ G	PLREKRR KR ↓ G	PQIEGRRRK RG ↓ G
NA	Stalk deletion	59-69del			Yes (30.8%)	
	Reduced susceptibility to zanamivir	V129A	A (80.9%)			
	Reduced susceptibility to oseltamivir	I203V	A (4.8%)			
PB2	Associated with reduced inhibition by NAIs	R430Q	Q (4.8%)			
	Enhanced polymerase activity, increased virulence in mice	T339K	K (42.1%)	K (94.4%)	K (61.5%)	K (100%)
	Reduction in replication efficacy in cell culture and virulence in mice and ferrets	R368Q	Q (42.1%)	Q (97.2%)	Q (68.2%)	
	Reduction in replication efficiency in cell culture and virulence in mice and ferrets	Q391E	E (100%)	E (100%)	E (100%)	E (100%)
	Increased replication efficiency in cell culture and enhanced virulence in mice; mammalian host adaptation	K627E	E (100%)	E (100%)	E (100%)	E (100%)

PB1	Decreased replication efficiency and virulence in ferrets	N328K	K (1.41%)			
	Decreased replication efficiency and virulence in ferrets	N375S	S (10.5%)			
PB1-F2	Increased pathogenicity in mice	Protein length	90 aa (80%) 57 aa (20%)	57 aa (100%)	57 aa (100%)	90 aa (100%)
PA		K356R	K/R???			
M2	Reduced susceptibility to amantadine/rimantadine	V27A	A (7.69%)			
	Reduced susceptibility to amantadine/rimantadine	A30S	S (1.41%)			
	Reduced susceptibility to amantadine/rimantadine	N31S	S (42.1%)	S (100%)	S (100%)	
NS1	Altered virulence in mice	80-84del D92E	Yes	Yes E (100%)	Yes E (100%)	Yes E (100%)
	Altered virulence in mice, PDZ motif	227–230				
NS2	Decreased antiviral response in host	T47A	A (100%)		A (100%)	A (100%)
	Decreased antiviral response in host	M51I				I (100%)

The PB2 residue K627 has been implicated in significant phenotypic changes in polymerase activity, which imparts replicative fitness advantages and virulence in mammals (Shinya *et al.*, 2004; Herfst *et al.*, 2012). PB2-E627 was identified in 111 viruses, suggesting a lack of mammalian adaptation in these viruses. Similarly, substitutions of R368Q have been associated with reduced replication efficiency in cell culture and virulence in mice and ferrets. This residue was identified in 91 viruses. A T339K substitution was found in 91 viruses, which enhanced polymerase activity and increased virulence in mice (CDC, 2014). An N328K or N375S substitution in the PB1 protein, which decreased replication efficiency and virulence in ferrets, was found in a single clade 2.3.2.1c virus and in two clade 1.1.2 viruses.

The M2 protein of one clade 2.3.2.4 virus had a V27A mutation and one clade 2.3.2.1c virus had an A30S mutation, both of which were associated with reduced susceptibility to rimantadine. The substitution N31K in the M2 protein, which may reduce susceptibility to rimantadine, was found in 8, 68 and 13 clade 1.1.2, 2.3.2.1c and 2.3.4.4 viruses, respectively (Table 3.3).

All viruses had NS1 proteins with the 80-84 amino acid deletion associated with altered virulence in mice. An D92E substitution in the NS1 protein, also associated with altered virulence in mice, was found in all viruses, except for clade 1.1.2. A T47A substitution in the NS2 protein, which was associated with decreased antiviral response in the host was found in all clade viruses, except for two clade 2.3.2.1c viruses. Finally, clade 7.2 viruses had mutation of M51I associated with decreased antiviral response.

3.3.7. Antigenic characterisation of A(H5) viruses circulating in Vietnam

The HI assay was used to assess the antigenic relatedness of poultry viruses in Vietnam (Figure 3.5a-c). Most clade 1 viruses tested with antiserum raised to the CVV A/Vietnam/1203/04 showed HI titres 4-fold lower than the homologous titre but approximately 20% of viruses tested had ≥ 8 -fold reductions in heterologous titres. Antiserum raised against A/Cambodia/X0810301/2013, the most recently developed clade 1.1.2 CVV, reacted with eight of eleven 2013-2014 viruses from Vietnam with HI titres that were within 4-fold or less of the homologous virus titre. Three 2013-2014 viruses from Vietnam reacted with antiserum at titres that were 8 to 32-fold lower than the homologous virus titre (Figure 3.5a). The 2012 to 2014 viruses tested differed by 8-12 amino acids from the A/Vietnam/1203/2004 and A/Vietnam/1194/2004 CVVs but by only 5 to 6 amino acids from the CVV, A/Cambodia/X0810301/2013 (Table 3.3, Figure 3.5a).

Antigenic analyses of 34 clade 2.3.2.1a avian isolates from Vietnam revealed that 23 of 34 viruses reacted within 8-fold of homologous antiserum titres of A/cm/HK/5052/2007. Twelve of 17 clade 2.3.2.1a viruses showed ≥ 8 -fold differences in HI titre with antiserum to the A/Hubei/1/10 vaccine candidate (relative to homologous antigen). These viruses also show reduced titres with antiserum to A/Vietnam/NCVD-700/2011, albeit it in the 4-fold

range. Antiserum produced to the A/Hubei/1/2010 CVV reacted with three viruses from Vietnam at titres ≥ 16 -fold compared to homologous titres. New antiserum produced to a more closely related Vietnamese clade 2.3.2.1a virus (A/chicken/Vietnam/NCVD-1255/2012) reacted within 2-fold or less with the same Vietnam viruses (Figure 3.5b). Six clade 2.3.2.1b viruses were analysed herein. The HI titres of antiserum to A/barn swallow/Hong Kong/D10-1161/2010 tested against these viruses showed differences ≤ 4 -fold of homologous antigen (Figure 3.5b).

Antiserum to A/HK/6841/2010 (clade 2.3.2.1c) showed a majority of viruses (33 of 34) with titres that differed from homologous virus by <4 -fold. Seventeen clade 2.3.2.1c avian isolates collected in 2013 from Vietnam were characterised. Ferret antiserum raised against the CVV, A/duck/Vietnam/NCVD-1584/2012, reacted with all viruses with HI titres that were within 2-fold or less of the titre with homologous virus. Ferret antisera against A/duck/Vietnam/NCVD-1584/2012 well-inhibited a majority of 2014 poultry viruses from Vietnam also with HI titres within 4-fold of the homologous virus titres. One exception was a poultry virus, which possessed the R189K HA1 mutation and exhibited an 8-fold reduced HI titre compared to the HI titre to the homologous virus (Figure 3.5b). Three 2013 clade 7.2 viruses collected in Vietnam revealed no detectable HI titres with sera produced against clade 7.1 CVVs (Figure 3.5a). Extensive amino acid sequence divergence continues to be detected in clade 7 viruses from 2013 relative to previous CVVs, A/chicken/Vietnam/NCVD-016/2008 and A/chicken/Vietnam/NCVD-03/2008 (Appendix 21), suggesting the reason for the antigenic distance between these viruses and the CVVs (Figure 3.5a).

Ferret antiserum raised against clade 2.3.4.4 A/gyrfalcon/Washington/41088-6/2014 [A(H5N8)] reacted within 2-fold or at equivalent titres to homologous and heterologous virus titres of related A(H5N8) viruses indicating antigenic similarity. In contrast, this serum did not cross-react well with A(H5N6) viruses from Vietnam. Similarly, antiserum raised to A/chicken/Vietnam/NCVD-14-A324/2014 [A(H5N6)] reacted poorly with A(H5N8) and A(H5N2) viruses detected in Europe and North America; heterologous virus HI titres were greater than 8-fold down to the homologous virus titre (Figure 3.5c). The HA1 proteins of A(H5N8) and A(H5N2) viruses detected in the USA differed from that of the A(H5N6) viruses by 11-12 substitutions identified at 4 potential antigenic sites (data not shown). The amino acid comparison of the HA1 proteins of recently circulating poultry A(H5N6) viruses in Vietnam, Laos and China differed from that of the A(H5N6) CVV by four or fewer amino acids but had on average 13 substitutions compared to the Re-5 poultry vaccine (Figure 3.5c). Notably, the Vietnamese A(H5N6) antiserum reacted within 4-fold to A/chicken/Laos/206/2014 [A(H5N6)]. HI titres of antisera raised to the A/Sichuan/26211/2014 [A(H5N6)] vaccine strain were reduced (>8-fold) against A(H5N2) and A(H5N8) viruses detected in the US. However, this A(H5N6) antiserum reacted within 2-fold to A/chicken/Laos/206/2014 [A(H5N6)] and well-inhibited A(H5N6) viruses detected in Vietnamese poultry (Figure 3.5c).

(A) Clades 1.1.2 and 7.2



(B) Clade 2.3.2.1c



(C) Clade 2.3.4.4

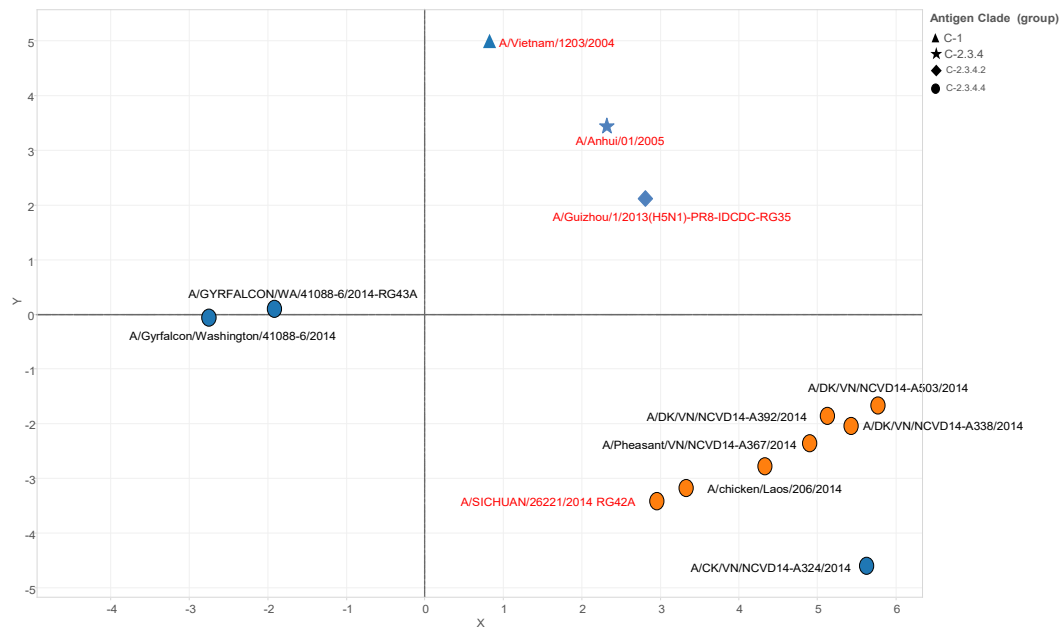


Figure 3.5: Antigenic mapping for HPAI A(H5N1) virus clades. For clades 1.1.2 and 7.2 (Figure 3.5a); Antigenic mapping for clade 2.3.2.1 (Figure 3.5b); Antigenic mapping for clade 2.3.4.4 (Figure 3.5c). Antisera are coloured blue and antigens are coloured in orange.

3.4. Discussion

Given the co-circulation of several clades and genotypes of A(H5N1) viruses in Vietnam during recent years and the difficulties in achieving optimum vaccination coverage under field conditions, there is a need to continuously monitor and characterise circulating A(H5N1) viruses to promptly detect changes in genetic and antigenic characteristics. This study focused on the molecular epidemiology of HPAI A(H5) viruses collected from poultry outbreaks and active surveillance at LBMs in Vietnam during the period from 2012 to 2015. The antigenic relatedness of newly circulating viruses was compared to older strains as well as pre-pandemic CVVs recommended by the WHO. Full genome sequencing and dated phylogenetic analysis were performed to identify further expansion and reassortment of A(H5) viruses in Vietnam, and to estimate the time periods during

which these events occurred. Molecular changes associated with the diversification of each major clade and the emergence of novel genotypes identified specific HA mutations associated with antigenic drift and substitutions previously correlated with increased pandemic risk. Through the study period, several key events, such as the emergence of clade 2.3.2.1c, likely disappearance of clade 1.1.2, and the emergence and reassortment of clade 2.3.4.4 viruses, were evaluated to assess the need for development of public health and poultry vaccines with improved antigenic match.

The comprehensive phylogenetic analysis of Vietnamese A(H5) viruses presented herein revealed significant diversification, reassortment, and lineage replacement between 2012 and 2015. Sequence analyses of Vietnamese viruses compared to other countries revealed that clade 1.1.2 viruses were closely related only to those found in Cambodia, confirming previous reports of sustained enzootic circulation of these viruses within the Mekong Delta region. Despite this apparent geographic restriction, clade 1.1.2 viruses continued to diversify genetically through 2013 and reassorted most likely with clade 2.3.2.1a viruses, acquiring their internal genes and giving rise to a novel genotype (VN49) also identified in Cambodia (Sorn *et al.*, 2013). Clade 1 viruses were not detected by either active or passive surveillance since April 2014 in Vietnam. It was unclear based on available sequence data whether or not this clade continued to circulate on the Cambodian portion of the delta beyond 2014. Interestingly, the most recent clade 1.1.2 viruses detected in Vietnam possessed all internal genes from clade 2.3.2.1 viruses, suggesting a possible loss of fitness of clade 1.1.2 viruses with this genome arrangement. Clade 2.3.2.1c viruses, most likely introduced into Vietnam in July 2012, became predominant throughout Vietnam during 2013 and gradually displaced clade 1.1.2 viruses from the Mekong delta region, where clade 1 (VN3) had been predominant since 2003.

The broad geographic distribution and genetic diversity of the three groups of clade 2.3.2.1 in Vietnam suggested multiple separate introductions rather than endemic genetic drift. The tMRCA estimates also support a series of independent introductions. Clade 2.3.2.1a viruses appeared as early as 2009 and continued to be detected from 2012 to 2013 throughout northern, central, and southern regions, during which time this group was also identified in Bangladesh, Bhutan, Laos, India, Nepal and Myanmar. Both clades 2.3.2.1a and 2.3.2.1b appeared to have been displaced by clade 2.3.2.1c viruses in 2014. During this surveillance period, clade 2.3.2.1c was dominant in both the south and centre of Vietnam and caused sporadic outbreaks and was identified in LBMs through August 2015. And 2.3.2.1c viruses disseminated widely throughout Southeast Asia, China, Japan, Korea, Mongolia, Russia and Europe, indicating widespread circulation likely mediated by large outbreaks in wild bird populations (Gilbert and Pfeiffer, 2012; Han *et al.*, 2012; Islam *et al.*, 2012; Nagarajan *et al.*, 2012; Sakoda *et al.*, 2012; Uchida *et al.*, 2012). Despite their detection in 37 of 63 provinces in Vietnam, clade 2.3.2.1c showed limited diversity, suggesting they originated from a single progenitor source. The long phylogenetic branch lengths separating the Vietnamese clade 2.3.2.1c from other countries make it difficult to speculate about potential origin.

The co-circulation of the three subgroups of clade 2.3.2.1 led to reassortment between these viruses and emergence of several novel genotypes with the HA of clade 2.3.2.1c and different combinations of internal genes derived from 2.3.2.1a and 2.3.2.1b (genotypes VN48, VN51, VN52, VN53). Although these reassortments generated genetic diversity at the genotype level, to date there has been relatively little drift within the HA and NA, nor much evidence for diversification of the internal genes of VN51-53, suggesting these

reassortants originated from a not so distant ancestor. Clade 2.3.4.4 viruses were detected in the northern and central regions of the country in April 2014 and have since gained dominance in many provinces throughout the country. This virus, which also circulated in China, Laos and South Korea during 2014 and 2015, generated several new reassortant 2.3.2.1 genotypes in Vietnam. The PB2 genes of several clade 2.3.4.4 viruses were derived from LPAI viruses indicating their co-circulation as well. The lack of reassortment between clade 1.1.2 and 2.3.4.4 viruses in Vietnam could be explained by the displacement of clade 1.1.2 from the Mekong region prior to or close to the incursion of clade 2.3.4.4, with no substantial period of co-circulation among the two viruses.

Although clade 1.1.2 viruses appeared to be displaced during this study period, antigenic testing of viruses revealed that existing pre-pandemic vaccine candidates belonging to this clade covered circulating viruses fairly well. The same was true for clade 2.3.2.1c viruses, which cross-reacted with the most closely related vaccine strain with little to no reduction in serum HI titres. The few viruses that were not well-inhibited by antisera raised against this clade had an R189K substitution in antigenic site B of the mature HA1 protein. This change has previously been associated with a loss of antigenic cross-reactivity.

Interestingly, characterisation of the dominant immunogenic portion of the 2.3.2.1c surface protein (mature HA1) indicated a significant number of amino acid substitutions in other positions that did not appear to affect antigenicity in relation to the WHO CVV. Clade 7.2 viruses were not inhibited by ferret antisera developed against earlier clade 7.1 viruses. As a consequence of this and evidence of continued circulation of the clade 7.2 in China in 2014 and 2015, a proposed WHO CVV against clade 7.2 is under development (WHO, 2015). Finally, clade 2.3.4.4 presents a unique challenge for optimising antigenic match due to the expanding range and diversity of these viruses. HI testing demonstrated that

recent clade 2.3.4.4 viruses from Vietnam reacted less well with post-infection ferret antiserum raised to the North American clade 2.3.4.4 A/gyrfalcon/Washington/41088-6/2014 [A(H5N8)], the virus from which a clade 2.3.4.4 CVV was developed. This finding indicated significant antigenic distances between North American and East Asian clade 2.3.4.4 viruses, and suggested low vaccine coverage by the North American CVV. In contrast, the A/Sichuan/26221/2014 strain, also developed as a CVV, cross-reacted well with the 2014 A(H5N6) viruses tested, demonstrating the need for more than one clade 2.3.4.4 vaccine candidate despite their recent global emergence and spread. In fact, increasing genetic and antigenic diversity among clade 2.3.4.4 viruses detected in China and Vietnam has also led to the WHO recommendation to develop two additional A(H5N6) clade 2.3.4.4 CVVs (WHO, 2016a). Because of the antigenic diversity even among genetically related WHO pre-pandemic vaccine candidates, these findings demonstrate that systematic antigenic testing of A(H5) clade variants in the context of current and proposed poultry vaccine strains is also urgently needed to assess potential impacts on poultry vaccine efficacy. Antigenic characterisation of circulating viruses with antisera raised specifically to vaccine strains in avian hosts (e.g., chickens and/or ducks) would allow for timely assessment of antigenic match. Routine identification of A(H5) antigenic variants may also help to guide selection of challenge viruses used in experimental vaccine efficacy trials in poultry. Together, these data have the potential to inform poultry vaccine strain selection in a manner akin to the WHO pandemic vaccine strain selection process. Unfortunately, to date there are no coordinated institutional mechanisms at the international level to promote such a systematic approach to veterinary vaccine development.

In summary, our analyses of contemporary A(H5) viruses circulating in Vietnamese poultry demonstrate the ever-changing genetic and antigenic characteristics of these viruses and their propensity to continue evolving both at the individual gene level and over the genome as a whole. Given the overlapping geographic distribution of A(H5) clade variants and their antigenic divergence, the vaccine efficacy of multivalent vaccine formulations containing representatives of both clade 2.3.2.1c and 2.3.4.4 viruses should be tested to determine their ability to protect poultry in Vietnam.

Utilisation of antisera generated in immunised chickens for antigenic characterisation of A(H5N1) viruses in Vietnam

Abstract:

To conduct an in-depth antigenic analysis of contemporary A(H5N1) HA clade variants in Vietnam, we generated reference chicken antisera against a panel of 85 Vietnamese A(H5N1) virus isolates and conducted HI assays to generate serological datasets for antigenic cartography. We examined the correlation between antigenic distance and phylogenetic distance, and compared the antigenic profiles of viruses using both ferret and chicken antisera. The data were used to evaluate the antigenic match between existing A(H5N1) poultry vaccines and circulating viruses. Using antigenic cartography methods, it showed that at least four antigenically distinct variants had circulated in Vietnam since 2003. Representative viruses that belonged to different phylogenetic clades were also generally antigenically distinct. The methods developed here provide a systematic approach to evaluating antigenic variation and may help inform vaccine strain selection for future A(H5) poultry vaccine intervention strategies in Vietnam.

4.1. Introduction

Poultry vaccination against A(H5) viruses has been an important component of HPAI A(H5) disease control measures in Vietnam since 2005 (DAH, 2013b; DAH, 2016b). One of the critical determinants of influenza vaccine efficacy is the antigenic match between vaccine strain and circulating field virus. Vaccine ‘matching’ refers to the antigenic similarity between vaccine strain and circulating viruses. Traditionally the degree of antigenic match is measured by raising post-infection immune sera to the vaccine strain in animal models of infection, followed by comparison of HI titres of the antisera with the vaccine strain itself (homologous titre) compared to titres tested against circulating field viruses (heterologous titres). Antigenic matching is considered a key first step when making decisions about vaccine usage. Indeed, for human influenza vaccine strain selection, the WHO has developed a global network of GISRS to systematically review and recommend antigen selection two times per year. Unfortunately, there is no similar coordinated review system for recommending poultry A(H5) CVVs, although GISRS does make recommendations for human pandemic A(H5) vaccines based on dominant clades.

In addition to antigenic matching, there are numerous other important factors that are known to influence immune responses to influenza vaccines, and the protective efficacy of particular formulations. These factors include the type of vaccine (live attenuated vs. inactivated, antigen concentration or potency, adjuvant), immunisation procedure (dosage and administration schedule), population coverage, and host-dependent factors (age, breed, maternal antibodies, co-infecting pathogens) (Pavade *et al.*, 2011; Swayne *et al.*, 2011; Swayne, 2012a; Swayne, 2012b). In the present research, we focused exclusively on measurements of antigenic relationships among wildtype viruses and vaccine strains, however these other important considerations will be further discussed in **Chapter 5**.

The HI assay is considered the gold standard for antigenic characterisation of influenza viruses and has been used for decades to measure variation among virus subtypes (Donald and Isaacs, 1954; OIE, 2009). Within the GISRS network of laboratories, antigenic characterisation of human seasonal influenza viruses and other animal-origin subtypes with the potential to infect mammals [e.g. HPAI A(H5) and LPAI A(H7N9)] are based on use of reference ferret antisera, since ferrets are considered the optimal animal model for studies of human influenza immunology and pathogenesis. Ferrets are susceptible to infection by a variety of influenza subtypes, and the clinical outcome and immune response are thought to closely mimic those of humans. As such, ferret antisera raised against influenza viruses are considered key reagents required for antigenic characterisation. Unfortunately, experimental studies using the ferret model are expensive, and comprehensive panels of A(H5N1) ferret antisera are rarely available in countries such as Vietnam, where the virus is enzootic in poultry. Importation of serologically influenza-naïve and specific pathogen free ferrets into Vietnam is complicated due to customs procedures and the possibility of infection during transport. In addition, available animal containment facilities within Vietnam are limited in space and resources, and to date, there are no commercial entities that raise ferrets under containment for research purposes. Thus, use of ferrets for experimental research on influenza is not yet feasible in Vietnam. Since chickens are the principle target species for AI virus poultry vaccine interventions, chickens are the logical choice as an animal model for vaccine efficacy testing and for production of reference antisera to evaluate response to immunisation and to measure antigenic variation among vaccines and field strains. In this study, we established techniques for production of reference chicken antisera to assess influenza A(H5N1) virus antigenic matching in Vietnam.

The HI assay is a valuable tool that utilises inexpensive reagents and common laboratory equipment. However, large datasets of cross-HI test results may become difficult to interpret. Computational techniques (e.g., antigenic cartography) are now used widely by researchers to visualise antigenic data and to facilitate comparisons among viruses and /or vaccines (Smith *et al.*, 2004; Smith *et al.*, 2009; Cai *et al.*, 2010; Fouchier and Smith, 2010). Antigenic maps involve transformation of pair-wise serological binding data into distance coordinates between homologous and heterologous titres using multidimensional scaling methods, and then plotting or projecting these distances in 2 or more dimensions. If a 2-dimensional plot is used, the resulting graphic is analogous to a geographical map. The greater the plotted distances on an antigenic map, the greater the antigenic differences between two viruses (Fouchier and Smith, 2010). Antigenic cartography not only provides a visualisation of HI data, but may facilitate identification of specific genetic changes in the HA gene that lead to phenotypic differences observed on the antigenic map (Nelson and Holmes, 2007). Combined genetic and antigenic characterisation can provide insight on which mutations alter the antigenicity of strains and which epitopes in the HA protein are sensitive to directional selection (i.e., immune selection or other host/environmental selective pressures).

When large numbers of strains are evaluated by cross-HI tests, antigenic cartography becomes a useful tool to assess antigenic relationships and patterns of clustering. Our primary motivation for visualising antigenic evolution of A(H5N1) viruses is to inform the evaluation of poultry vaccines (Smith *et al.*, 2004; Smith *et al.*, 2009; Cai *et al.*, 2010; Fouchier and Smith, 2010). To date, the systematic use of ‘cartography’ for vaccine strain selection has been largely applied to analysis of human seasonal influenza, where the pattern of antigenic drift displays a remarkable (and almost predictable) pattern of cluster

shifts. Although these tools have yet to be widely applied to development of veterinary vaccines, research funded by the US Defence Threat Reduction Agency (DTRA) is currently ongoing to use genetically engineered viruses for exploring ‘antigenic space,’ with the goal of designing human pre-pandemic A(H5) CVVs for maximal immune coverage.

In this study, we aimed to 1) produce reference antisera in chickens to identify A(H5N1) clade variants using standard operating procedures previously established at the Southeast Poultry Research Laboratory at the US Department of Agriculture (SEPRL/USDA, Athens, GA); 2) antigenically characterise circulating A(H5N1) viruses in Vietnam using in-country produced reagents; 3) compare quantitative antigenic distances based on chicken antisera versus complementary ferret antisera-based HI datasets; 4) determine antigenic relationships between current circulating viruses and available poultry vaccines to facilitate selection of challenge viruses for use in vaccine efficacy studies and, ultimately, selection of vaccine viruses for use in the field.

4.2. Materials and methodologies

4.2.1. Selection of viruses for antisera production and HI test antigens

Representative Vietnamese A(H5N1) viruses (Table 4.1) were selected for inclusion in the study based on genetic diversity of HA clusters/clades, preliminary data on antigenic diversity (i.e., differential HI titres to vaccine antisera), and presence/absence of amino acid substitutions at known antigenic sites of HA1, as presented in **Chapter 3** (Figure 3.2, Figure 3.3, Figure 3.4, Table 3.3). Additional viruses were included to represent ancestral (prototype) strains of major A(H5N1) clades circulating in Vietnam and other A(H5N1) endemic countries. When vaccine viruses were not available for study, we included the

available wild-type strain with highest HA protein homology.

4.2.2. Propagation of viruses and production of inactivated antigens for chicken immunisations

The selected AI virus isolates were grown in 9 or 10-day old influenza-free, clean-embryonated chicken eggs (C-ECE) (antibody free). 10 C-ECEs per isolate were used and the allantoic fluid was harvested after incubation at 37 °C for 48–72 h under Biosafety Level 2 plus conditions. After harvesting, up to 30 ml of pooled infectious allantoic fluid was transferred into a 50ml tube. The viruses were inactivated by adding beta - propiolactone (BPL) at a final concentration of 0.05% for 4 hours at 37 °C with intermittent stirring and then keeping at 4 °C overnight for hydrolysis of the BPL followed by adjustment of pH to 7.2 using sterile 7.5% NaHCO₃ solution (w/v). The inactivation was confirmed by inoculation in the chorioallantois of 9-day old C-ECE for three passages without any evidence of virus growth as detected by HA test.

The antigens for generating chicken antisera were prepared as close to the time of use as possible (e.g. max. 24-48 hours ahead). Inactivated viral antigen was emulsified with adjuvant Montanide ISA70 (3:7 v:v ratio) by sonication for 15-20 seconds as recommended by the manufacturer (<http://www.seppic.com/animal-health/vaccine-adjuvant/montanide-isa/-montanide-isa-70-vg>). The prepared antigens were transferred to a 50 ml vaccine bottle and stored at 4 °C. Poultry vaccines (Re-1, Re-5, Re-6) were purchased from Harbin (China) and reconstituted prior to use according to manufacturer's instructions.

In contrast to the inactivated chicken immunogens, production of ferret antisera was conducted at the US CDC and involved inoculation of naïve ferrets with live wildtype A(H5N1) viruses; all experiments were conducted under BSL-3 containment with enhancements to meet USDA/Animal and Plant Health Inspection Service (APHIS) biocontainment/biosafety regulations.

4.2.3. Production of sera

Chicken antisera

Chickens were obtained as day-old chicks from a Hanoi commercial hatchery certified free of avian influenza viruses by the DAH in Vietnam. Serum samples were collected from 5 chickens in each experiment to ascertain that the birds were serologically negative for antibodies to the NP protein of influenza A viruses by the commercial ELISA test Flock Check AI (Idexx Laboratories, Westbrook, ME). Clean chickens were kept under standard management practices in the bio-containment (BSL 2 plus) animal facility at the NCVD and provided autoclaved feed and water before and during the experiments. Five-week-old chickens (5 chickens per group) were immunised subcutaneously in the nape of the neck with 0.5 ml of immunogen/adjuvant (with at least 1280 haemagglutinating units (HAU) of virus mixed with MontanideTM ISA 70 VG adjuvant) using a 5ml syringe and 22g needle. Sera were collected at 3 weeks post inoculation. Chickens were sedated by intravenous inoculation of ketamine/xylazine based on body weight. Blood was collected by cardiac puncture using a 10ml or 20ml syringe and 18g needle, depending on the size of the bird. Tubes with whole blood were incubated for 3-5 hours at 37°C on their sides and placed at 4 °C overnight to maximise serum volume. Sera were harvested by centrifuging to pellet the clot. Sera were decanted and stored at -20 °C in small aliquots till further use.

Procedures for animal work at the NCVD were adapted from the OIE (available at:

http://web.oie.int/eng/normes/mcode/en_chapitre_1.7.8.htm) and approved by the DAH of Vietnam.

Ferret antisera

Outbred, male ferrets with body weights ranging from 900-1800 g and less than one year of age were obtained from Triple F Farms (Sayre, PA). Ferrets were tested for HI antibody against human influenza A and B viruses. Virus-specific polyclonal antisera against 14 viruses were generated using serologically naïve ferrets inoculated with an intranasal (i.n.) dose of 10^6 EID₅₀ of live virus diluted in PBS. At 14 days post-inoculation, each ferret was boosted by subcutaneous injection in both hind legs with at least 2048 HAU of virus mixed with Titermax Gold Adjuvant. Antisera were collected 14 days post-boost. All work with animals was performed with protocols approved by US CDC's Institutional Animal Care and Use Committee.

4.2.4. HI assays comparing chicken versus ferret antisera

The influenza virus haemagglutinin (HA) protein mediates receptor binding to sialic acids on the membranes of specific cell types, such as epithelial cells, in the respiratory tract (Baum and Paulson, 1990; Couceiro *et al.*, 1993; Skehel and Wiley, 2000) and red blood cells (RBCs). Binding of sialic acids on RBCs in the presence of viral antigen results in haemagglutination. Titrering the presence of HA-specific antibodies through an inhibition assay requires selection of appropriate source of RBC (e.g. turkey, guinea pig, horse, chicken), since the affinity of the HA globular head for sialic acid varies among species and among influenza subtypes (Rogers and Paulson, 1983; Ryan-Poirier and Kawaoka, 1991; Connor *et al.*, 1994; Imai and Kawaoka, 2012). For detection of human antibodies to

A(H5), horse RBCs have traditionally been used because they are thought to have the appropriate sialic acid moieties (a predominance of $\alpha(2,3)$ -linkages), and have been observed to yield best sensitivity, particularly for detecting low titres. For titration of chicken antisera to detect antibodies to A(H5), we chose to use chicken RBCs because chickens express a mixture of sialic acid $\alpha(2,3)$ -galactose and sialic acid $\alpha(2,6)$ -galactose linkages (Connor *et al.*, 1994; Ito *et al.*, 1997; Ducatez *et al.*, 2011); the observed results from past experimental manipulations at NCVD demonstrated good sensitivity; we were following recommendations of the USDA SEPRL protocols. For practical reasons, there was not sustainable supply of alternative RBCs from horse, turkey, or guinea pig. Finally, similar to other published work, we had noted that the agglutination phenotype when using chicken RBCs tended to yield a clearer result (more compact button with non-agglutinated RBCs compared to well-dispersed lattice formation due to viral agglutination) when compared to mammalian cells, thus facilitating reading and interpretation of results (Pawar *et al.*, 2012). For all HI results produced at NCVD, chicken RBCs were used to determine antibody titres to influenza A(H5) viruses.

Antigenic characterisation was performed using post-infection ferret antisera and immunised chicken sera raised against the selected viruses used in this study. Both chicken and ferret sera were heat inactivated at 56 °C for 30 minutes, absorbed with packed chicken red blood cells (CRBCs) to remove non-specific agglutinators, and tested by HI assay with 0.5% CRBCs. All antisera were diluted so that the lowest dilution was 1:10 in PBS. The HI assays were performed in accordance with standard procedures (USDA, 2013). Titres were calculated as the reciprocal of the last HI positive serum dilution.

4.2.5. Antigenic cartography construction

Antigenic maps were used to infer antigenic differences among virus strains, with each titre in the HI table specifying the relative distance for the virus and serum points in the antigenic map (Smith *et al.*, 2004).

Antigenic cartography was performed using the HI tables containing titres of a set of sera against a panel of antigens. An antigenic map enables a quantitative and visual interpretation of the binding assay data, incorporating the multiple serum-virus relation measurements to enable reliable inferences about antigenic differences among strains (Smith *et al.*, 2004). An HI titre between an antibody (i.e. serum) and antigen (i.e. virus) is converted into a distance by calculating the difference between the $\log_2$ (HI titre) of a virus-serum pair and the maximum $\log_2$ (HI titre) of that serum against any virus. Therefore, a serum with a high HI titre will have a shorter distance, and be placed more closely to that virus, than a serum with a lower HI titre. The viruses and sera are subsequently positioned in the map using multidimensional scaling by minimising the difference between the target distances and the distances in the antigenic map (500 dimensional annealing runs were performed as random restarts to avoid bias from local optima). Titre differences are shown in two-fold dilutions (denoted as 1 antigenic unit) with 2 grid units corresponding to a four-fold dilution in the HI assay, 3 units equal to an 8-fold dilution, and so on. When calculating an antigenic map, it is not necessary for the HI table to include the homologous sera matching the viruses tested, because any three different (serum) data points can triangulate a virus data point, as long as the titres are numeric. We have more than three ferret serum data points for all viruses, and thus the redundancy in the data allow good coordination of the viruses and sera (as indicated by small symbol sizes in the figures).

The tabulated HI results for each dataset (chicken sera, ferret sera, and combined chicken and ferret sera) were formatted for processing and imported to the Antigenic Cartography server (<https://acmacs-web.antigenic-cartography.org/>) to generate antigenic maps.

Metadata associated with all reference and test antigens were prepared in Microsoft Office Excel 2010 spreadsheets with an antigen table (test, antigen ID, strain name, clade, and abbreviation); antisera table (test, serum ID, serum strain, serum clade, abbreviation, serum type). Antigenic maps were visualised using Tableau version 9.1 by importing x/y coordinate output files and linking each coordinate with reference or test antigen metadata. Tableau reader version 9.1 was used to visualise cartography.

4.3. Results

4.3.1 Viruses used in this study and HI data matrices

The viruses included in the analysis comprised a total of 85 Vietnamese A(H5N1) viruses collected from 2001 to 2014 and an additional 15 representative A(H5N1) CVVs from other countries collected between 1996 and 2010 (Table 4.1). Together these represent 21 A(H5N1) clades. Poultry vaccine viruses included were Re-1 (A/goose/Guangdong/1/1996) and viruses demonstrated to be genetically related to vaccine strains Re-4 (A/chicken/Shanxi/2/2006), Re-5 (A/duck/Anhui/1/2006), and Re-6 (A/duck/Guangdong/S1322/2006).

In this study, we selected representative genetically divergent Vietnamese A(H5N1) viruses for comprehensive antigenic characterisation, including all 16 A(H5N1) clades that have circulated in Vietnam since 2005. However, clade 7.2 viruses were removed from the antigenic maps since there were no available antisera raised against this clade and they showed little cross-reactivity to heterologous antisera.

The HI data generated using 18 different ferret antisera involved cross-HI tests with 15 homologous antigens and 25 test antigens. The HI data generated using chicken antisera involved cross-HI tests between each of the experimentally produced reference chicken antisera ($n = 29$) with their homologous antigen, plus testing of 65 heterologous antigens (circulating field viruses). Post vaccination sera of Re-1, Re-5, and Re-6 vaccines were titrated against the full complement of antigens (29 reference viruses and 65 test antigens). For 14 viruses, we had access to both ferret and chicken antisera raised against the same virus; these sera were titrated against the 14 homologous antigens plus 28 test antigens.

Table 4.1: Viruses used in the study. Antigens were produced in embryonating eggs for all strains. Chicken antisera were produced for strains indicated in red, and are thus designated ‘reference’ antigens.

	Isolate name	Abbreviation	Viral status*	Clade	Antigen type
1	A/goose/Guangdong/1/1996	GD/1/96	WT	0	Reference
2	A/Vietnam/1203/2004	VN/1203/04	WT	1	Reference
3	A/duck/Vietnam/NCVD-016/2007	DK/VN/016/07	WT	1.1	Reference ^a
4	A/Cambodia/R0405050/2007	CB/R0405050/07	WT	1.1	Reference
5	A/chicken/Vietnam/NCVD-1192/2012	CK/VN/1192/12	WT	1.1	Reference ^a
6	A/Indonesia/5/2005	INDO/5/05	WT	2.1.3.2	Test
7	A/Indonesia/5/2005 (CDC-RG2)	INDO/5/05 RG2	RG	2.1.3.2	Reference
8	A/turkey/Turkey/1/2005	TK/1/05	WT	2.2.1	Reference
9	A/Egypt/321-Namru3/2007	EG/321/07	WT	2.2.1	Reference
10	A/Egypt/321-Namru3/2007 (IDCDC-RG11)	EG/321/07 RG11	RG	2.2.1	Reference ^a
11	A/Egypt/N03072/2010 (IDCDC-RG29)	EG/N03072/10 RG29	RG	2.2.1	Reference
12	A/Common magpie/Hongkong/5052/2007	CM/HK/5052/07	WT	2.3.2.1	Reference ^a
13	A/Hubei/1/2010 (IDCDC-RG30)	HB/1/10 RG30	RG	2.3.2.1a	Reference
14	A/chicken/Vietnam/NCVD-675/2011	CK/VN/675/11	WT	2.3.2.1a	Reference ^a
15	A/duck/Vietnam/NCVD-1207/2012	DK/VN/1207/12	WT	2.3.2.1a	Reference ^a
16	A/barn-Swallow/Hongkong/D10-1161/2010	BS/HK/1161/10	WT	2.3.2.1b	Reference
17	A/duck/Vietnam/NCVD-672/2011	DK/VN/672/11	WT	2.3.2.1b	Reference
18	A/duck/Vietnam/NCVD-1163/2012	DK/VN/1163/12	WT	2.3.2.1b	Reference ^a
19	A/Barheaded goose/Mongolia/X53/2009	BG/MG/X53/09	WT	2.3.2.1c	Reference ^a
20	A/Hongkong/6841/2010	HK/6841/10	WT	2.3.2.1c	Reference
21	A/duck/Vietnam/NCVD-1648/2012	DK/VN/1648/12	WT	2.3.2.1c	Reference ^a
22	A/duck/Vietnam/NCVD-1544/2012	DK/VN/1544/12	WT	2.3.2.1c	Reference
23	A/duck/Vietnam/NCVD-1584/2012	DK/VN/1584/12	WT	2.3.2.1c	Reference ^b
24	A/duck/Vietnam/NCVD-003/2008	DK/VN/003/08	WT	2.3.4	Reference ^a
25	A/duck/Vietnam/NCVD-391/2009	DK/VN/391/09	WT	2.3.4	Reference ^a
26	A/Japanese-white-eye/Hongkong/1038/06	JW/HK/1038/06	WT	2.3.4	Reference ^a
27	A/chicken/India/NIV33487/2006 (IBCDC-RG7)	CK/IND/NIV33487/06 RG7	RG	2.3.4	Reference ^a
28	A/Anhui/1/2005	ANH/1/05	RG	2.3.4	Reference
29	A/duck/Vietnam/NCVD-293/2009	DK/VN/293/09	WT	2.3.4.1	Reference
30	A/chicken/Vietnam/NCVD-35/2008	CK/VN/35/08	WT	2.3.4.2	Reference ^a
31	A/chicken/Vietnam/NCVD-279/2009	CK/VN/279/09	WT	2.3.4.3	Reference ^a
32	A/chicken/Vietnam/NCVD-016/2008	CK/VN/016/08	WT	7.1	Reference ^a
33	A/chicken/Vietnam/NCVD-016/2008 (IDCDC-RG12)	CK/VN/016/08 RG12	RG	7.1	Reference ^a
34	A/chicken/Vietnam/NCVD-3/2008 (IDCDC-RG25A)	CK/VN/3/08 RG25A	RG	7.1	Reference
35	A/goose/Vietnam/113/2001	GS/VN/113/01	WT	3	Test
36	A/duck/Vietnam/NCVD-1/2002	DK/VN/1/02	WT	5	Test
37	A/Whooper Swan/Mongolia/244/2005 (Ag8)	WS/MG/244/05 AG8	WT	2.2	Reference ^b
38	A/duck/Vietnam/10/2005	DK/VN/10/05	WT	2.3.2	Test
39	A/duck/Vietnam/NCVD-19/2003	DK/VN/19/03	WT	1	Test
40	A/duck/Vietnam/NCVD-2153/2012	DK/VN/2153/12	WT	1.1.2	Test
41	A/chicken/Vietnam/NCVD-2750/2013	CK/VN/2750/13	WT	1.1.2	Test
42	A/chicken/Vietnam/NCVD-1096/2013	CK/VN/1096/13	WT	7.2	Test
43	A/chicken/Vietnam/NCVD-93/2008	CK/VN/93/08	WT	7.2	Test
44	A/chicken/Vietnam/NCVD-2749/2013	CK/VN/2749/13	WT	2.3.2.1a	Test
45	A/chicken/Vietnam/NCVD-2693/2013	CK/VN/2693/13	WT	2.3.2.1b	Test
46	A/duck/Vietnam/NCVD-2745/2013	DK/VN/2745/13	WT	2.3.2.1c	Test
47	A/chicken/Vietnam/NCVD-14-A318/2014	CK/VN/A318/14	WT	2.3.2.1c	Test
48	A/duck/Vietnam/NCVD-14-A332/2014	DK/VN/A332/14	WT	2.3.2.1c	Test

* WT: Wild type; RG: Reverse genetics produced strains, PR8 attenuated constructs with HA inserts of wildtype virus, with polybasic cleavage site removed.

^a Reference antigen for chicken model only

^b Reference antigen for ferret model only

4.3.2. Collection and preliminary analysis of chicken antisera

The average volume of antisera harvested from individual chickens was 6.5ml (ranging from 4 to 13 ml). Antisera were subsequently tested in HI assays using all reference viruses. Mean HI titres induced by heterologous viruses varied from 1:40 to 1:640 (Table 4.2). The homologous HI titres varied from 1:160 to 1:1280 in ferrets and from 1:40 to 1:1280 in chickens (Table 4.2). For 4 of 14 viruses (29%) the homologous HI titres raised in chickens and ferrets were equivalent (with titres ranging from 1:320 to 1:1280); for 8 of 14 viruses (57%) the HI titre raised in ferrets was higher than chickens (ranging from 2-fold to 8-fold higher); and for 2 of 14 viruses (14%), the antisera raised in chickens yielded higher homologous HI titres (ranging from 2- to 4- fold difference) (Table 4.2).

Table 4.2: Preliminary analysis of chicken antisera

	Isolate name	Abbreviation	Clade	Chicken antisera		Ferret sera	
				# lots* (volume ml)	HI titre GMT (range)	HI titre	HI fold difference
1	A/goose/Guangdong/1/1996	GD/1/1996	0	2 (7)	80 (80-80)	640	8
2	A/Vietnam/1203/2004	VN/1203/04	1	5 (36)	304 (80-640)	320	1
3	A/duck/Vietnam/NCVD-016/2007	DK/VN/016/07	1.1	5 (38)	40 (40-40)		
4	A/Cambodia/R0405050/2007	CB/R0405050/07	1.1	4 (34)	40 (40-40)		
5	A/chicken/Vietnam/NCVD-1192/2012	CK/VN/1192/12	1.1	5 (29)	144 (80-160)		
6	A/Indonesia/5/2005 (CDC-RG2)	INDO/5/05 RG2	2.1.3.2	4 (36)	160 (160-160)	1280	8
7	A/turkey/Turkey/1/2005	TK/1/05	2.2.1	4 (32)	260 (80-320)	640	2
8	A/Egypt/321-Namru3/2007	EG/321/07	2.2.1	5 (35)	160 (160-160)	160	4
9	A/Egypt/321-Namru3/2007 (IDCDC-RG11)	EG/321/07 RG11	2.2.1	3 (26)	40 (40-40)		
10	A/Egypt/N03072/2010 (IDCDC-RG29)	EG/N03072/10 RG29	2.2.1	5 (37)	288 (160-320)	1280	1
11	A/Common magpie/Hongkong/5052/2007	CM/HK/5052/07	2.3.2.1	4 (28)	80 (80-80)		
12	A/Hubei/1/2010 (IDCDC-RG30)	HB/1/10 RG30	2.3.2.1a	5 (22)	80 (80-80)	640	8
13	A/chicken/Vietnam/NCVD-675/2011	CK/VN/675/11	2.3.2.1a	5 (30)	136 (40-160)		
14	A/duck/Vietnam/NCVD-1207/2012	DK/VN/1207/12	2.3.2.1a	5 (34)	80 (80-80)		
15	A/barn-Swallow/Hongkong/D10-1161/2010	BS/HK/1161/10	2.3.2.1b	5 (26)	72 (40-80)	160	2
16	A/duck/Vietnam/NCVD-672/2011	DK/VN/672/11	2.3.2.1b	4 (29)	420 (80-640)	160	4#
17	A/duck/Vietnam/NCVD-1163/2012	DK/VN/1163/12	2.3.2.1b	5 (36)	136 (40-160)		
18	A/Barheaded goose/Mongolia/X53/2009	BG/MG/X53/09	2.3.2.1c	3 (17)	745 (320-1280)		
19	A/Hongkong/6841/2010	HK/6841/10	2.3.2.1c	5 (34)	288 (80-640)	640	1
20	A/duck/Vietnam/NCVD-1648/2012	DK/VN/1648/12	2.3.2.1c	5 (32)	256 (160-320)		
21	A/duck/Vietnam/NCVD-1544/2012	DK/VN/1544/12	2.3.2.1c	5 (33)	288 (80-640)	160	2#
22	A/duck/Vietnam/NCVD-003/2008	DK/VN/003/08	2.3.4	5 (41)	80 (80-80)		
23	A/duck/Vietnam/NCVD-391/2009	DK/VN/391/09	2.3.4	5 (27)	80 (80-80)		
24	A/Japanese-white-eye/Hongkong/1038/06	JW/HK/1038/06	2.3.4	5 (30)	240 (80-320)		
25	A/chicken/India/NIV33487/2006 (IBCDC-RG7)	CK/IND/NIV33487/06 RG7	2.2	5 (26)	80 (80-80)		
26	A/Anhui/1/2005	ANH/1/05	2.3.4	5 (31)	128 (80-160)	640	4
27	A/duck/Vietnam/NCVD-293/2009	DK/VN/293/09	2.3.4.1	4 (24)	80 (80-80)	640	8
28	A/chicken/Vietnam/NCVD-35/2008	CK/VN/35/08	2.3.4.2	5 (32)	160		
29	A/chicken/Vietnam/NCVD-279/2009	CK/VN/279/09	2.3.4.3	4 (25)	70 (40-80)		
30	A/chicken/Vietnam/NCVD-016/2008	CK/VN/016/08	7.1	3 (15)	80 (80-80)		
31	A/chicken/Vietnam/NCVD-3/2008 (IDCDC-RG25A)	CK/VN/3/08 RG25A	7.1	4 (27)	560 (320-640)	640	1
32	A/goose/Guangdong/1/1996 (Re-1)	Re-1 vaccine	0	5 (31)	136 (40-160)		
33	A/duck/Anhui/1/2006 (Re-5)	Re-5 vaccine	2.3.4	5 (26)	144 (80-160)		
34	A/duck/Guangdong/S1322/2010 (Re-6)	Re-6 vaccine	2.3.2.1b	5 (34)	144 (80-160)		

* Each lot represented sera harvested from an individual chicken; # Ratio of chicken/ferret HI titter.

4.3.3. Antigenic analysis of specified A(H5N1) virus isolates

Following the conventions and terminology of antigenic characterisations of serological datasets, we refer to ‘two-way’ versus ‘one-way’ analyses of HI titre matrices. Two-way analyses refer to interpretations of antigenic relationships based on complete datasets, in which each virus is represented by both an antigen and its homologous antiserum. For two-way analyses, the spatial coordinates for a given antigen or antiserum reflect the differential reactivity to both homologous and heterologous viruses. For the purposes of comparing chicken versus ferret models, we first performed two-way analyses, to assess whether the chicken and ferrets models generated antisera with comparable specificities. In contrast to ‘two-way’ analyses, a ‘one-way’ analysis involves interpretation of antigenic relationships when a given virus is represented solely by the viral antigen, because no homologous antiserum has been produced (in this case the antigens are called ‘test’ antigens). For one-way analyses, the spatial coordinates for a given antigen are derived from the combined reactivity of a given antigen to a panel of heterologous antisera. The most common scenario for cartography is to have a small number of reference antigens, and a much larger number of test antigens, because antisera are expensive and time-consuming to generate.

4.3.3.1. Chicken-based map

Antigenic distances between viruses based on HI analysis using chicken antisera are displayed in Figure 4.1. Each square grid represents the 2-fold dilution of HI titres. The two-way HI analysis of chicken antisera shows that viruses within clades 1, 2.3.2.1, 2.3.4 and 7.1 reacted strongly with antisera against viruses from the same clade, indicating close antigenic relationships within each clade. This can also be seen from the chicken based-map (Figure 4.1). Clade 1 viruses clustered together within 4-fold distances. Similarly, the

majority (73%, $n = 8/11$) of clade 2.3.2.1 viruses mapped within 4-fold distances of each other, however, some viruses (27%, $n = 3/11$) showed 8-fold reduced titres to other viruses within clade 2.3.2.1. All clade 2.3.4 viruses showed strong intra-clade clustering within 4-fold of 6/8 clade 2.3.4 antisera, whereas the 2 remaining clade 2.3.4 antisera had 8-fold reduced titres to the clade 2.3.4 viruses. Average clustering of subclade 2.3.2.1b viruses was less evident than for viruses of subclades 2.3.2.1a and 2.3.2.1c. The estimated coordinates of Re-6 vaccine virus (based on nearest homologous strain of 2.3.4.1b) exhibited an average 5-fold titre difference to other viruses within the subclade.

The Re-1 vaccine antigen [with HA and NA genes from clade 0 A/goose/Guangdong/1/1996 A(H5N1) virus (GS/1)] showed strong reactivity with antisera raised against both clade 1 and 2.3.4 viruses, and the antisera raised with the Re-1 vaccine showed reciprocal strong reactivity to both clade 1 and 2.3.4 antigens. In contrast, antisera against Re-1 showed little to no inhibition of clade 2.3.2.1 and 7.1 viruses. The Re-5 antisera [clade 2.3.4; A/duck/Anhui/1/2006 (AH/1)] reacted well against clade 1 and clade 2.3.4 antigens, whereas Re-6 antisera (clade 2.3.2.1b, A/duck/Guangdong/S1322/2006) antiserum reacted well with only the clade 2.3.2.1 antigens. Clade 7.1 viruses demonstrated relatively close antigenic relationships to one another but were outliers compared to all other clades; the three data points on the chicken-based map demonstrated relatively close antigenic relationships to one another on the chicken-based map.

For the one-way HI analyses that included many more antigen data points (Appendix 23), comparable titre profiles were observed for all members of clade 1, and subclades 1.1.1 and 1.1.2 viruses. Similarly, the addition of thirty-nine test antigens from clade 2.3.2.1 viruses (comprising 10 clade 2.3.2.1a, 7 clade 2.3.2.1b and 22 clade 2.3.2.1c viruses),

demonstrated moderate reactivity to most of within-clade heterologous antisera (64%, $n = 7/11$), at titres within 4-fold of each other, but had no reactivity to clade 1, 2.3.4 and 7 viruses. The clade 2.3.2.1a viruses were antigenically separated into two clusters, whereas the clade 2.3.2.1c viruses overlapped within 2-fold on the chicken-based antigenic map and also clustered with some clade 2.3.2.1a viruses (Figure 4.1). The clade 2.3.2.1b viruses were antigenically distinct from the other clade 2.3.2.1 viruses. Similar to the two-way analyses, clade 2.3.2.1a and 2.3.2.1b viruses clustered well together within 4-fold changes, but had greater antigenic distances compared to clade 2.3.2.1c viruses (within 2-fold difference). Clade 7.2 viruses had no reactivity to the clade 7.1 antisera tested. Although the clade 2.3.2.1b A/duck/Vietnam/NCVD-672/2011 virus reacted well with the genetically related clade 2.3.2.1b viruses, it did not react well with antisera raised against clade 2.3.2.1a or 2.3.2.1c viruses (with reduction of 8- to 64-fold difference compared to heterologous HI titres of clade 2.3.2.1a and 2.3.2.1c). These relationships can be observed from the HI data matrices as well as the chicken-based antigenic map.

Similar to results of the two-way HI titre comparison, vaccine antisera Re-1 and Re-5 showed reactivity to both clade 1 and 2.3.4 antigens. Re-6 antiserum showed specific reactivity with only the clade 2.3.2.1 viruses. Clade 7.1 viruses were antigenically related to each other and remained outliers compared to all other clades. For viruses that have never circulated in Vietnam (i.e. clade 2.2, 2.2.1, 2.1.3), little reactivity was observed to Vietnamese clade viruses. In general, the results of two-way and one-way analyses of chicken antisera were entirely compatible.

4.3.3.2. Ferret-based map

The antigenic distance between viruses based on HI analysis of ferret antisera is displayed in Figure 4.2.

The two-way analyses of ferret antisera and the ferret based-map (Figure 4.2; Appendix 22) show a similar trend of clade clustering to the chicken-based map (Figure 4.1) for clades 1, 2.3.2.1, 2.3.4 and 7.1. Clade 1 viruses reacted well against heterologous antisera within 2 or 4 fold antigenic distance in both ferret and chicken-based maps. Clade 2.3.2.1 viruses in the ferret-based map are clustered relatively closely, reflecting the high cross-reactivity among viruses of this group, which produced heterologous titres within a two-fold difference within the subclade, but exhibited lower reactivity to genetically more distant clades. Because the ferret HI included only two clade 2.3.4 viruses compared with eight clade 2.3.4 viruses used in the chicken-based map, a direct comparison between chicken sera and ferret sera was not possible. A(H5N1) viruses included on the map that have never circulated in Vietnam (i.e., the Egyptian clade 2.2.1, and Indonesian clade 2.1.3 viruses), did not react strongly against heterologous antisera and thus did not show a consistent pattern of clustering across the chicken and ferret maps.

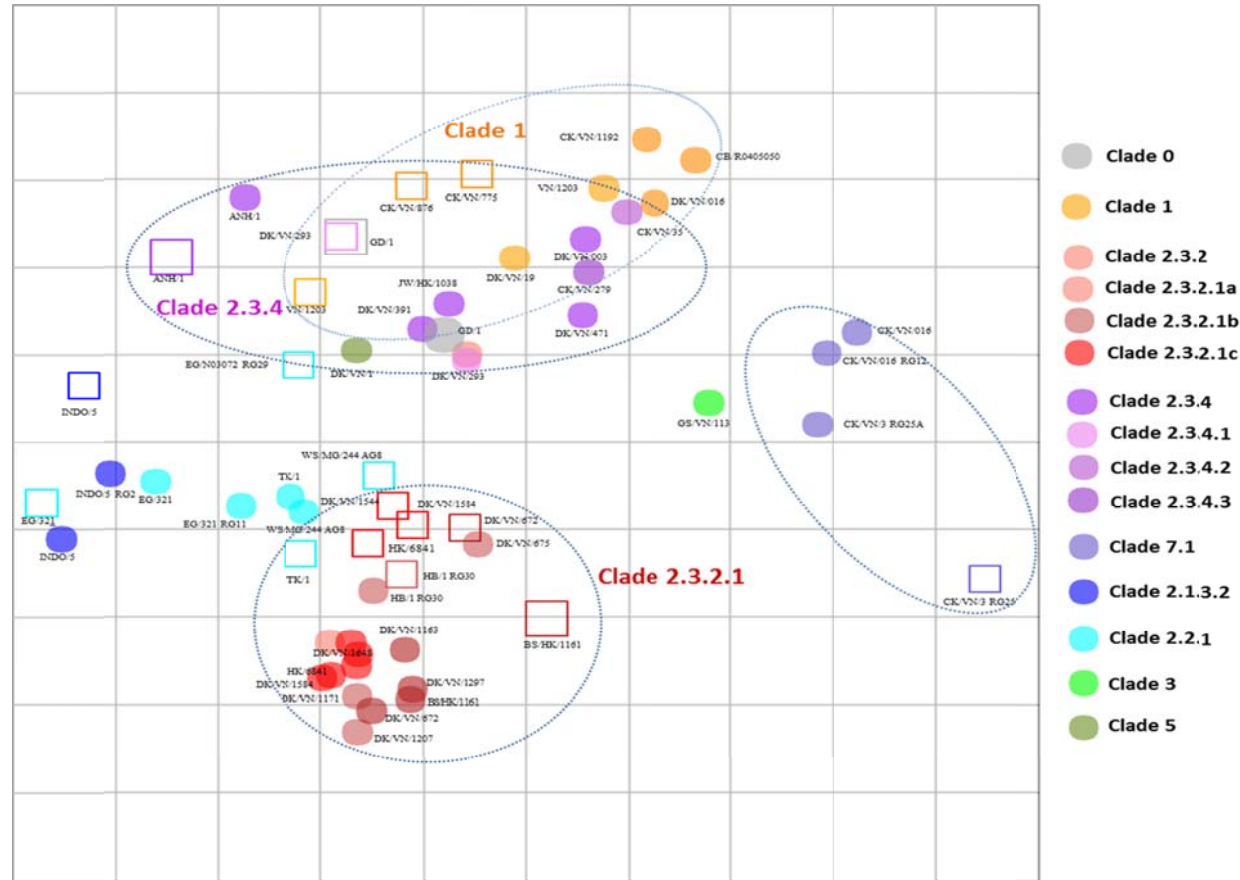


Figure 4.2: Antigenic maps of influenza A(H5N1) HA based on HI immune responses to ferret antisera. Both vertical and horizontal axes represent antigenic distance. Grid lines represent 1 antigenic distance unit, corresponding to a 2-fold dilution in the HI assay. Different antigenic clusters are indicated with different colours. Filled circles indicate antigens, open squares indicate antisera.

Using ferret antisera for one-way analysis of additional test antigens yielded heterologous HI titres of sera tested against related and unrelated A(H5N1) clade viruses ranging from 1 to 64-fold difference compared to homologous titres. From the ferret-based map (Figure 4.2), we see the majority of clade 2.3.2.1 (86%, $n = 12/14$) and clade 7.1 (67%, $n = 2/3$) viruses clustered closely together with heterologous titres within two-fold of homologous titres of genetically related viruses. Clade 2.3.4 and 1 viruses clustered close to each other, however variation was still observed within each clade and subclade. The clade 1 viruses tested using chicken antisera displayed shorter antigenic distances (4-fold difference) than HI tests with ferret antisera (8-fold distances), which may be explained by the addition of clade 1.1.1 and 1.1.2 antigens included in the chicken HI test. Eight out of nine of clade 2.3.4 viruses in the ferret-based map were inhibited by antisera against other clade 2.3.4 viruses and homologous antisera at titres within 4-fold of each other, in contrast, 9/10 clade 2.3.4 viruses in the chicken-based map were inhibited within only 2-fold distance.

4.3.3.3. Combined chicken and ferret antisera-based map

In general, the homologous HI titres indicated that four of fourteen pairs (28%) of chicken/ferret antisera had identical homologous HI titres (with titres ranging from 1:320 to 1:1280); eight pairs (57%) had HI titres higher in ferrets than chickens, ranging from 2- to 8-fold higher (2 pairs had 2-fold, 2 pairs had 4-fold and 4 pairs had 8-fold differences) and 2 pairs (14%) had higher HI titres in chickens compared to ferrets (1 pair had 2-fold difference, 1 pair had 4-fold difference).

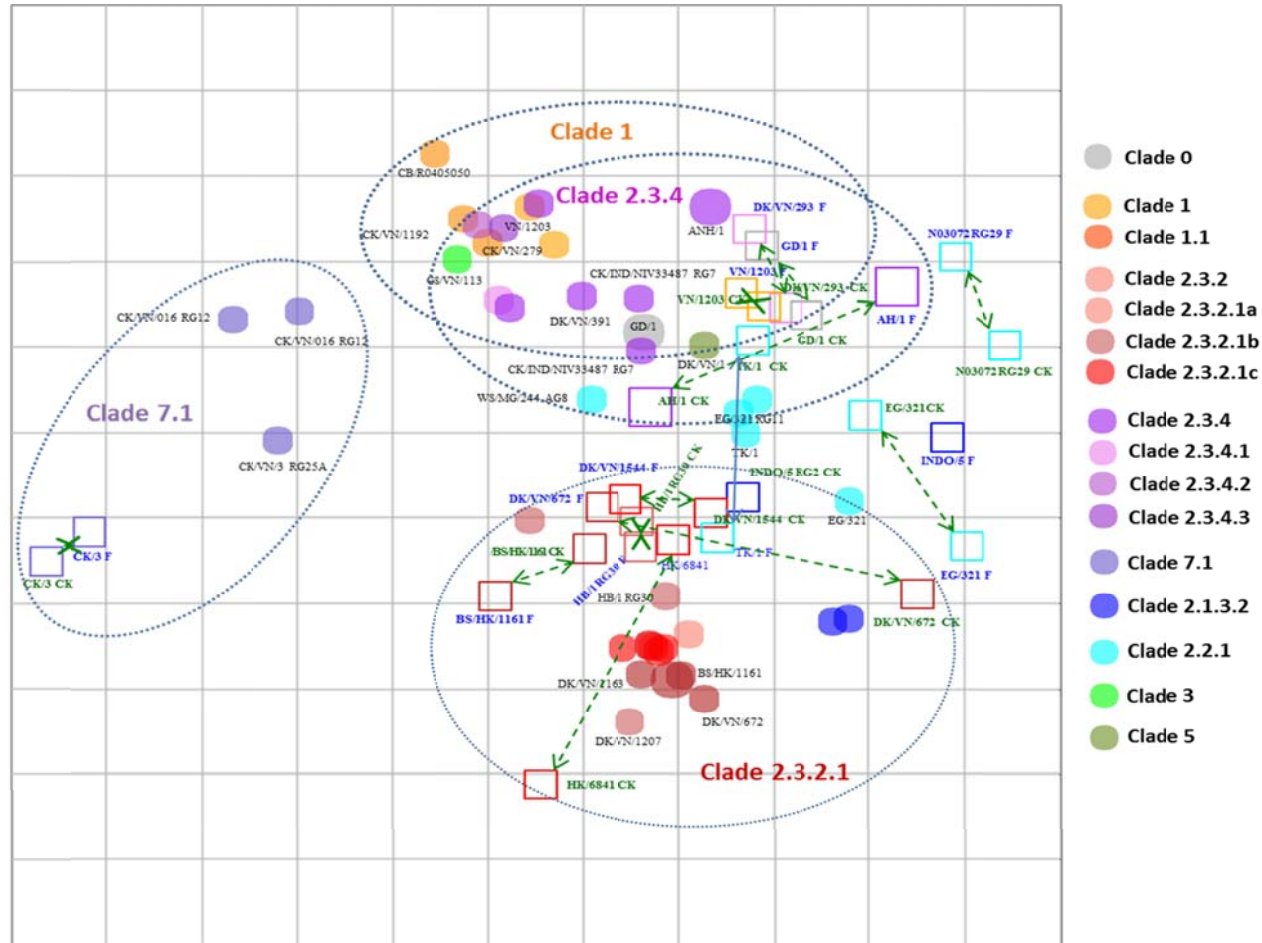


Figure 4.3: Map showing inferred relationships (antigenic distance units) between pairs of chicken and ferret reference antisera raised against the same viruses. Grid lines represent 1 antigenic distance unit, corresponding to a 2-fold dilution in the HI assay. Different antigenic clusters are indicated with different colours. Filled circles indicate antigens, open squares indicate antisera. Green arrows with dotted lines indicate chicken and ferret antisera pairs.

Side by side comparisons of chicken and ferret antisera run in the same HI test are shown in Figure 4.3. The majority of antiserum pairs mapped to within proximate locations of each other (71%, $n = 10/14$). Antiserum pairs for clade 7.1 ($n = 1$), clade 1 ($n = 1$) and clade 0 ($n = 1$) showed nearly identical positions, as did most pairs in clade 2.3.2.1 ($n = 3/5$) and 2.3.4 ($n = 1/2$). However, three antigens that had homologous HI titres within 4- to 8-fold to chicken and ferret antisera, displayed greater fold reductions in side by side comparison of heterologous titres. For instance, the clade 2.3.2.1 A/Hong Kong/6841/2010 (HK/6841) antigen had identical homologous titres to the chicken/ferret antiserum pair, however, the ferret antiserum displayed broad reactivity against unrelated clade viruses, whereas the chicken antiserum did not. Similarly, the clade 2.3.2.1 A/duck/Vietnam/NCVD-672/2011 (DK/VN/672) antiserum pairs had homologous HI titres within 4-fold of each other, while heterologous titres with non-clade 2.3.2.1 viruses varied between 8- to 32- fold as expected. In particular, DK/VN/672 ferret antiserum was able to inhibit clade 2.3.4 viruses, whereas DK/VN/672 chicken antisera did not. Together, these results suggest that the DK/VN/672 virus did not produce clade-specific antisera in either the chicken or ferret model. The clade 2.3.4 A/duck/Vietnam/NCVD-293/2009 (DK/VN/293) antigen also displayed greater fold reductions (between 8- to 32-fold differences) when comparing reactivity to chicken versus ferret antisera. Closer analysis of the HI table indicated that 90% of all antigens ($n = 38/42$) were broadly inhibited by the A/duck/Vietnam/NCVD-293/2009 chicken antiserum, thus confounding the relative distance measures to different strains. There are many possible explanations for this pattern of non-specific inhibition: e.g. the chicken antisera production could have been compromised due to contamination of the inoculum with multiple viral subtypes, bacterial agents, or other immunogenic materials; or non-specific inhibitors may have been present in this antiserum. Another virus, clade 2.1.3.2 A/Indonesia/5/2005 virus, also displayed

greater fold reductions (between 8- to 32-fold differences) when comparing reactivity to the homologous chicken and ferret antisera. The HI table shows it produced a very high homologous HI titre of 1:1280 in the ferret, whereas the immune response in the chicken produced a titre of 1:160 (8-fold difference). This could be an anomaly in the ferret antiserum produced against this virus, suggesting that careful evaluation of initial homologous titres is warranted prior to use in routine HI testing.

4.4. Discussion

Vietnam is one of four countries where poultry A(H5) vaccination has become routine practice in the fight against HPAI (the other countries are China, Indonesia, and Egypt). To date, most government-supported poultry vaccination programs have employed inactivated oil-in-water emulsion recombinant PR8-backbone vaccines imported from the Chinese company Harbin (the so-called Re-X vaccines). These vaccines contain the internal genes from the high growth attenuated A/Puerto Rico/8/34 (PR8) virus, and the HA and NA genes from various A(H5N1) clade variants. Phylogenetic analyses of whole genome sequences of A(H5N1) viruses isolated from poultry outbreaks in Vietnam have identified a total of 56 genotypes and circulation of at least 16 distinct clades (Wan *et al.*, 2008; Davis *et al.*, 2010; Tung *et al.*, 2012; Creanga *et al.*, 2013; Dau *et al.*, 2013; Nguyen *et al.*, 2014; Nguyen *et al.*, 2016a). From 2012 to 2015, six clades (1.1.2, 2.3.2.1a-c, 2.3.4.4 and 7.2) circulated, and the most recent surveillance data show that clades 2.3.2.1c and 2.3.4.4 viruses predominated in 2015 and 2016. Given the rapid evolution and frequent introduction of new A(H5) clade variants, evaluating and updating vaccine formulations presents a continual challenge. Maintaining a comprehensive panel of appropriate reference antisera for strain characterisations could greatly facilitate vaccine evaluations

through improved understanding of relationships between HI titres, cross-immunity, and protection profiles in vaccinated birds.

Ferret antisera against wildtype A(H5N1) viruses are generally only produced in WHO collaborating centres and are produced against a limited number of representative variants. These antisera may not be readily available to laboratories in A(H5N1) endemic countries until several months post-emergence. Production of ferret antisera in resource-limited settings such as Vietnam is currently not feasible. In this study, we explored using post-immunisation chicken sera for in-depth antigenic characterisations of A(H5N1) influenza viruses, and report on a limited number of side-by-side comparisons of ferret and chicken antisera raised against the same viral antigen.

In general, antigenic distance measures (using multidimensional scaling) and ‘mapping’ approaches applied to both the chicken and ferret HI datasets suggested similar patterns of clustering that corresponded well to clade differences as measured by HA phylogenies. Clade 2.3.2.1 viruses were inhibited by antisera at titres within 4-fold of each other, but exhibited little or no reactivity to the clade 1, 7 and 2.3.4 viruses. Cross-HI tests for the group of 2.3.2.1 viruses yielded heterologous titres within two-fold of homologous titres of genetically related viruses; these HI results translated into average intra-clade antigenic distance measures of 2.13 ADU (ranged from 0.15 to 4.95), versus inter-clade differences of 3.84 ADU (ranged from 0.53 to 9.01). While clade 2.3.2.1c viruses exhibited a high degree of homogeneity in antigenic profile (both serum and antigen points were tightly clustered), the 2.3.2.1a and 2.3.2.1b subclades were not consistently inhibited by 2.3.2.1c antisera and vice versa (Figure 4.1). The clade 7.1 viruses, which in Vietnam have historically only sporadically been found in asymptomatic market chickens, were

antigenically related to each other but remained outliers compared to all other clades.

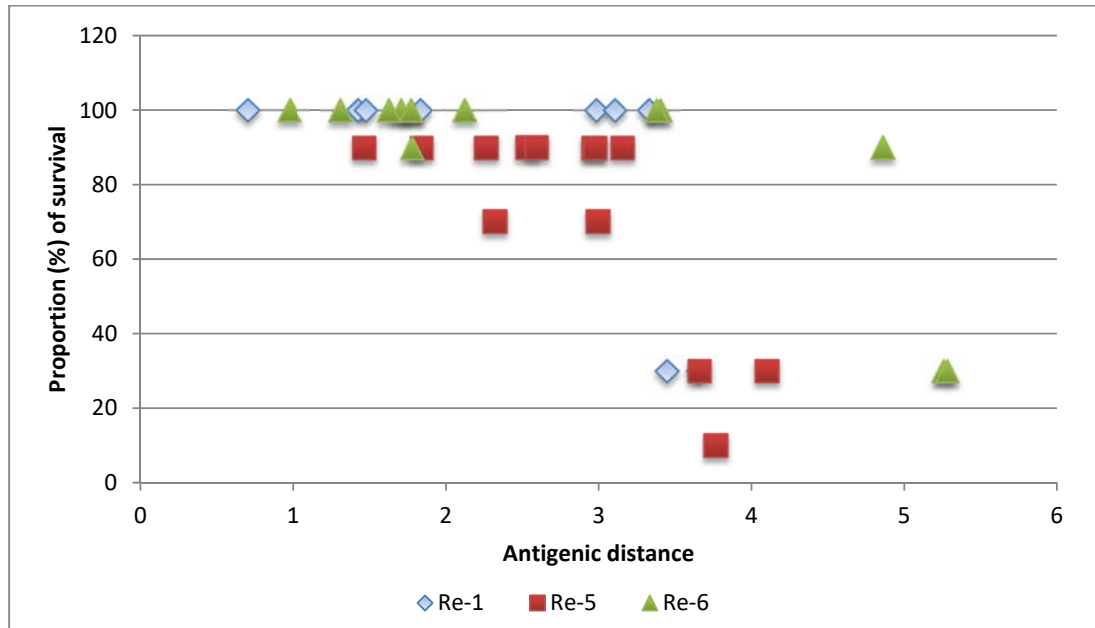


Figure 4.4: Plot showing the correlation between antigenic distances versus vaccine efficacy (percent of survival), as determined in annual challenge studies conducted at NCVD. Routine annual challenge studies use 10 birds/challenge virus, intranasal inoculation with a standardised dose of 10^6 TCID₅₀.

Regarding current poultry vaccines, our analysis of chicken antisera raised against Re-1 vaccine (clade 0, A/goose/Guangdong/1/1996 A(H5N1)) and Re-5 vaccine strains (clade 2.3.4; A/duck/Anhui/1/2006) demonstrated similar activities to both clade 1 and 2.3.4 viruses. Likewise, ferret antisera raised to the parental wild type viruses used to construct Re-1 and Re-5 also showed strong inhibition of both clade 1 and 2.3.4 viruses. In contrast, neither Re-1 nor Re-5 antisera showed significant reactivity with clade 2.3.2.1 and 7.1 viruses (i.e., heterologous titres were reduced by 8-fold or more compared to homologous virus titres; Figure 4.1, Figure 4.2, Figure 4.3), and this was true for both chicken and ferret antisera. Chicken Re-6 antiserum showed strong reactivity within clade 2.3.2.1 but not with other clades. Importantly, the observed antigenic relationships provide some explanatory support for results from *in-vivo* vaccine efficacy challenge studies (NCVD, 2014; NCVD, 2015; NCVD, 2016), in which the majority of the Re-1 vaccinated poultry

survived or did not develop clinical disease (protected) although they were challenged with both clade 1 (100%) and 2.3.4 viruses (90%) (Figure 4.4). In general, Figure 4.4 confirms a general trend towards lower survival rates as the antigenic distance between challenge virus and vaccine virus increases. However, the rate of drop-off in survival rates varies substantially between vaccines. Proportion of the vaccinated poultry that survived after the challenge with the clade 2.3.2.1 viruses was slightly smaller (50-70% to clade 2.3.2.1a, 30-40% to clade 2.3.2.1b, 50-70% to clade 2.3.2.1c). Similar to Re-1, the Re-5 vaccine provided good protection in vaccinated poultry against both clades 1 and 2.3.4 (90-100%), but only poor protection against clade 2.3.2.1 (50-70% to clade 2.3.2.1a, 10-30% to clade 2.3.2.1b and 50-70% to clade 2.3.2.1c viruses). The Re-6 vaccine protected against all three subclades 2.3.2.1a, b and c, but showed only 30-50% protection to against clade 1. The reactivity profile with clade 2.3.4.4 viral antigens is of particular interest, as this was the predominant clade circulating in Vietnam in 2016, as evidenced by the extensive national active surveillance system in LBMs (DAH, 2016b). The antigenic profiling described herein supports current recommendations for use of Re-5 vaccine in response to outbreaks of clade 2.3.4.4, and use of Re-6 vaccine for outbreaks of clade 2.3.2.1c. A new vaccine formulation under evaluation, Re-8 (clade 2.3.4.4 A/chicken/Guizhou/4/13), is not yet licensed for use in Vietnam, but is under evaluation in both a monovalent and bivalent formulation (mixture of Re-6 and Re-8).

Of the paired chicken and ferret antisera, 70% showed almost identical (within 2-fold) titre patterns of HI reactivity. However, potentially significant differences in reactivity (and relative map positions) were observed for 4 of the 14 chicken and ferret antisera pairs. Variation in the reactivity profiles of chicken versus ferret antisera may be attributed to important differences in the methodology for antisera production. First, in contrast to the

inactivated viruses used to immunise chickens, production of ferret antisera involved inoculation with live virus. Live viruses efficiently trigger the activation of the innate and cell-mediated immune system, presumably through pathogen associated signals (viral RNA) allowing their recognition by pattern recognition receptors. Following infection, viral particles rapidly disseminate throughout the host cells and reach their target tissues. This pattern is identical to that occurring during natural infection. Thus, these differences in live virus infection versus exposure to an inactivated antigen may explain the generally higher reactivity observed for ferret antisera compared to chicken antisera. A second important methodological difference was the use of different adjuvants in the inoculums for chickens and ferrets. An adjuvant is an agent that stimulates the immune system and increases the host response to an antigen without itself conferring a specific antigenic effect (Bowersock and Martin, 1999). For production of ferret antisera, our collaborators at US CDC used Titermax Gold adjuvant, while production of chicken antisera at NCVD used MontanideTM ISA 70 VG. TiterMax Gold forms stable depots of water-in-oil emulsion in tissue and may produce long-lasting responses and particularly produces high titres following a boost (<http://www.titermax.com/technical-information.html>).

MontanideTM ISA 70 VG is a mineral oil based adjuvant which has been developed for the manufacture of water-in-oil (W/O) emulsions. Vaccine formulations with Montanide ISA 70 VG induce a strong and long-lasting immunity and are very stable and easy to inject (<http://www.seppic.com/file/galleryelement/pj/71/e5/87/36/3546-technical-bulletin-mtd-isa-70-vg8240265333756822307.pdf>).

Results of antigenic characterisation of avian influenza A(H5N1) isolates with ferret sera were consistent with titre patterns in HI tests with chicken antisera (Fouchier and Smith, 2010; Koel *et al.*, 2014). Similarly, our study demonstrated that antigenic characterisations

using chicken antisera are feasible, reproducible and frequently recapitulate patterns of clade clustering as observed with HI tests performed using ferret antisera. Systematic cross-HI datasets could substantially contribute to design of animal challenge experiments (i.e. laboratory-based vaccine efficacy investigations), and to further investigations of avian immunity to influenza infections. Improved understanding of avian immune responses and the relationships between HI titres and antigenic distances as measured by cartography may lead to more robust predictions of cross-protection afforded by antigenically related or diverse vaccines.

CHAPTER 5

General discussion

The overall objective of this thesis was to evaluate the genetic and antigenic evolution of HPAI A(H5) viruses in poultry to contribute to studies relating to poultry vaccine usage in Vietnam. Both **Chapter 2** and **Chapter 3** focused on the molecular epidemiology of HPAI A(H5) viruses collected under passive and active surveillance in Vietnam between 2012 and 2015. **Chapter 2** presented analyses of overall influenza A(H5) detection rates and geo-temporal distribution, using data from the 44 provinces included in LBM surveillance. The market prevalence of influenza A virus was 75.4% (95% CI 70.8 - 79.6), A(H5) subtype virus was 32.2% (95% CI 27.6 - 37.1) and A(H5N1) virus was 26.4% (95% CI 22.1-31.0). The analysis identified a striking lack of correlation between virus prevalence rates as detected in markets versus the number of reported poultry outbreaks, thus raising a number of questions about the interpretation of prevalence/distribution data from active versus passive surveillance, and suggesting that crowding of birds in LBMs encourages increased transmission. **Chapter 2** also presented analysis of HA H5 genes detected between 2011 and 2013, revealing the persistence and *in situ* evolution of clade 1.1.2 viruses in the Mekong region, and the contrasting incursion and dispersal of clade 2.3.2.1 (a, b and c) subgroups across Vietnam. In **Chapter 3**, I used whole genome sequences to investigate the significant diversification, reassortment, and lineage replacement of HPAI A(H5) over the 2012 to 2015 time period. Molecular clock analyses in the BEAST software package were used to generate dated phylogenetic trees for estimating the time

during which specific genetic groups emerged and spread in Vietnam. This study also revealed inter-clade reassortant viruses (clade 1.1.2 and 2.3.2.1c) that were detected with different combinations of internal genes derived from 2.3.2.1a and 2.3.2.1b viruses, indicating extensive co-circulation. The majority of gene constellations detected corresponded to the HA clade. For example, clade 1.1.2, clade 2.3.2.1 a-c, clade 7.2 and clade 2.3.4.4 A(H5N6) each formed genome constellations that were unique to that clade. Five previously described genotypes (VN3, VN12, VN45, VN43, VN48) were identified (Creanga, Thi, et al., 2013; T. Nguyen et al., 2012; Wan et al., 2008) and eight new genotypes were identified (VN49-VN56) (Nguyen *et al.*, 2016a). Although these reassortants generated diversity at the genotype level, the analyses revealed relative genetic stasis within the HA and NA genes associated with each clade. There was also little evidence for diversification of the internal genes of genotypes VN51-53, suggesting these reassortants originated from a not so distant ancestor. The broad geographic distribution and genetic diversity of the three groups of clade 2.3.2.1 in Vietnam suggested multiple, separate introductions rather than endemic genetic drift. Antigenically, clade 1.1.2, 2.3.2.1a, 2.3.2.1b, 2.3.2.1c viruses remained related to earlier viruses including the WHO recommended pre-pandemic vaccine strains representing these clades. Clade 7.2 viruses, although only detected in small numbers in northern Vietnam, were the exception as indicated by introduction of a genetically and antigenically diverse strain in 2013. Antigenic analyses of clade 2.3.4.4 viruses compared to existing clade 2.3.4 CVVs also indicated the need for an updated vaccine virus.

Chapter 4 provided a systematic antigenic analysis of A(H5) clade variants detected in Vietnam between 2003 and 2015, using panels of antisera raised in both ferrets and chickens to better understand how strains are antigenically matched to available poultry

vaccines. Our results of antigenic characterisation of A(H5N1) isolates with antisera raised in ferrets are consistent with our results using antisera raised in chickens. This demonstrated that antigenic characterisations by the HI assay using chicken antisera are feasible to conduct within national laboratories of Vietnam, with reproducible test results and with patterns of cross-reactivity similar to that determined using ferret antisera.

The key conclusions arising from the various studies in this thesis are presented below. These are discussed in the wider context of HPAI A(H5) molecular epidemiology and disease control, as well as recommendations for policy and further research.

5.1. HPAI A(H5) surveillance in Vietnam

The findings of our systematic surveillance have proven that HPAI A(H5) viruses circulated widely throughout Vietnam with high prevalence and caused sporadic outbreaks among poultry in Vietnam (DAH, 2015; DAH, 2016b). The high prevalence of HPAI A(H5) virus at LBMs revealed the threat of transmission to humans as well as difficulties in designing appropriate and effective poultry disease management strategies in Vietnam. This high prevalence can be influenced by several important aspects of influenza virus ecology, poultry farming and cultural practices. In particular, Vietnam has a large poultry population of which the majority (more than 70%) is reared in backyard flocks of households where minimum biosecurity measures are applied. The conditions of these production systems may facilitate endemic transmission of both HPAI and LPAI virus among domestic and wild species, and sporadic occurrences of HPAI outbreaks. Vietnam has the second largest duck population (after China) in the world, which means it has a large population of the natural reservoir for influenza A viruses that facilitates the interaction of viruses from various sources, thereby promoting continual shifts in the

genetic diversity of field influenza viruses (FAO, 2003). Moreover, the influenza ecosystem in Vietnam has itself undergone substantial changes with several avian species such as quails and swiftlets potentially playing an increasing role in AI transmission (DAH, 2015). These birds may play a role as intermediate hosts for interspecies transmission from avian to mammals, or as ‘mixing vessels’ to facilitate reassortment events for viruses with different origins (Humberd et al., 2006; Liu et al., 2003; Makarova et al., 2003; Matrosovich et al., 2001; Perez et al., 2003a; Perez et al., 2003b).

In Vietnam, movement of poultry is highly dynamic, both within the production system (for ducks), and along the value chain for retailing and slaughter in LBMs (Long, 2013). Duck populations are routinely moved to graze in multiple places at the end of the rice harvest (Desvaux *et al.*, 2007; Minh, 2010). Because infected ducks very often do not have clinical signs, movements of iterant (free-running) duck flocks likely leads to spreading the virus from one location to another. LBMs are commonly present in suburban areas, rural villages of all provinces, and within large urban cities. LBMs often do not have a well-designed infrastructure, and regulations regarding daily cleaning and disinfection have only recently been introduced. Infrastructure and biosecurity of a small number of LBMs were improved under various projects such as the Vietnam - Avian and Human Influenza Control and Preparedness Project (VAHIP), or the ongoing Livestock Competitiveness and Food Safety Project (LIFSAP). However, many markets have not maintained good biosecurity practice after the introduction of the specific VAHIP interventions (Chu *et al.*, 2017). Lack of strong biosecurity practices may increase the risk of AI virus maintenance and transmission within LBMs. Given the large volume of live poultry brought to these markets, it is thought that LBMs play a central role as transmission hubs and as locations

for high levels of environmental contamination (Long, 2013; Chu et al., 2016; Chu et al., 2017).

Vaccination has been one of the key tools to control HPAI A(H5) over the years, with national massive vaccination campaigns carried out between late 2005 and 2012. Evaluations of poultry vaccination programmes conducted in 2010-2011 by DAH concluded that vaccination had contributed significantly to reducing the number of AI poultry outbreaks (MARD, 2011). Subsequently, based on a variety of justifications, the biannual mass campaigns and federal subsidies for routine preventative vaccination were stopped in 2013. Since 2013, central government funding has only been provided for ring vaccination in response to reported poultry outbreaks (MARD, 2014). As a result of reduced central government funding, A(H5N1) poultry vaccination coverage most likely declined in many provinces after 2013. Within larger commercial operations, vaccine use has undoubtedly continued with private funding sources; however estimates of these coverage rates are extremely difficult to obtain, since data on vaccine use within the private sector are not collected by the DAH. Between 2013 and 2016, 70% of HPAI A(H5) outbreaks were reported from unvaccinated flocks (DAH, 2017). Low vaccine coverage may be one factor contributing to high prevalence of HPAI A(H5) virus in certain provinces of Vietnam, and differences in vaccine use between provinces may also partially explain observed patterns of HPAI geographical distribution. Continued research efforts are needed to evaluate the relationships between vaccination coverage levels, trends in outbreak reporting, and changes in prevalence at the farm and market level. Although the passive and active surveillance systems generate enormous amounts of data on virus parameters (genetic distribution and diversity), it remains challenging to conduct rigorous evaluations of the impact of vaccination programmes due to a relative lack of information

about vaccine practice and performance in the field. Thus, systematic investigation of vaccine efficacy/effectiveness and impact on epidemiology of HPAI transmission will likely require specific targeted projects to collect more in-depth data on vaccine use, as well as prospective case controlled studies.

Vietnam shares a long border of more than 1,000 km with China and daily movement of animal and animal products are highly dynamic across the border. To date there is no official agreement on animal quarantine policies between the two countries and movement of live animals without quarantine is considered illegal. However, because prices of live poultry and poultry products greatly vary between Vietnam and neighbouring countries, there are frequent economic incentives to import chickens, day old birds (DOB) and chicken eggs across the northern border with China, whereas ducks are frequently exported across southern borders with Cambodia (Van Kerkhove *et al.*, 2009; FAO, 2014; FAO, 2015). It is thought that illegal movement of live poultry and poultry products, mainly from China, has resulted in multiple incursion events of new HPAI A(H5) clades, including past introductions of clade 2.3.2.1c A(H5N1) viruses in 2012 and clade 2.3.4.4 A(H5N6) viruses in 2014 (Creanga *et al.*, 2013; Nguyen *et al.*, 2016a). Therefore, the role of cross-border movement of poultry and poultry products should be investigated. The continuation of the national active surveillance is critical for Vietnam to detect variants or new introduction of AI viruses early and develop proper responses. This may be especially important given the expanded range of LPAI A(H7N9) virus in China during 2016/2017.

Emergence of A(H7N9) virus in southern China and occurrence of annual outbreaks of large numbers of human cases of A(H7N9) infections since 2013 has raised alarm concerning further cross-border introductions. In the effort to ensure early detection of

A(H7N9) incursion, DAH/FAO/US CDC has engaged in intensive surveillance activities in border provinces, with >200,000 swabs and environmental samples collected at many LBMs and poultry trade points (>200 collection sites), however, to date no A(H7N9) viruses have been detected (Nguyen *et al.*, 2016b; DAH, 2017). The lack of incursion of A(H7N9) viruses thus far may be attributed to successful cross-border patrolling, which may have limited the number of smuggled poultry and poultry products imported into Vietnam. From January 2016 to February 2017, an increased amount of illegal poultry and products that have been confiscated and processed comprised 356,072 breeding poultry, 2,365 broiler birds, 62,406 kg of poultry meat and 212,080 chicken eggs (DAH, 2017). Continuation of these intense surveillance activities is extremely costly, yet it is viewed as critical for early detection, and will likely be necessary for prolonged periods in the future, especially given the sporadic detection in China of highly pathogenic variants within the A(H7N9) lineage.

The research presented in this thesis would not have been possible without the framework of the current active surveillance system, which generated the majority of the isolates used for sequence analysis and antigenic characterisations. The utility and efficiency of the national active surveillance programmes has periodically been criticised by scientists, civil servants and policy makers who have questioned whether the data generated is actually being used to inform intervention strategies. There are many possible improvements to optimise the system. In particular, as the active surveillance programme has mainly focused on LBMs since 2011, to estimate the true prevalence of HPAI A(H5) virus, the current surveillance strategy should be reviewed and extended to poultry raising households where the prevalence of the virus is likely much lower (Nguyen *et al.*, 2016c). Implementation of active surveillance would be greatly improved if more relevant

information could be collected regarding the origin of birds sampled, i.e. information on farm type, flock size, vaccination status. Currently, epidemiological data (that can be aggregated in space, time, species and relevant factors) are not collected systematically. The cold chain during the sampling period and transportation from the field to laboratory is also an area that needs improvement, which would likely improve virus isolation rates. Past surveillance efforts have focused on testing exclusively for A(H5) subtype, and more recently also for A(H7) from border provinces. Many additional subtypes are also of interest, such as A(H9) and A(H10) because these subtypes have caused either disease in poultry or in humans in China recently (Bi *et al.*, 2015b; Su *et al.*, 2015). However, systematic typing for these is not yet routinely performed. Finally, although the focus on sampling at LBMs has proven highly effective in raising detection levels, surveillance strategies should also consider sampling at the farm level in order to monitor the differences between detection rates in farms and their practices versus markets (Nguyen *et al.*, 2016c). Specifically targeted surveillance activities in LBMs could be designed to address questions about optimising cleaning and biosecurity protocols in LBMs (e.g. number of required rest days), and alternative ways to minimise persistent contamination of the market environment.

5.2. Molecular study of HPAI A(H5) in Vietnam

Since the first clade 1 A(H5N1) viruses were introduced in Vietnam in 2003, there have been 16 distinct clades detected and an average of at least one clade introduced each year into Vietnam between 2003 and the present. HPAI A(H5) viruses have been repeatedly introduced into the north of Vietnam and then spread rapidly to the centre and south of Vietnam. In particular, clade 2.3.4 was first detected in the north in 2005 and then gradually spread to the centre and south of Vietnam in 2010 (Tung *et al.*, 2012). Similarly,

clade 2.3.2.1c was detected in Bac Ninh, a northern province of Vietnam in July 2012 (Creanga *et al.*, 2013), and it appeared and caused outbreaks in the southern provinces by late 2012 (Nguyen *et al.*, 2014). The rapid dispersal of clade 2.3.2.1 variants (2.3.2.1a and 2.3.2.1c) to the southern latitudes during 2012-2013 (**Chapter 3**) indicated increased intensity of poultry trade along the north-south axis, the leakiness of transport barriers for infected or non-vaccinated flocks, and/or particularly high transmissibility of clade 2.3.2.1 variants in poultry. Our most recent study also estimated that clade 2.3.4.4 viruses [A(H5N1) and A(H5N6)] were likely introduced in April 2014 and appeared to gain dominance across northern and central regions (Nguyen *et al.*, 2016a). Our molecular studies indicated that clades 2.3.2.1c [A(H5N1)] and 2.3.4.4 [A(H5N6)] are currently the most prevalent in Vietnam. The exception is clade 7, which has only been detected in the north (Nguyen *et al.*, 2009b; Nguyen *et al.*, 2014). Interestingly, unlike all other A(H5) clade variants, clade 7 has never been associated with any poultry outbreaks in Vietnam, perhaps due to an attenuated phenotype in poultry species (Nguyen *et al.*, 2009b), which may have slowed transmission as well. Clade 1.1 and its sub-clades (1.1.1 and 1.1.2) have caused outbreaks only in the south since 2004 (Nguyen *et al.*, 2009b; Nguyen *et al.*, 2014; Nguyen *et al.*, 2016a). There are several possible explanations for why clade 1 remained endemic in the Mekong Delta for a prolonged period of time. Clade 1 viruses may be particularly well adapted to duck populations, which are much larger in southern than northern Vietnam. In addition, the weather (hot and wet) in the south may favour year-round transmission cycles, in contrast to the stronger seasonal trends in transmission in the north, as has been demonstrated for human seasonal influenza (Thai *et al.*, 2015), and promote the persistence of viruses in the environment. Of relevance to mapping geographical distribution patterns is the fact that ducks are rarely moved from the south to the centre and north of Vietnam, whereas there is considerable movement from north to

south. Surveillance data has indicated the disappearance of clade 1 since 2014, which may be explained by a loss of fitness of these viruses caused by genome arrangement of the most recent clade 1.1.2 viruses detected in Vietnam that possess all internal genes from clade 2.3.2.1 viruses .

Chapter 3 showed evidence for emergence of several reassortant viruses over the past three years. This reassortment yielded major genetic changes in A(H5) viruses creating multiple novel genomes. The emergence of divergent AI virus reassortants in Vietnam could be due to the practice of backyard farming and LBMs, which results in constant close proximity of various poultry (chicken, ducks, geese, and numerous other species) and mammalian species (dogs, humans, pigs, tigers). The practice of backyard farming is gradually being replaced by more vertically integrated production unit, with larger commercial flock sizes that are housed in enclosed premises (i.e. western industrial style operations). LBMs, however, still provide ample opportunities for domestic poultry, humans and other mammals to interact and potentially transmit influenza viruses, sometimes resulting in the emergence of novel viruses. In shared wetlands or LBMs, the wild bird-derived viruses or their genes might be inherited in domestic poultry by reassortment with a poultry adapted AI virus, such as novel A(H7N9) and/or A(H10N8) viruses as has occurred in China (Su *et al.*, 2015). HPAI A(H5N1) viruses have been isolated previously in apparently healthy chickens from LBMs, but these viruses are highly pathogenic for specific-pathogen-free (SPF) chickens (Shortridge *et al.*, 1998; Chen *et al.*, 2009). These observations suggest that apparently healthy chickens in LBMs may also act as mixing vessels for AI virus reassortment and shed virus without any clinical signs of the disease (Liu and Gao, 2014). Combined with enzootic circulation of A(H5N1) and A(H9N2) strains, this pool of genetically diverse AI viruses, and their genes, poses

significant risks to public health and may lead to a greater chance for the emergence of novel viruses.

Poultry vaccinations attempt to control HPAI A(H5) outbreaks and limit their impact by reducing inter- and intra-species viruses transmission (Leung *et al.*, 2013). Although A(H5N1) vaccination programmes in Vietnam have been largely viewed as successful, and remain a key component of outbreak response, there have been some examples of ‘vaccine breakthrough,’ i.e. virus transmission within vaccinated flocks (Long *et al.*, 2011; Bui *et al.*, 2014b; Cuong *et al.*, 2016). One of the unwanted intrinsic consequences of mass vaccination may be vaccine failure due to incomplete vaccination coverage, and this partial population immunity may contribute to selection pressure for immune escape and antigenic drift. It has been hypothesised that poultry vaccine use may increase opportunities for viral reassortment (Smith *et al.*, 2006a; Pu *et al.*, 2015). Since current vaccines against A(H5N1) cannot completely eliminate AI virus infections in poultry (Smith *et al.*, 2006a; Leung *et al.*, 2013; Pu *et al.*, 2015), the virus is still able to replicate in immunised domestic birds without apparent clinical signs or mortality. The vaccinated animals may then act as silent carriers or even as mixing vessels for AI viruses, thus enabling virus dissemination to naive animals through poultry transports or LBMs and potentially increasing opportunities for reassortment to occur in co-infected animals (Shortridge *et al.*, 1998; Chen *et al.*, 2009; Guan and Smith, 2013; Bi *et al.*, 2015a; Pu *et al.*, 2015).

The molecular analyses in this thesis indicated substantial *in-situ* evolution of clade 1 and 2.3.4 viruses, over the period 2010-2012, followed by lineage replacement with clade 2.3.2.1 and 2.3.4.4 viruses during 2014-2015. The combination of frequent introductions of new viruses as well as ongoing *in-situ* evolution of endemic lineages resulted in a very

high level of genetic diversity. Given the co-circulation of multiple clade variants, there is a great need to develop effective vaccine strategies to induce broadly cross-reactive immune responses against the diverse set of currently circulating strains and future A(H5) variants. Similarly, if traditional inactivated vaccines are used, formulations that are bivalent or trivalent may be required rather than relying mainly on imported, monovalent vaccines from China.

5.3. Antigenic characterisation to inform vaccine selection

Selection of the best matched poultry vaccines has been a challenge to Vietnamese veterinary authorities because of the multiple HPAI A(H5) virus clades circulating and causing outbreaks in poultry in Vietnam. In particular, between 2003 and early 2006, clade 1 was the predominant virus and caused outbreaks throughout Vietnam (Nguyen *et al.*, 2008; Wan *et al.*, 2008). The Government of Vietnam used the Re-1 vaccine, which has HA and NA genes derived from the clade 0 A/goose/Guangdong/1/1996 virus, for the first vaccination campaign (DAH, 2007). Between 2007 and 2010, three lineages of clade 2.3.4 (2.3.4.1, 2.3.4.2, 2.3.4.3) were predominant in the north of Vietnam, and, consequently, the Re-5 vaccine with HA and NA genes derived from the clade 2.3.4, A/duck/Anhui/1/2006, was used in this region. In contrast, two lineages of clade 1.1 (1.1.1 and 1.1.2) were predominant in southern Vietnam from 2007 to 2012. Thus, the Re-1 vaccine was used throughout this period in southern provinces. Between 2010 and 2012, clade 2.3.2.1 viruses diversified (a, b and c subclades) and complicated the epidemiologic and vaccination status in the whole country. The failure of the Re-1 vaccine (clade 0) and Re-5 vaccine (clade 2.3.4) to provide an efficacious protective immune response against clade 2.3.2.1b and c viruses led Vietnam to import the Re-6 vaccine with the HA and NA genes derived from the clade 2.3.2.1b A/duck/Guangdong/S1322/2006 virus.

In Vietnam, registration and licensing of poultry A(H5) vaccines are under management of the DAH. Annually, DAH uses results of molecular epidemiology studies to inform their vaccine selection. For imported vaccines (mainly from China), the National Centre for Veterinary Medicine and Control conducts verifications of vaccine quality, and then carries out field trials at the flock level (small-scale), which are then evaluated by the DAH's scientific committee. Licenses and marketing authorisation are granted based on these assessments. For national campaigns, DAH also requests the NCVD to carry out laboratory challenge studies for licenced vaccines using various field collected viruses. DAH then develops technical guidelines to instruct which province should use which vaccine based on the challenge study results. As of March 2017, three poultry vaccines: Re-5, Re-6 (imported vaccines) and Navet Vifluvac (developed by the NAVETCO company in Vietnam using HA and NA genes derived from clade 1, A/Vietnam/1194/2004), are being used following the above described procedures. However, because of the limited capacity for molecular analyses in Vietnam, analyses of full genome sequences only can be done at overseas laboratories (such as the US CDC), so results are not sufficiently timely to develop the molecular epidemiology maps to be used for vaccination. In addition, vaccine efficacy experiments are only periodically implemented once or twice annually, which is often not sufficient for timely selection of vaccines to be used at the provincial level, especially when there are several distinct clades detected at the same time.

Several vaccination strategies have been implemented since late 2005 through the National Vaccine Programme in five phases (2005-2007, 2008-2010, 2010-2012, 2013-2014 and 2014-2018). National mass vaccination was applied to a number of high risk provinces

(which had a large number of outbreaks and high density of poultry) between 2005 and 2012. However, because the disease has been under control recently with a small number of outbreaks sporadically occurred in backyard flocks, currently the national strategy is only to apply ring vaccination.

Ring vaccination is conducted in response to outbreaks with the cost of vaccine covered by the government. As discussed previously, the preventive routine use of vaccines within production cycles has not been subsidised since the suspension of the mass biannual vaccination campaigns in 2012. Currently, each province exercises discretionary authority over budget allocations to support vaccination campaigns, and there is considerable variation in practice; some high risk provinces continue to promote routine age-based vaccination within the commercial and semi-commercial sector. Importantly, financial compensation for compulsory culling conducted during outbreaks is only provided to farmers who provide evidence of flock vaccination. A number of initiatives have been launched to improve tracking and documentation of flock vaccination status, however this remains a major challenge, given the informal nature of poultry retailing through wet markets.

The observed antigenic relationships between existing poultry vaccines and circulating viruses described in **Chapter 4** indicated similar results to *in vivo* vaccine efficacy (VE) challenge studies. In particular, through a novel contextualised strategy for assessment of potential vaccine efficacy against circulating viruses, we generated reference chicken antisera against a panel of 85 Vietnamese A(H5N1) virus isolates and conducted HI assays to generate serological datasets for antigenic cartography in Vietnam, the first time this has been done for Vietnam. These data suggest antigenic characterisation by HI analysis is an

informative and rapid *in vitro* surrogate to challenge studies, providing comparable antigenic profiles as those observed for VE protection profiles. For example, antigenic profiling supported current recommendations for use of the Re-5 vaccine in response to A(H5N6) outbreaks of clade 2.3.4.4, and the use of Re-6 vaccine for A(H5N1) outbreaks of clade 2.3.2.1c. The results strongly suggest that these antigenic data could be used as a surrogate (or as a precursor) to challenge studies that take time and resources to conduct. These data could be used to make fast decisions about vaccine selection for Vietnamese poultry. Chinese vaccines currently in use in Vietnam may not offer the optimal antigenic match due to the rapidly evolving A(H5) viruses detected in Vietnam and hence selection of vaccine candidates for producing local vaccines could be based on the type of data/results provided herein. The relationship between antigenic matching of field variants and vaccines and its influence on vaccine efficacy are exceedingly complex, and may be complicated by different immunologic responses in avian species or breeds (e.g. commercial vs. village chickens, Peking ducks, Muscovy ducks, geese, and spent hens). Further research targeting development of appropriately cross-protective and highly efficacious A(H5) poultry vaccines is urgently needed to facilitate the continued use of vaccination as part of integrated programmes of HPAI control in Vietnam. The study of Prabakaran (2014) indicated that a combination of three carefully selected HAs in a single vaccine formulation could efficiently induce neutralizing antibody titres against distinct clades of A(H5N1) strains. The study employed a single multivalent MVA (Modified Vaccinia virus Ankara) vector as a delivery mechanism for antigen presentation; a major advantage of this approach is the potential to provide broad cross-protection against a range of A(H5N1) strains within a single manufactured product (Prabakaran *et al.*, 2014). The strategy for vaccine strain selection and vaccine candidate design indicates the

feasibility of the development of a broadly protective A(H5N1) vaccine for pandemic preparedness.

Given the rapid evolution and frequent introduction of new A(H5) clade variants into Vietnam, maintaining a comprehensive panel of appropriate antisera for strain characterisations presents a continuous challenge. Appropriate ferret antisera are not often available in-country and as discussed previously, the conditions and infrastructure for producing ferret antisera have not yet been established. However, our study demonstrated the feasibility of producing reference chicken antisera within NCVD facilities. Our results of antigenic characterisation of A(H5N1) viruses using panels of antisera raised in ferrets were consistent with characterisation using antisera raised in chickens. The results demonstrated that antigenic characterisation by the HI assay using chicken antisera were highly reproducible yielding patterns of cross-reactivity similar to those determined with ferret antisera.

The use of antigenic cartography has become an important tool for the study of antigenic relatedness of circulating viruses and vaccine strain selection for AI vaccines in poultry. Antigenic cartography was used in these studies to assess antigenic relatedness between circulating A(H5N1) viruses and the A(H5N1) viruses used in poultry vaccines, and considerable antigenic variation was observed within and between A(H5) clades. For evaluation of post-vaccination sera, the three critical parameters which need to be considered as surrogate markers for the potency of the vaccine are: (1) the magnitude of the antibody response, (2) the focus of the antibody response, and (3) the breadth of the antibody response (Fouchier and Smith, 2010). Antigenic maps do not evaluate the magnitude of the antibody response but facilitate evaluation of focus and breadth of

antibody response. Hence, the magnitude of antibody response (measured by HI titre) needs to be considered in addition to the antigenic cartography results when selecting the vaccine strain. For breadth, HI or neutralisation assays can be used to see how well antisera cover diverse strains.

The protective efficacy of a given vaccine depends on many factors in addition to ‘antigenic matching’, and several studies have shown that a strong antibody response may compensate for a poor antigenic match (Coudeville *et al.*, 2010; Wikramaratna and Rambaut, 2015; Sitaras *et al.*, 2016). The most suitable strain should be based on comprehensive evaluation, which would also include the extent/duration of the HI antibody titre, protection from morbidity/mortality, and virus shedding in vaccinated and challenged chickens. This stepwise approach for selection of vaccine candidate virus is ideal since it is first based on genotypic data, followed by phenotypic (antigenic) data and challenge studies.

5.4. Future studies

The continuing emergence and diversification of the HPAI A(H5) virus lineages poses a huge challenge for influenza control programmes. Efforts to understand influenza virus evolution require continued input of resources and further investigations. Suggested priorities are summarised below as follows:

- 1) Sustained support for AI surveillance programmes in Vietnam. As the pandemic threat of HPAI A(H5) viruses still persists, it is essential to maintain government commitment to both active and passive influenza surveillance activities, to track the continued emergence and re-emergence of viruses with pandemic potential and to provide an early warning

system for global public health. Early detection of outbreaks/infections and rapid response to those detections are essential components of the effective control of an HPAI outbreak.

2) Support for whole genome sequencing of contemporary AI viruses from Vietnam. Thus far, the approach to monitoring genetic diversity of AI virus threats in Vietnam has focused on partial HA sequencing of A(H5) viruses. Given the vast diversity of AI virus subtypes and the continual generation of new HPAI genotypes, as well as the complexity of LPAI co-circulating variants, there is a need to modernise the process of rapidly generating sequence data from surveillance and outbreak samples. A number of methods could be considered for more systematic sequencing of large amounts of influenza viruses, including both promotion of in-country capacity as well as establishing ‘pipelines’ for out-sourcing the production of next generation sequence data.

3) Experimental and epidemiological vaccine efficacy studies. While the ability to use virus sequence data to inform risk assessment of pandemic potential is improving, it remains essential to complement sequence databases with other phenotypic data on virus diversity, derived from both experimental and epidemiological investigations. The impact of ongoing industrialisation of the poultry sector merits research, i.e. research into the agroecology of backyard production systems as compared to larger more vertically integrated production units. Investigations to compare production systems will require a combination of prospective case-control studies as well as modelling and interdisciplinary approaches that address socio-economic issues.

4) Clade distribution and abundance assessments. Phylogenetic clade identification is a practical means for risk prediction of influenza viruses. However, in species where reassortment is frequent, phylogenetic data must be considered on a gene by gene basis. Viruses circulating in poultry, swine or other domestic animals with extensive human interactions should be prioritised for surveillance and further study. Further study will then be required to assess the abundance and prevalence of different virus subtypes, clades, and genotypes along with geographic spread and overlap with ecological risk factors (Hill et al., 2015; Gilbert et al., 2014), e.g. live animal markets, cohabitation of humans and animals, and biosecurity in animal processing facilities.

5) Support for accessing antigenic relatedness of circulating viruses and vaccine strain selection. With rapid evolution of HPAI A(H5) viruses in Vietnam, it is essential to produce more reference antisera in chickens to identify A(H5) clade variants and to determine antigenic relationships between circulating viruses and available poultry vaccines to facilitate selection of challenge viruses for use in vaccine efficacy studies and to inform vaccine selection for use in the field. The use of antigenic cartography should become routine to access antigenic relatedness of circulating viruses and vaccine strain selection for AI poultry vaccines in Vietnam.

6) Multivalent vaccines. Given the overlapping geographic distribution of A(H5) clade variants and their antigenic divergence, the vaccine efficacy of multivalent vaccine formulations containing representatives of both clade 2.3.2.1c and 2.3.4.4 viruses should be tested to determine their ability to protect poultry in Vietnam. Antigenic cartography could also become an useful tool to interpret the output from studies using multivalent vaccines.

7) Data sharing. Continued circulation of the A(H5) viruses of various subclades, which are more adapted to land based poultry in a highly populated region, such as Southeast Asia, may lead to evolution and emergence of potential pandemic strains with devastating consequences. Hence, there is an urgent need for faster sharing of data in the public domain or through bilateral/international agreements.

8) Universal vaccines. In areas of high endemicity of HPAI, vaccination of domestic poultry provides one of the most feasible preventative strategies to mitigate production losses associated with avian influenza infection, and is the most readily acceptable solution within the context of the rapidly growing Vietnamese poultry industry. However, current poultry influenza vaccines have many limitations, such as limited local capacity for vaccine manufacturing; relatively long production times for registration/ commercial availability of updated strains; only moderate efficacy in key reservoir populations (i.e. ducks); and lack of strong protective cross-reactivity among clade variants. Therefore, there is a great need to apply novel technologies to improve the quality of current poultry vaccines, and to ensure that the best possible vaccines can be made rapidly available to the poultry producers in a timely manner. A ‘universal’ vaccine within the context of HPAI would provide broad and durable protection against all clades of highly pathogenic A(H5) viruses when delivered as a single dose, and that could delivered in a non-invasive manner (orally through feed, or in ovo at hatchery). To move in the direction of broader cross-H5-clade protection, vaccine research on introduction of novel antigens from highly conserved regions of HA (i.e. stem region) or other immunogenic non-HA, non-NA antigens could prove promising, but will require the need for validating novel immune correlates of protection. The incorporation of more conserved influenza antigens in live vectored

formulations could be used to supplement the humoral anti-HA and anti-NA immunity provided by currently licensed inactivated vaccines (Soema et al., 2015). Looking ahead to a future world in which ‘universal’ cross-clade and cross-subtype protection vaccines are available, one great advantage is that those vaccines could be produced continuously, which would greatly increase available supply and help to promote more consistent and systematic uptake, thus leading to higher population coverage. Such a cross-protective vaccine against all A(H5) clade variants would indeed be a major achievement. The ability to provide further broad protection against all 16 influenza HA subtypes could be envisioned for the long term future and could make use of similar principles, but such research is considered beyond the scope of what may be feasible with current technology. Perhaps the most significant technical obstacle for development of novel poultry vaccines is the need for separate vaccine delivery systems for ducks versus chickens. In this regard, vaccination for ducks would be different from vaccination for chickens. Viral vectored vaccines may use similar platforms for production, however they would need to pursue species-specific formulations that may prove challenging from a regulatory perspective, and that may also pose particular challenges for vaccine implementation in the field. In addition to the grand challenge of achieving broader cross-clade (and eventually cross-subtype) immune protection, more applied research is needed on how to scale up and produce vaccines faster, especially since the timely availability of reagents for vaccine potency testing remains a major time-delaying factor for regulatory purposes (Soema et al., 2015).

This thesis generated novel data and used innovative analytical tools to conduct an in-depth investigation of molecular epidemiology of HPAI A(H5) virus in Vietnam. Study results presented in this thesis provide scientific evidence for Vietnamese veterinary

authorities to justify or change current and future control measures. Several major constraints and issues need to be addressed in order to develop more feasible interventions for AI virus control in the future, since HPAI A(H5) viruses will likely remain in Vietnam for many years. Our findings enrich the international community's understanding about AI circulation in endemic regions of Vietnam, where a number of interventions, including vaccination, have been applied for more than a decade.

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Appendix

Appendix 1: Protocol for real-time RT-PCR for detection and subtyping of avian Influenza A virus (National Centre for Veterinary Diagnosis – NCVD)

Materials

1. One-step qRT-PCR kit: Invitrogen Superscript III platinum one-step qRT-PCR kit: 11732-020 (100rxn x 50µl) or 11732-088 (500rxn x 50µl)
2. Primers and probes: sequence and final concentration are in Table 2. (Primers and probe mix will be provided as ready-to-use. These primers may be changed when needed)
3. PCR tubes and filtered tips: molecular biology grade, nuclease free

Testing procedure

1. Select primer-probe mix of the target gene.
(Note: Choose N1-3 for N1 gene as the first choice. Use N1-2 when both N1 and N6 are negative. Currently there is no primer-probe set available that detect all N1 of H5N1)
2. Prepare the master mix as in Table 1.

Table 1. Master mix for one reaction of total of 15ul volume

Reagent	Volume (ul)
DW	3.3
2x Reaction Mix	7.5
Primer-Probe Mix	0.9
Enzyme mix	0.3

3. Dispense 12µl of master mix to PCR tubes.
4. Add 3µl of sample RNA to tubes.
5. Place tubes to PCR machine.
6. Run the thermal cycling program as follows.
 - A) 1 cycle of [50C for 15min, and 95C for 2min]
 - B) 40 cycles of [95C 15sec, and 60C for 45sec]

Interpretation of results

1. Test is valid: when positive control gives expected Ct value and negative control gives no Ct value
2. Sample is positive: when sample RNA gives Ct value ≤ 35
3. Sample is negative: when sample RNA gives Ct value >35 or no Ct value

Table 2: List of primers and probes

PPP name on tube	Target gene	Primer code	Sequence 5'-3'	Final conc (nM)
M-5	M	M-4F InfA CDC	GACCRATCCTGTACCTCTGAC	800
		M-4R InfA CDC	AGGGCATTYTGACAAACGCTCTA	800
		M-4P InfA CDC	TGCAGTCCTCGCTCACTGGGCACG	200
	M	M-11F D161M RP	AGGGCATTYTGACAAADCCTCTA	900
		M-11R D162M RP	TGCAANACATCYTCAAGTCTCTG	900
		M-11P D231 RP	TCAGGCCCCCTCAAAGCCGA	250
H5-9M	H5	H5-9F1 LH1 VLA	ACATATGACTACCCACARTATTAG	400
		H5-9R1 RH1 VLA	AGACCAGCTAYCATGATTGC	400
		H5-9P2 PRO VLA	TCAACAGTGGCGAGTTCCTAGTG	100
	H5	H5-9F3 VLH1 VLA	ACGTAYGACTACCCYARTATTAG	400
		H5-9R3 RH1 VLA	AGACCAGCCACRATGATTGC	400
		H5-9P3 PRO VLA	TCAACAGTGGCGAGTTCCTARCA	100
H7-6	H7	H7-6F CODA RP	GYAGYGGYTACAAAGATGTG	800
		H7-6R CODA RP	GAAGACAAGGCCCATGCAA	800
		H7-6P CODA RP	TGGTTTAGCTTCGGGGCATCATG	200
H7-CN	H7CN	H7CN-5F CNIC RP	AGAAATGAAATGGCTCCTGTCAA	800
		H7CN-5R CNIC RP	GGTTTTTCTGTATTTTATATGACTTAG	800
		H7CN-5P CNIC RP	AGATAATGCTGCATTCCCGCAGATG	400
H9-1	H9	H9-1F JCM2008 RP	ATGGGGTTTGCTGCC	900
		H9-1R JCM2008 RP	TTATATACAAATGTTGCAYCTG	900
		H9-1P JCM2008 RP	TTCTGGGCCATGTCCAATGG	250
N1-3	N1	N1-3F 1316F RP	GYGGGAGCAGCATATCYTT	900
		N1-3R 1379R RP	CCGTCTGGCCAAGACCAA	900
		N1-3P 1336P RP	TGTGGTGTAAYAGTGACAC	250
(N1-2)	N1	N1-2F 1330F JVM2008	TGGACTAGTGGGAGCAGCAT	800
		N1-2R 1426R JVM2008	TGTCAATGGTTAAGGGCAACTC	800
		N1-2P 1383P JVM2008	TGGTCTTGCCAGACGGTGC	200
N6-1	N6	N6-1F D721 RP	CCC CAC CAA TGG GAA CTG	900
		N6-1P D723 RP	CC AAT AAC AGG AGG GAG CCC AGA CCC	300
		N6-1R D722 RP	TCT AGG AAT GCA AAC CCT TTT ACC	250
N8-1	N8	N8-1F 1296F FLI RP	TCC ATG YTT TTG GGT TGA RAT GAT	800
		N8-1P 1354F FLI RP	TCH AGY AGC TCC ATT GTR ATG TGT GGA GT	400
		N8-1R 1423R FLI RP	GCT CCA TCR TGC CAY GAC CA	800
N9-1	N9	N9-1F CNIC RP	TGGCAATGACACACACTAGTCAGT	800
		N9-1P CNIC RP	AGACAATCCCCGACCGAATGACCC	400
		N9-1R CNIC RP	ATTACCTGGATAAGGGTCGTTACACT	800

Appendix 2: Protocol for PCR to Big Dye procedures for Sanger Sequencing (US CDC)

1. Materials

1. 96-well cooler racks for 0.2mL and 1.5mL PCR reaction tubes
2. Pipettes (1 μ L, 10 μ L, 20 μ L, 200 μ L and 1000 μ L) (Rainin)
3. Aerosol barrier pipette tips
4. 0.2 mL nuclease free PCR reaction tube strips or plates (Phoenix)
5. Sterile, nuclease free 1.5mL microcentrifuge tubes
6. 96-well Optional Reaction Plate (Applied Biosystems Cat. No.4306737)
7. Disposable powder-free gloves
8. ABI High-Capacity cDNA RT Kit (Life Technologies Cat. No.4374966)
9. Nuclease-free sterile distilled water (Ambion Cat. No. AM9937)
10. Taq PCR Master Mix (2X) (Affymetrix, Cat. No. 71162) or AccessQuick RT-PCR System (Promega, Cat. No. A1702)
11. E-Gel® Low Range Quantitative DNA Ladder (Life Technologies, Cat. No. 12373031)
12. E-Gel Agarose Gels (Life Technologies, 24-well 2% double comb G6018-02; 48-well 2% double comb G8008-02 ; 96 well 2% G7008-02)
13. ExoSAP-IT® for PCR Product Clean-Up (Affymetrix, Cat. No.78202)
14. BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Cat. No.4337457)
15. Forward and reverse gene specific primers (20 μ M for RT-PCR, 1 μ M for sequencing) *for complete list see appendix)

2. Assay Preparation

1. All reagents should be kept on a +4 to +8°C cold rack or ice during assay preparation.

3. Procedure

1. Reverse Transcription PCR

1. Thaw reagents on ice (High-Capacity cDNA RT Kit 2X Reaction buffer, water, random primer mix, dNTP mix). Keep enzyme mix on ice
2. Vortex and spin down all reagents and place in cold rack.
3. Prepare Master Mix by adding each reagent as denoted in Table 1. Prepare n+1 volume of master mix. (n = number of reactions. Example: If 5 reactions, prepare master mix for 6 reactions.)

Reagent	Amount (μ L)
Nuclease free water	28.0
RT Buffer	5.0
Random primer mix	5.0
dNTP mix	2.0
RNase Inhibitor	2.5
MultiScribe RT	2.5
Template (RNA)	5.0
Total	50.0

4. Invert reaction mix tube several times to mix. Briefly centrifuge reaction mix.
5. In the nucleic-free assay set-up area, dispense 50µL of each master mix into each 0.2 mL reaction strip tube or plate well.
6. Prepare the NTC reactions in the nucleic-free assay set-up area before moving to the nucleic acid handling area. Pipette 5µL of nuclease free water into the specified NTC wells.
7. Securely cap NTC wells after addition of nuclease free water and continue to the nucleic acid handling area to proceed with assay preparation.
8. Pipette 5 µL of previously extracted nucleic acid samples into specified tubes labelled for the samples. Change tips after the addition of each sample.
9. Change gloves when necessary to avoid contamination.
10. Centrifuge tube strips in flash centrifuge for 10-15 seconds. If using plates, centrifuge at 500 x g for 30 seconds.

Reverse Transcription reaction thermal profile:

Temperature Time (minutes)

Step 1 25°C 10:00

Step 2 37°C 120:00

Step 3 85°C 5:00

Step 4 4°C ∞

Place cDNA on ice and keep frozen at -20°C for storage

2. RT-PCR Amplification with M13 Tagged Primers

1. Thaw reagents, keep on ice and prepare master mix for n+1 samples.

Reagent	Amount (µL)
AQ master mix	6.25
dH ₂ O	3.5
AMV RT (10 U/µL)	0.25
Forward primer (20 µm)	0.25
Reverse primer (20 µm)	0.25
Template RNA	2.0
Total	12.5

PCR Thermal Profile for Access Quick RT-PCR System:

Step	Temp (°C)	Time (minutes)
1	48	45:00
2	94	2:00
3	94	0:20
4	50	0:30
5	72	1:00
6	Go to step 3	34X
7	72	7:00
8	4	∞

3. PCR Amplification with M13 Tagged Primers

1. Thaw reagents, keep on ice and prepare master mix for n+1 samples.

Reagent	Amount (μL)
AQ master mix	6.25
dH ₂ O	4.75
Forward primer (20 μm)	0.25
Reverse primer (20 μm)	0.25
Template cDNA	1.0
Total	12.5

PCR Thermal Profile:

Step	Temp (°C)	Time (minutes)
1	94	2:00
2	94	0:30
3	50	1:00
4	68	1:30
5	Go to step 2	34X
6	68	8:30
	4	∞

4. Conventional PCR with Non-M13 Tagged Primers

1. Thaw reagents, keep on ice, and prepare Master Mix for n+1 samples

Reagent	Amount (μl)
AQ master mix	6.25
dH ₂ O	4.75
Forward primer (20 μm)	0.25
Reverse primer (20 μm)	0.25
Template cDNA	1.0
Total	12.5

PCR Thermal Profile:

Step	Temp (°C)	Time (minutes)
1	48	45:00
2	94	2:00
3	94	0:20
4	50	0:30
5	72	1:00
6	Go to step 3	34X
7	72	10:00
8	4	∞

5. Visualization of PCR Products

1. Use nitrile gloves to handle E-Gels
2. Add 18μL of purified water in gel chambers except lane M (for 96-well E-Gel)
3. Add 2μL of PCR product
4. Add 5-6μL of standard (DNA ladder) to gel in the lanes labelled with M
5. Run gel for 5 minutes (96-well), 10-15 minutes (24-well or 48 well gels)

6. Wearing a protective shield or goggles, visualize on a UV light table; or take a picture with a UV light illuminator platform.
- 6. ExoSAP-IT® for PCR Product Clean-Up**
1. Manufacturer: Affymetrix Cat. No.78200
 2. PCR Product - 12.5µL
 3. ExoSAP-IT® - 3µL (or 1µL per every 5µL PCR Product)
 4. Add 3µL of the 1X ExoSAP-IT® mix to each PCR product of 12.5µL.
 5. Incubate samples at 37°C for 15 minutes and then at 85°C for 15 minutes in a PCR cycler.
 6. Store samples at +4°C or -20°C until needed.
Note: It is a good idea to aliquot the ExoSAP-IT® into smaller volumes (~500µL) and store at -20°C to avoid repeated freeze-thaw cycles.

7. Big Dye Sequencing

1. Reagents
 1. Big Dye Terminator v3.1
 2. 5X Sequencing Buffer (Life Technologies)
 3. Nuclease free water
 4. Sequencing Primer (1 µm)
2. Use the following volumes of reagents for each reaction:
 1. 2µL of 5X Sequencing Buffer
 2. 0.5µL of Big Dye Terminator v3.1
 3. 3µL of PCR template
 4. 1µL of 1 µM Primer
 5. 3.5µL of purified water
 6. Final volume per reaction: 10µL
 7. Thermal Profile:

Step	Temp (°C)	Time (minutes)
1	96	1:00
2	96	0:10
3	50	0:05
4	60	1:15
5	Go to step 2	34X
	4	∞

Note: If you have poor sequencing quality or low concentration of PCR product, volumes of Big Dye Terminator can be increased in increments of 0.5µL up to 2.0µL and the volume of water adjusted according to final volume of 10µL per reaction.

PCR Primers

M13 Labeled

M13 Forward Tag	TGT AAA ACG ACG GCC AGT
M13 Reverse Tag	CAG GAA ACA GCT ATG ACC

H5N1

H5

Fragment	CDC Primer Name	Sequence
1	SZ_HA+	TGTAAAACGACGGCCAGT CTC GAG AGC AAA AGC AGG GG
	H5_F1_R508_2012	CAGGAAACAGCTATGACC CTT TTT GAK AAG CCA YAC CAC ATT TC
2	H5_F2_F214_2012	TGTAAAACGACGGCCAGT AGT GTA GCW GGA TGG CT
	H5_F2_R797_2012	CAGGAAACAGCTATGACC GAG CAA TGA AAT TTC CRT TAC TCT C
3	H5_F3_F482_2012	TGTAAAACGACGGCCAGT GAA ATG TGG TRT GGC TTM TCA AAA AG
	H5_F3_R973_2012	CAGGAAACAGCTATGACC TGM TTT CAC ATA TTT GGG
4	H5_F4_F722_2012	TGTAAAACGACGGCCAGT GAG AGT AAY GGA AAY TTC ATT GCT C
	H5_F4_R1174_2012	CAGGAAACAGCTATGACC CTA TWG CCT TTT GAG TRG ATT C
5	H5_F5_F955_2012	TGTAAAACGACGGCCAGT CCC AAA TAT GTG AAA KCA
	H5_F5_R1591_2012	CAGGAAACAGCTATGACC GCC ACT GTW GAA TAA ATT GAC ART ATT TG
6	H5_F6_F1333_2012	TGTAAAACGACGGCCAGT CTG GTT CTM ATG GAA AAT GAG AGA ACT
	SZ_HA-	CAGGAAACAGCTATGACC AGT AGA AAC AAG GGT GTT TTT

N1

Fragment	CDC Primer Name	Sequence
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2	N1_F2_384For_fix	TGTAAAACGACGGCCAGT TGY CAA AGA YAG RAG CCC TC
	N1_F2_679Rev_fix	CAGGAAACAGCTATGACC TTG GTC CAT CWG TCA TYA CA
3	N1_F3_474For_fix	TGTAAAACGACGGCCAGT TTG GTC RGC AAG YGC TTG CCA TGA TGG
	N1_F3_1001Rev_fix	CAGGAAACAGCTATGACC CCG TAT YTA AAT GAR AAC CCT T
4	N1_F4_556For_fix	TGTAAAACGACGGCCAGT TTG AAA TAY AAY GGC ATA ATA ACA GAC AC
	N1_F4_1181Rev_fix	CAGGAAACAGCTATGACC CCT GTC ART TCY GGR TGC TGG ACA A
5	N1_F5_941For_fix	TGTAAAACGACGGCCAGT TAG GAT ATA TWT GCA GTG GRG TTT TCG G
	SZ_NA-	CAGGAAACAGCTATGACC AGT AGA AAC AAG GAG TTT TTT

PB2

Fragment	CDC Primer Name	Sequence
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	PB2_R1_562-fix	CAGGAAACAGCTATGACC CTT TYG TTA TTG TCA ATT GYG A
2	PB2_F2_352_fix	TGTAAAACGACGGCCAGT TAC TTY GAG AAG RTT GAA AGG TTA A
	PB2_R2_773_Fix	CAGGAAACAGCTATGACC CTC TGG TCA RCA TCG TCA TTT CT
3	PB2_F3_451-fix	TGTAAAACGACGGCCAGT CAT GCA GAY CTC ART GCT AAA GAA GC
	PB2_R3_1096-fix	CAGGAAACAGCTATGACC ACC ATT GTR AAT TCY TCA TAC CC
4	PB2_F4_670-fix	TGTAAAACGACGGCCAGT ACA AGC AGY GTR TAC ATT GAR
	PB2_r4_1248-fix	CAGGAAACAGCTATGACC CCTCGGACTGCYTTTATCATRCAATC
5	PB2_F5_908-fix	TGTAAAACGACGGCCAGT CTG ARG ARC AAG CYG TGG ATA TAT G
	PB2_R5_1590-fix	CAGGAAACAGCTATGACC TCA TTG ATG AYG AAT ATG TTA TTG TCA
6	PB2_F6_1125-fix	TGTAAAACGACGGCCAGT AAA GCW ACT AGR AGG CTD ATT CA
	PB2_R6_1673-fix	CAGGAAACAGCTATGACC TCC CAR TTT CTG ATR ATC CAY TG
7	PB2_F7_1444-fix	TGTAAAACGACGGCCAGT AAA ATG GGA GTR GAT GAR TAT TCC
	PB2_r7_2149_fix	CAGGAAACAGCTATGACC CAA GAY TGC TCA RTT CAT TGA TGC
8	PB2_F8_1664-fix	TGTAAAACGACGGCCAGT CAR TGG ATC ATC AGA AAY TGG G
	SZ_PB2-	CAGGAAACAGCTATGACC AGT AGA AAC AAG GTC GTT TTT AAA C

PB1

Fragment	CDC Primer Name	Sequence
1	SZ_PB1+	TGTAAAACGACGGCCAGT AGC AAA AGC AGG CAA ACC AT
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2	PB1_F2_319Fw_2013	TGTAAAACGACGGCCAGT TGT CTT GAA ACG ATG GAA ATT GTY CAA CA
	PB1_F2_809Rv_2013	CAGGAAACAGCTATGACC CCA GAT TGC TCA AGY TTY TCA CAG ATA
3	PB1_F3_424Fw_2013	TGTAAAACGACGGCCAGT GCT TTG GCC AAC ACT ATA GAA ATY TTC AGA
	PB1_F3_968Rv_2013	CAGGAAACAGCTATGACC GTT ATC ATY GCC AGA AAC ATY CTA GG
4	PB1_F4_721Fw_2013	TGTAAAACGACGGCCAGT ATT GCR ACA CCC GGA ATG CAA ATC AG
	PB1_F4_1130Rv_2013	CAGGAAACAGCTATGACC TCA ATG TTT GCR AGC AYT TCT GCT G
5	PB1_F5_853Fw_2013	TGTAAAACGACGGCCAGT GTC GTR AGG AAG ATG ATG ACY AAC TCA CA
	PB1_F5_1478Rv_2013	CAGGAAACAGCTATGACC GTG AAT TCA AAT GTC CCT GTC CGA TTT AT
6	PB1_F6_1199Fw_2013	TGTAAAACGACGGCCAGT CAG CCT CAT TGA GYC CTG GAA TGA
	PB1_F6_1679Rv_2013	CAGGAAACAGCTATGACC CGG TAT GTG TAY CTG TAG TCC TTG ATG AA
7	PB1_F7_1510Fw_2013	TGTAAAACGACGGCCAGT AAT TTC AGT ATG GAG CTR CCC AGY TTT G
	PB1_F7_1985Rv_2013	CAGGAAACAGCTATGACC GTT GCA ACR GCA TCA TAY TCC ATA CT
8	PB1_F8_1825Fw_2013	TGTAAAACGACGGCCAGT GTC TGC TTR AAA TGG GAA TTG ATG GAT G
	SZ_PB1-	CAGGAAACAGCTATGACC AGT AGA AAC AAG GCA TTT TTT CAT

PA

Fragment	CDC Primer Name	Sequence
1	SZ_PA+	TGTAAAACGACGGCCAGT CTC GAG CAA AAG CAG GTA CTG AT
	PA_F1-608Rv_2013	CAGGAAACAGCTATGACC TCT TCA ATT GTY TCT TCG CCT CTC TC
2	PA_F2-222Fw_2013	TGTAAAACGACGGCCAGT CCG ATT YGA RAT AAT TGA AGG RAG AGA CC
	PA_F2-757Rv_2013	CAGGAAACAGCTATGACC CTT CYT TTG ACA TTT GAG AAA GCT TGC CC
3	PA_F3-583Fw_2013	TGTAAAACGACGGCCAGT GAG AGA GGC GAA GAR ACA ATT GAA GA
	PA_F3-947Rv_2013	CAGGAAACAGCTATGACC CCG AAA AAW GTC TTC ATG CAT TTG ATT GC
4	PA_F4-685Fw_2013	TGTAAAACGACGGCCAGT TTT AGA GCC TAT GTG GAT GGA TTC RAA CCG
	PA_F4-1238Rv_2013	CAGGAAACAGCTATGACC TTG TTG AAT TCD CTC TGR ATC CAG CTT G
5	PA_F5-919Fw_2013	TGTAAAACGACGGCCAGT GCA ATC AAA TGC ATG AAG ACW TTT TTC GG
	PA_F5-1472Rv_2013	CAGGAAACAGCTATGACC GTY CTG CAY TTG CTT ATC ATT GGA ATC A
6	PA_F6-1211Fw_2013	TGTAAAACGACGGCCAGT CAA GCT GGA TYC AGA GHG AAT TCA ACA A
	PA_F6-1748Rv_2013	CAGGAAACAGCTATGACC CGY CTC ATY TCC ATG CCC CAT TTC AT
7	PA_F7-1363Fw_2013	TGTAAAACGACGGCCAGT GCY ACT GAA TAC ATA ATG AAG GGD GTG TA
	PA_F7-2036Rv_2013	CAGGAAACAGCTATGACC CCA GGT TCC AGG TTG TCC CTA AGT G
8	PA_F8-1723Fw_2013	TGTAAAACGACGGCCAGT ATG AAA TGG GGC ATG GAR ATG AGR CG
	SZ_PA-	CAGGAAACAGCTATGACC AGT AGA AAC AAG GTA CTT TTT TGG AC

NP

Fragment	CDC Primer Name	Sequence
1	NP_F1_28	TGTAAAACGACGGCCAGT CAG GGT AGA TAA TCA CTC AC
	NP_R1_553	CAGGAAACAGCTATGACC AGA GCA CAT YCT GGG ATC CAT
2	NP_F2_296	TGTAAAACGACGGCCAGT ATG GTR CTC TCT GCT TTT GAT GA
	NP_R2_757	CAGGAAACAGCTATGACC TTT GTG CAG CTG TTT GAA ATT TYC CTT T
3	NP_F3_513	TGTAAAACGACGGCCAGT TGG CAT TCH AAT TTR AAT GAT
	NP_R3_1042	CAGGAAACAGCTATGACC CTG RCT CTT GTG TGC DGG
4	NP_F4_619	TGTAAAACGACGGCCAGT GCT GCA GTC AAR GGA RT
	NP_R4_1177	CAGGAAACAGCTATGACC AAG CRA TTT GTA CYC CTC TAG T
5	NP_F5_872	TGTAAAACGACGGCCAGT TTC TGA GRG GRT CAG TTG CTC
	NP_R5_1565	CAGGAAACAGCTATGACC AGT AGA AAC AAG GGT ATT TTT C
SZ	SZ_NP+	TGTAAAACGACGGCCAGT CTC GAG AGC AAA AGC AGG GT
	SZ_NP-	CAGGAAACAGCTATGACC AGT AGA AAC AAG GGT ATT TTT C

M

Fragment	CDC Primer Name	Sequence
1	M_F1_0F	TGTAAAACGACGGCCAGT AGC AAA AGC AGG TAG
	M_F1_467Rev_2012	CAGGAAACAGCTATGACC TCT GCA ATC TGC TCA CAR GT
2	M_F2_223F	TGTAAAACGACGGCCAGT CAC CGT GCC CAG TGA GCG
	M_F2_762Rev_2012	CAGGAAACAGCTATGACC GGA TCA CTT GAA YCG YTG CA
3	M_F3_448For_2012	TGTAAAACGACGGCCAGT ACY TGT GAG CAG ATT GCA GA
	M_R3_1027_R	CAGGAAACAGCTATGACC AGT AGM AAC AAG GTA GT
SZ	SZ_M+	TGTAAAACGACGGCCAGT CTC GAG CAA AAG CAG GTA GAT
	SZ_M-	CAGGAAACAGCTATGACC AGT AGA AAC AAG GTA GTT TTT T

NS

Fragment	CDC Primer Name	Sequence
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1	SZ_NS+	TGTAACACGACGGCCAGT AGC AAA AGC AGG GTG ACA AA
	NS_F1_R442_2012	CAGGAAACAGCTATGACC CTT CTG TGA AAG CTC TRA G
2	NS_F2_F262_2012	TGTAACACGACGGCCAGT ATG ACT CTC GAR GAR ATG TCA AGG GA
	NS_F2_R799_2012	CAGGAAACAGCTATGACC TCT TAT CTC TTG CTC CAC TTC AAG C
3	NS_F3_F374_2012	TGTAACACGACGGCCAGT TGA AAG CAA ACT TCA GTG TGA
	SZ_NS-	CAGGAAACAGCTATGACC AGT AGA AAC AAG GGT GTT TTT T

PCR Primers

Conventional

Gene	Reaction #	CDC Primer Name	Sequence
HA (H5)	1	H5F-1	AGC AAA AGC AGG GGT YTA AT
		H5R-1111	CCA TAC CAA CCA TCT AYC ATT CC
	2	H5F-751	AYG CMT AYA ARA TTG TCA AG
		H5R-1773	AGT AGA AAC AAG GGT GTT TTT AAC TAC AAT
PB2	3	SZPB2+	CTC GAG CAA AAG CAG GTC AA
		PB2R1418	AAT ATY CCD ATC ATY CCC A
	4	PB2F1110	GAA TTC ACA ATG GTT GG
		SZPB2-	AGT AGA AAC AAG GTC GTT TTT AAA C
PB1	5	SZPB1+	AGC AAA AGC AGG CAA ACC AT
		PB1R1274	TTR AAC ATG CCC ATC ATC ATY CC
	6	PB1F991	CGT ACA TCA CAA GRA AHC
		SZPB1-	AGT AGA AAC AAG GCA TTT TTT CAT
PA	7	PAUF	AGC AAA AGC AGG TAC TGA T
		PAR1265	CCT TGT TGA ACT CAT TCT GCA GAA TGA GTT C
	8	PAF743	GGY TRC ATT GAG RGC AAG C
		SZPA-	AGT AGA AAC AAG GTA CYT TTT TGG AC
NP	9	NPF1	AGC AAA AGC AGG GTT AAT AAT CAC TCA C
		NPR1029	GAC TCT AAG GTC CTC AAA TGC
	10	NPF859	AGT GGA TAT GAC TTT GAG AGA GAA
		NPR1565	AGT AGA AAC AAG GGT ATT TTT CCT TAA TTG TCG
NA (N1)	11	N1F1	AGC AAA AGC AGG AGA TTA AAA TGA ATC CAA
		N1R835	CAC ACA TGT GAT TTC GCC AGC
	12	N1F458	GGC AAG TGC TTG CCA TGA TGG
		NA-	AGT AGA AAC AAG GAG TTT TTT
M	13	MF1	AGC AAA AGC AGG TAG ATA TTG AAA GAT GAG
		MR1027	AGT AGA AAC AAG GTA GTT TTT TAC
NS	14	NSF1	AGC AAA AGC AGG GTG ACA AAG ACA
		NSR890	AGT AGA AAC AAG GGT GTT TTT TAT

Sequencing Primers

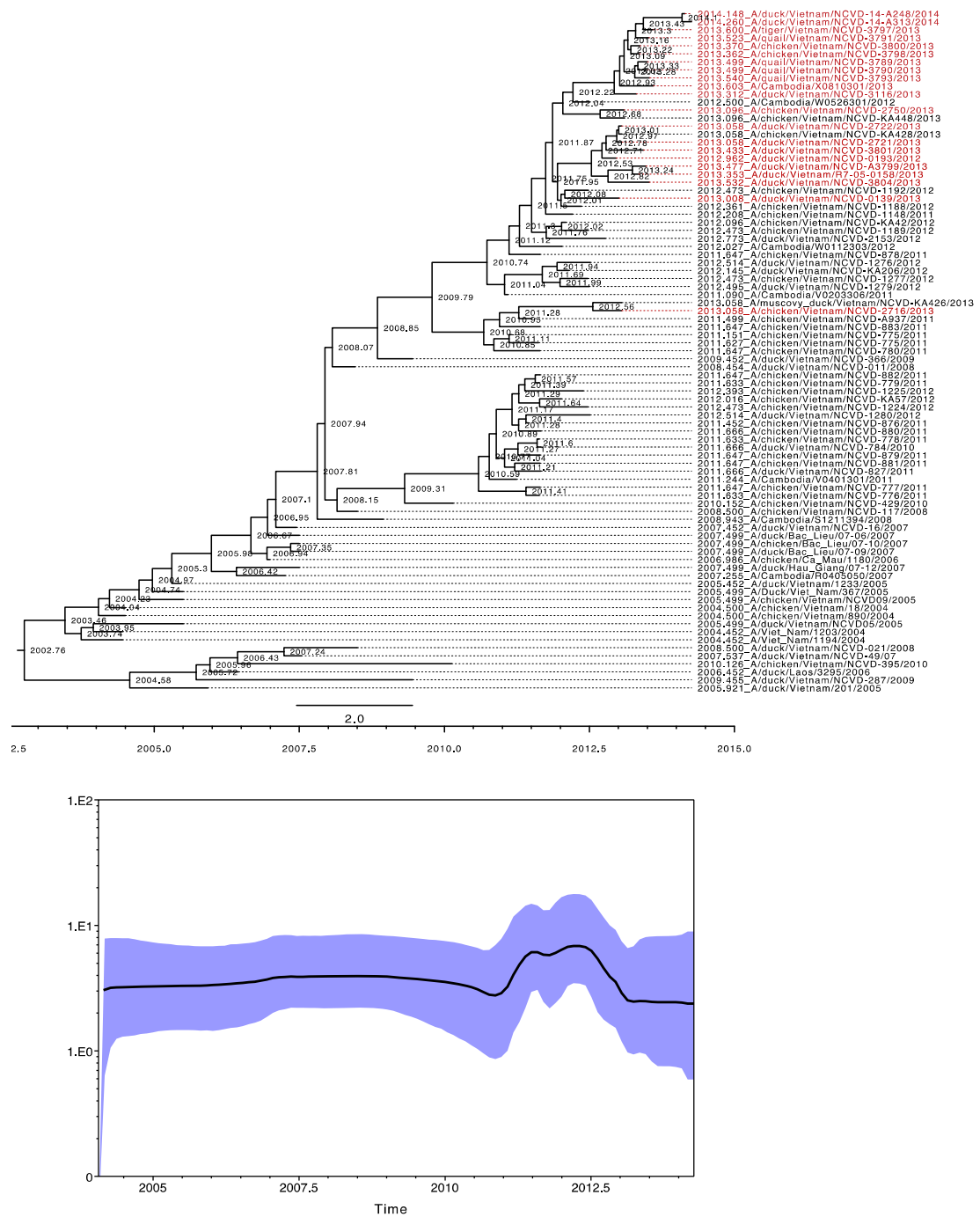
For use with M13 Amplicons

M13 Forward	TGT AAA ACG ACG GCC AGT
M13 Reverse	CAG GAA ACA GCT ATG ACC

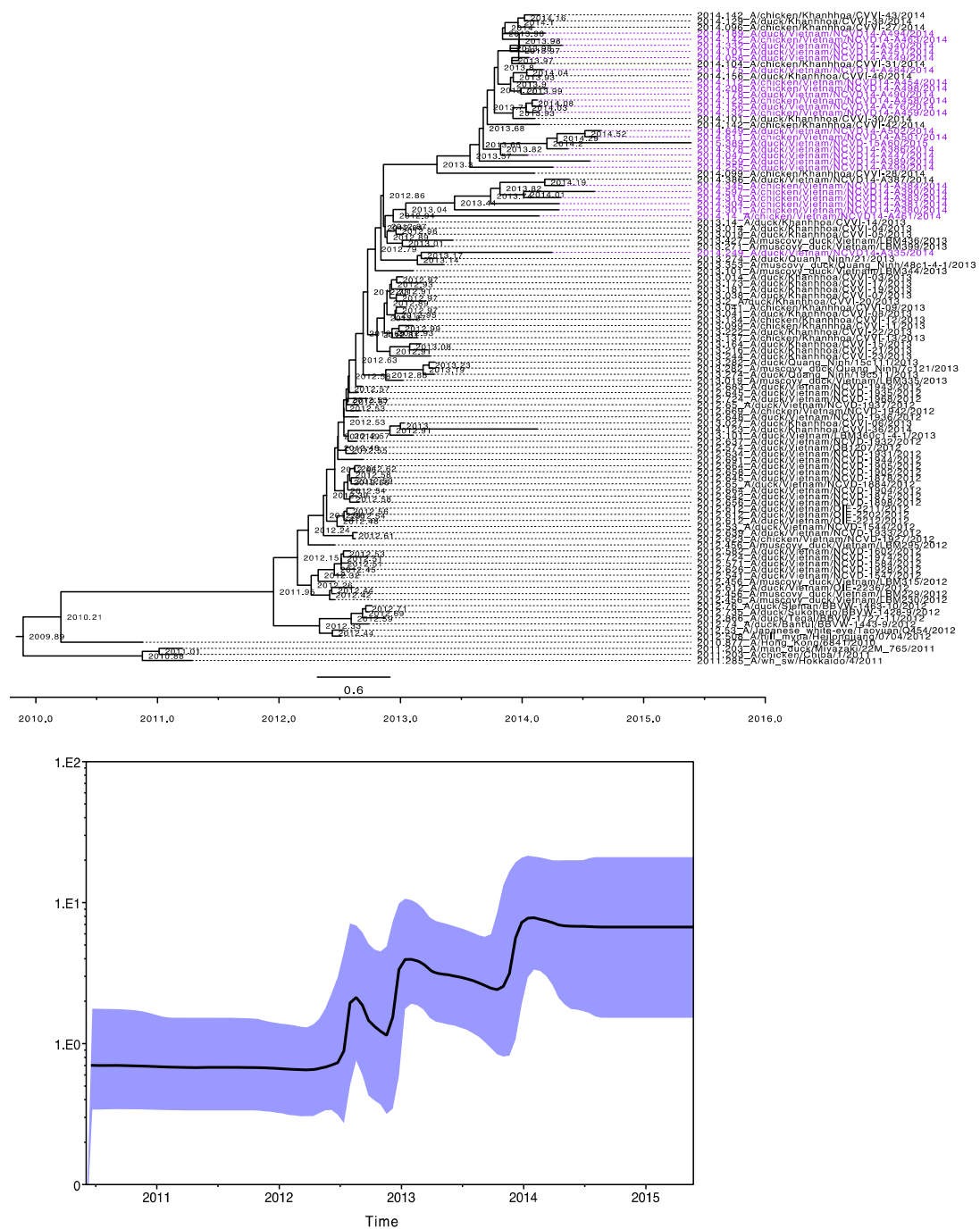
For use with Conventional Amplicons

PCR Amplicon	Seq Reaction #	CDC Primer Name	Sequence
1	1	H5F-219	GAT CTT CTT GRT TGG TRT TAT TGT ARC T
	2	H5R-515	GAG TGA AGC CTC TRA TYT T
	3	H5R-1000	TGA TTT CAC RTA YTT GGG RCA TTC
2	4	H5F-751	AYG CMT AYA ARA TTG TCA AG
	5	H5F-945	CAT TCC ACA AYA TMC AYC C
	6	H5F-1150	CAA YGA GCA GGG GAG TGG RTA
3	7	PB2F254	GAG CAA RRC AAA TGA TGC TG
	8	PB2R370	TTC AAC CTT CTC AAA GTA TG
	9	PB2F770	CAG AGT YTG ATC ATH GCT GC

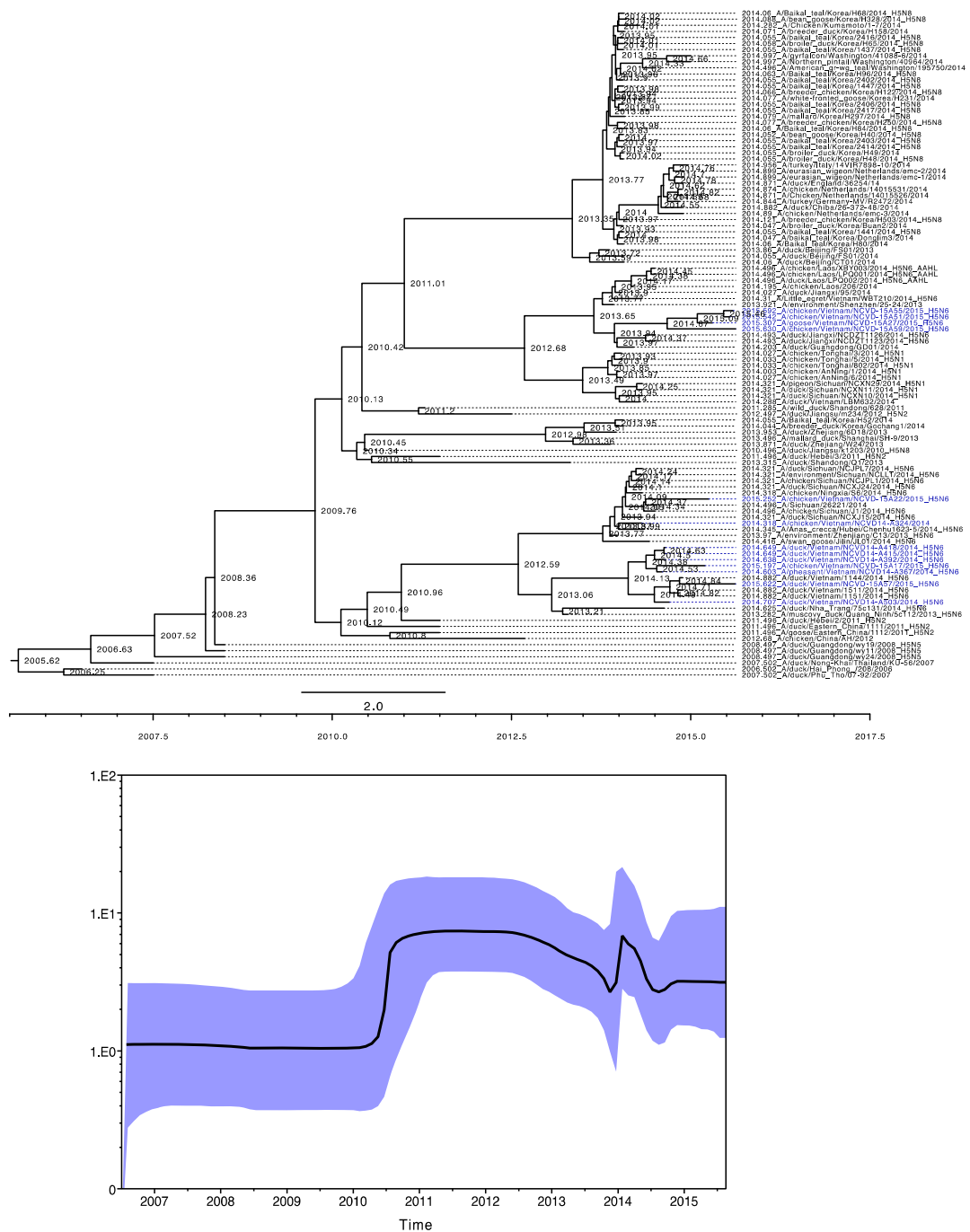
4	10	PB2F1143	GAT TCA RTT GAT AGT AAG TGG AA
	11	PB2R1621	TCC CAC ATC ATT GAK GAT GA
	12	PB2F1860	GAT ACT GTY CAR ATA ATA AAG
5	13	PB1F300	GGC TTT CCT TGA AGA ATC CCA TCC
	14	PB1R473	CTG TTA GAC CGT TCG ATC TG
	15	PB1R1274	TTR AAC ATG CCC ATC ATC ATY CC
6	16	PB1F1230	ACT GAG TCC TGG CAT GAT GAT GGG
	17	PB1R1654	CTG TTG CTG GTC CAA GGT C
	18	PB1F1830	CCA AAT CTA TAC AAT ATC CG
7	19	PAF30	AAG AYT TTG TGC GAC AAT GCT
	20	PAR774	GAC ATT TGA GAA AGC TTG CC
	21	PAF587	TGG GAT TCC TTT CGT CAA TC
8	22	PAF1074	ATG AGG AGA AAA YYC CAA
	23	PAF1877	ACA TGG CCG ATT GGA GAG T
	24	PAR2234	TGG ACA GTA TGG ATA GCA A
9	25	NPF1	AGC AAA AGC AGG GTT AAT AAT CAC TCA C
	26	NPR403	TTG GAA TGC CAT ATC ATC AGR TG
	27	NPF517	TGG RAT GGA CCC CAG AAT GTG
	28	NPR1029	GAC TCT AAG GTC CTC AAA TGC
10	29	NPF859	AGT GGA TAT GAC TTT GAG AGA GAA
	30	NPF1009	GCA TTT GAG GAC CTT AGA GTC
	31	NPR1164	TGT TTC CAT GTT CTC ATT TGA AGC
	32	NPR1565	AGT AGA AAC AAG GGT ATT TTT CCT TAA TTG TCG
11	33	NAF1	AGC AAA AGC AGG AGA TTA AAA TGA ATC CAA
	34	N1R561	GAC CAA GCA ACA GAC TCA AAC C
	35	N1F458	GGC AAG TGC TTG CCA TGA TGG
	36	N1R835	CAC ACA TGT GAT TTC GCC AGC
12	37	N1F458	GGC AAG TGC TTG CCA TGA TGG
	38	N1F955	TCA AAT AGG ATA YAT ATG CAG TGG
	39	N1R1112	AAC TGA TTG GTC AGG RTA YAG YGG
	40	NA-	AGT AGA AAC AAG GAG TTT TTT
13	41	AvMF476	TGT GAG CAG ATT GCA GATTCAC
	42	AvMR560	TTA GCT GTA GTG CTG GCC AGC ACC
	43	MF1	AGC AAA AGC AGG TAG ATA TTG AAA GAT GAG
	44	MR1027	AGT AGA AAC AAG GTA GTT TTT TAC
14	45	AvNSF467	TRG GAG AAA TCT CAC CAT TAC C
	46	AvNSR580	AAC TGT GTT ATC ATT CCA TTC AAG
	47	NSF1	AGC AAA AGC AGG GTG ACA AAG ACA
	48	NSR890	AGT AGA AAC AAG GGT GTT TTT TAT



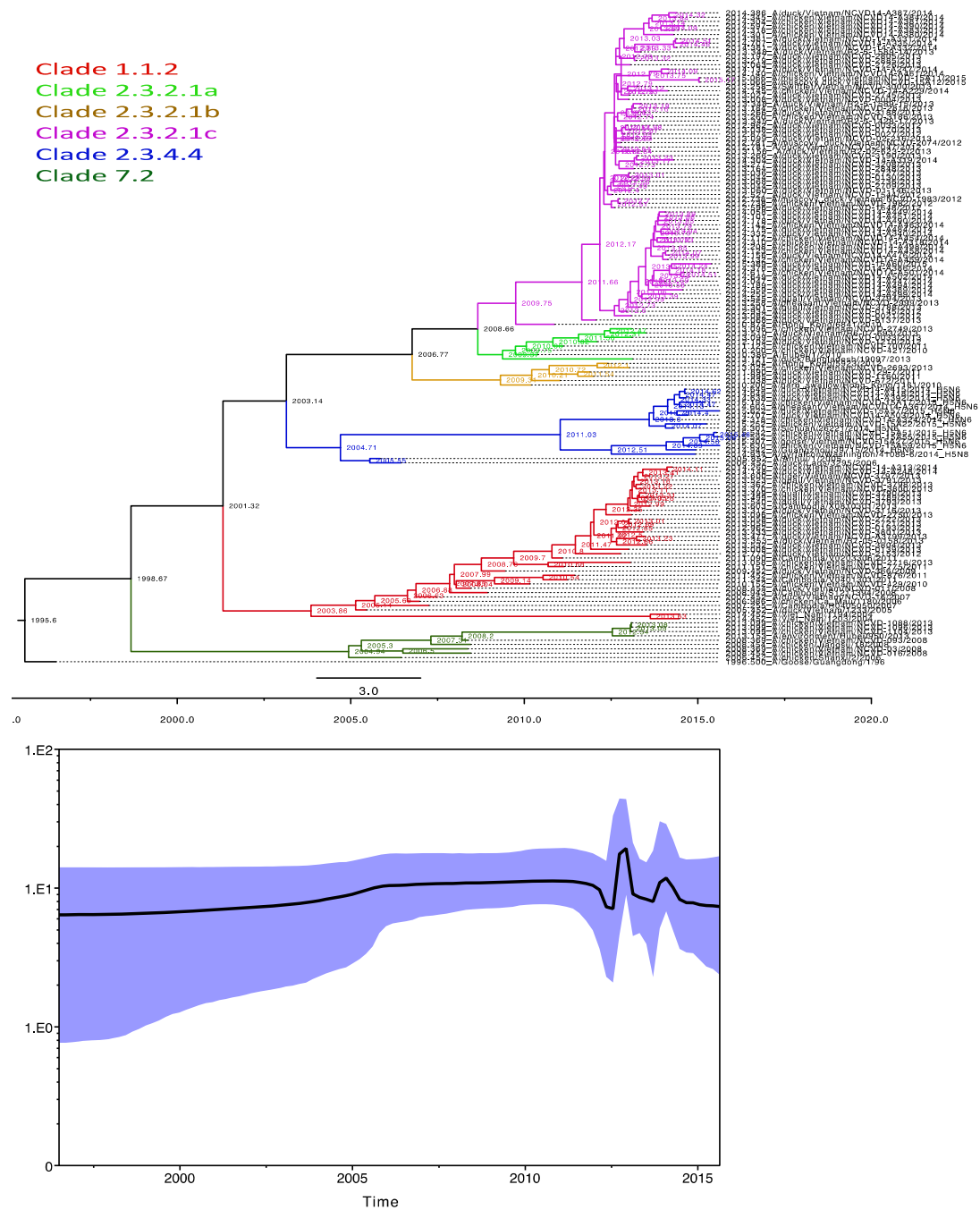
Appendix 3: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of influenza A(H5) HA genes of viruses belonging to clade 1.1.2 in Vietnam, 2012-2015. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. Viruses sequenced for this study are shown in colour.



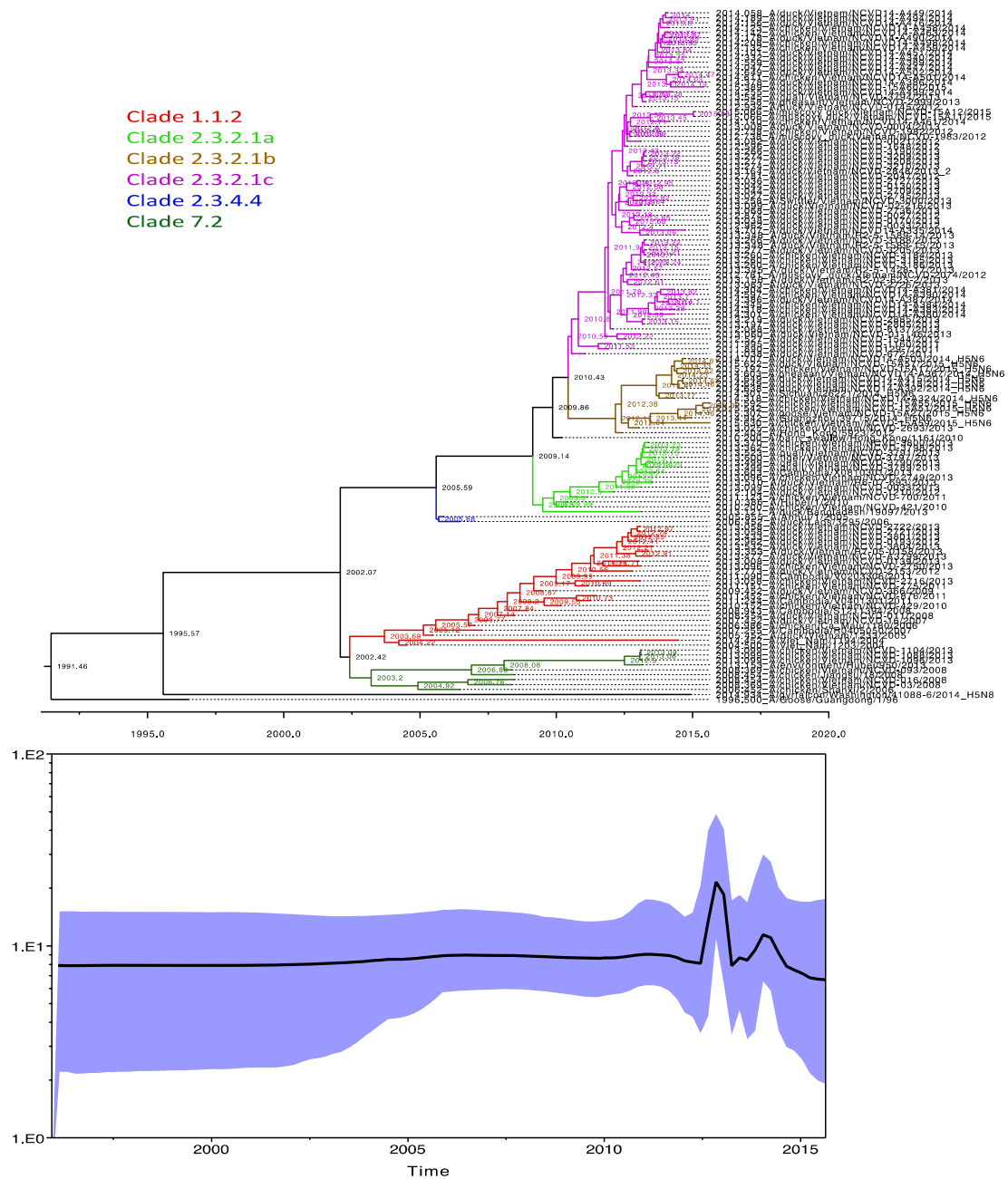
Appendix 4: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of influenza A(H5) HA genes of viruses belonging to clade 2.3.2.1c in Vietnam, 2012-2015. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. Viruses sequenced for this study are shown in colour.



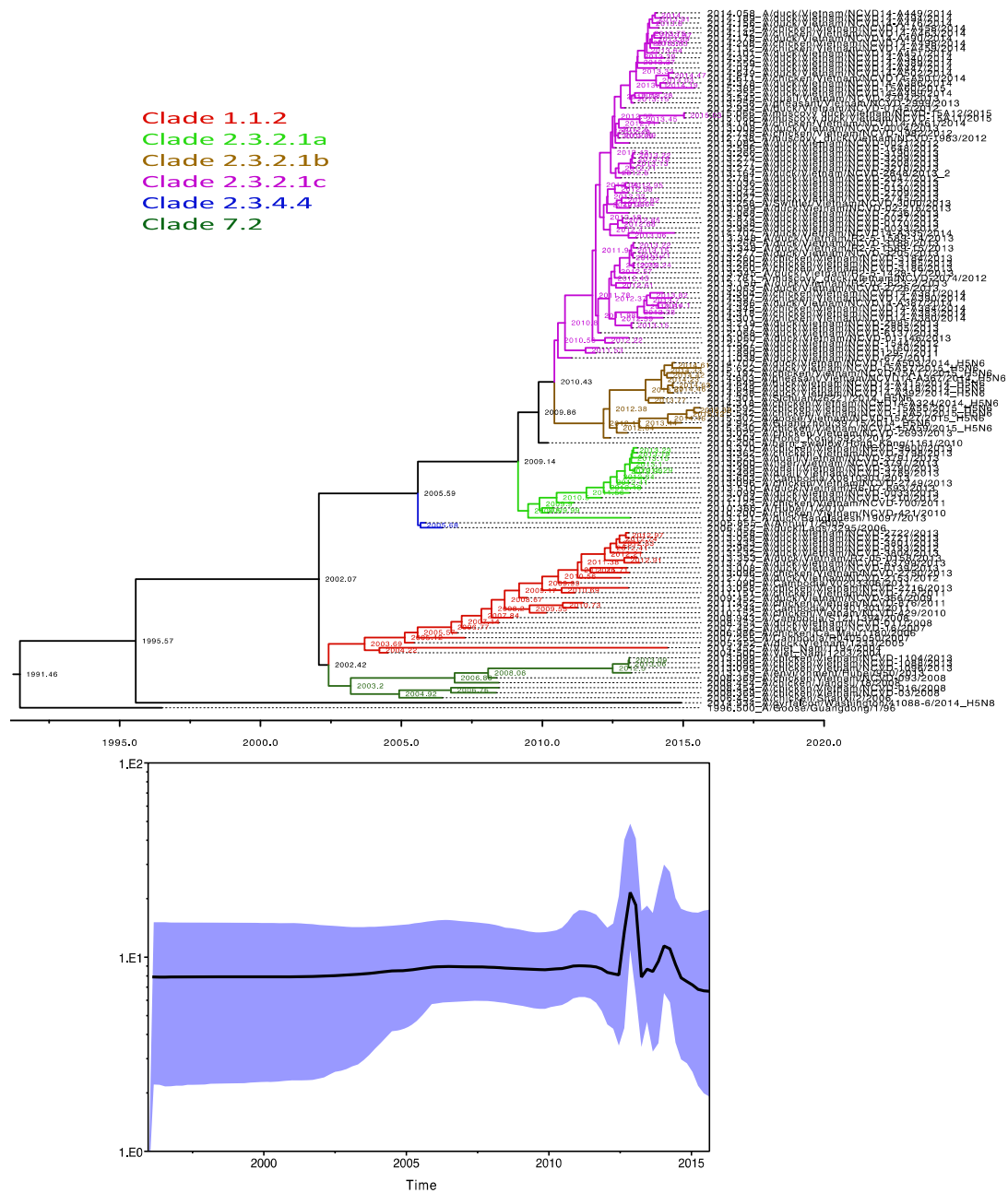
Appendix 5: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of influenza A(H5) HA genes of viruses belonging to clade 2.3.4.4 in Vietnam, 2012-2015. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. Viruses sequenced for this study are shown in colour.



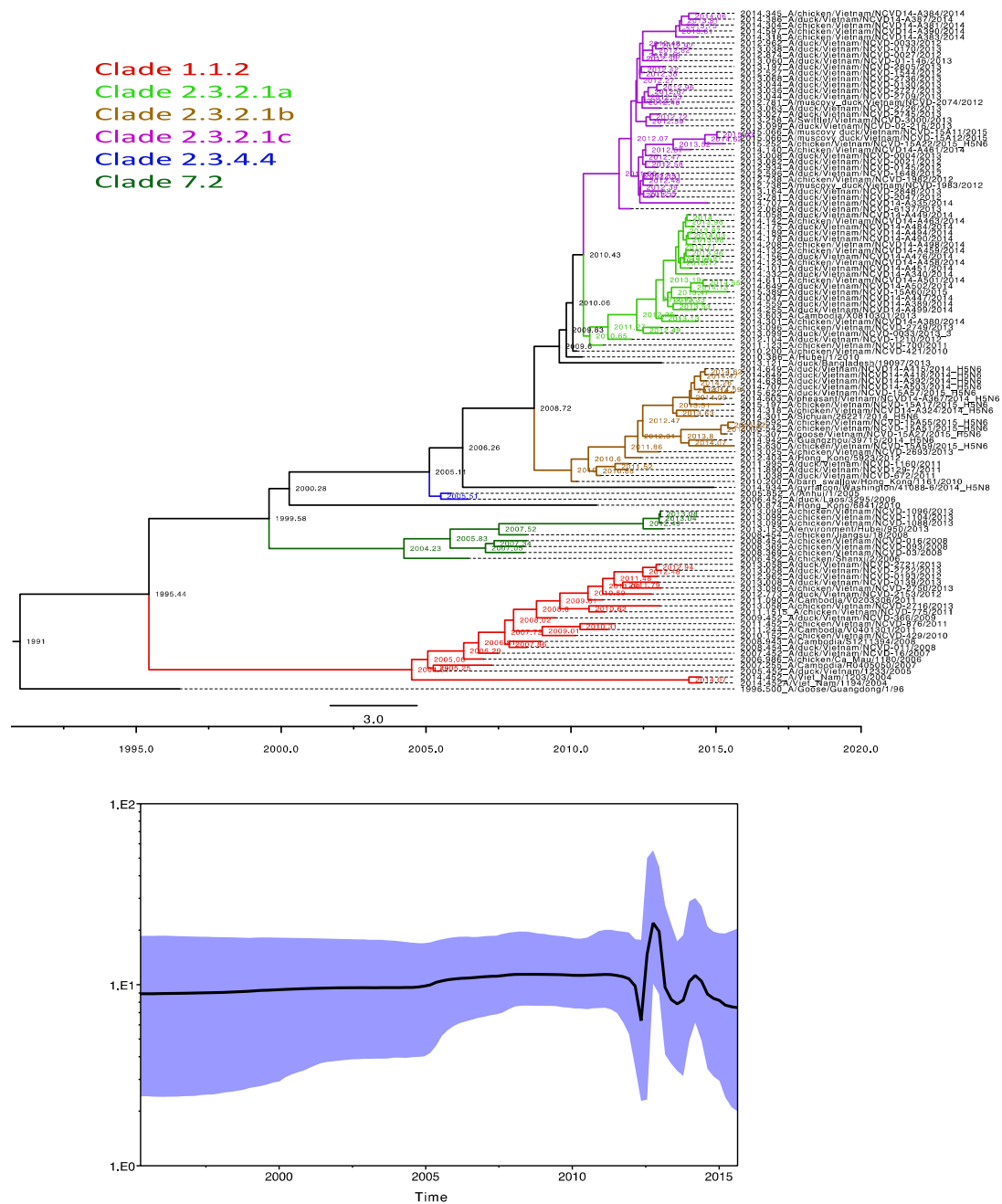
Appendix 6: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of HA gene segment of all clade A(H5) viruses. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. The gene segments of each virus are color-coded based on the origin of the HA gene. Tree branches for clade 1.1.2 viruses are shown in red, 2.3.2.1a in light green, 2.3.4.4 A(H5N6) in brown (except for the N1 NA gene tree), 2.3.2.1c in purple, 2.3.4 in blue, and 7.2 in dark green.



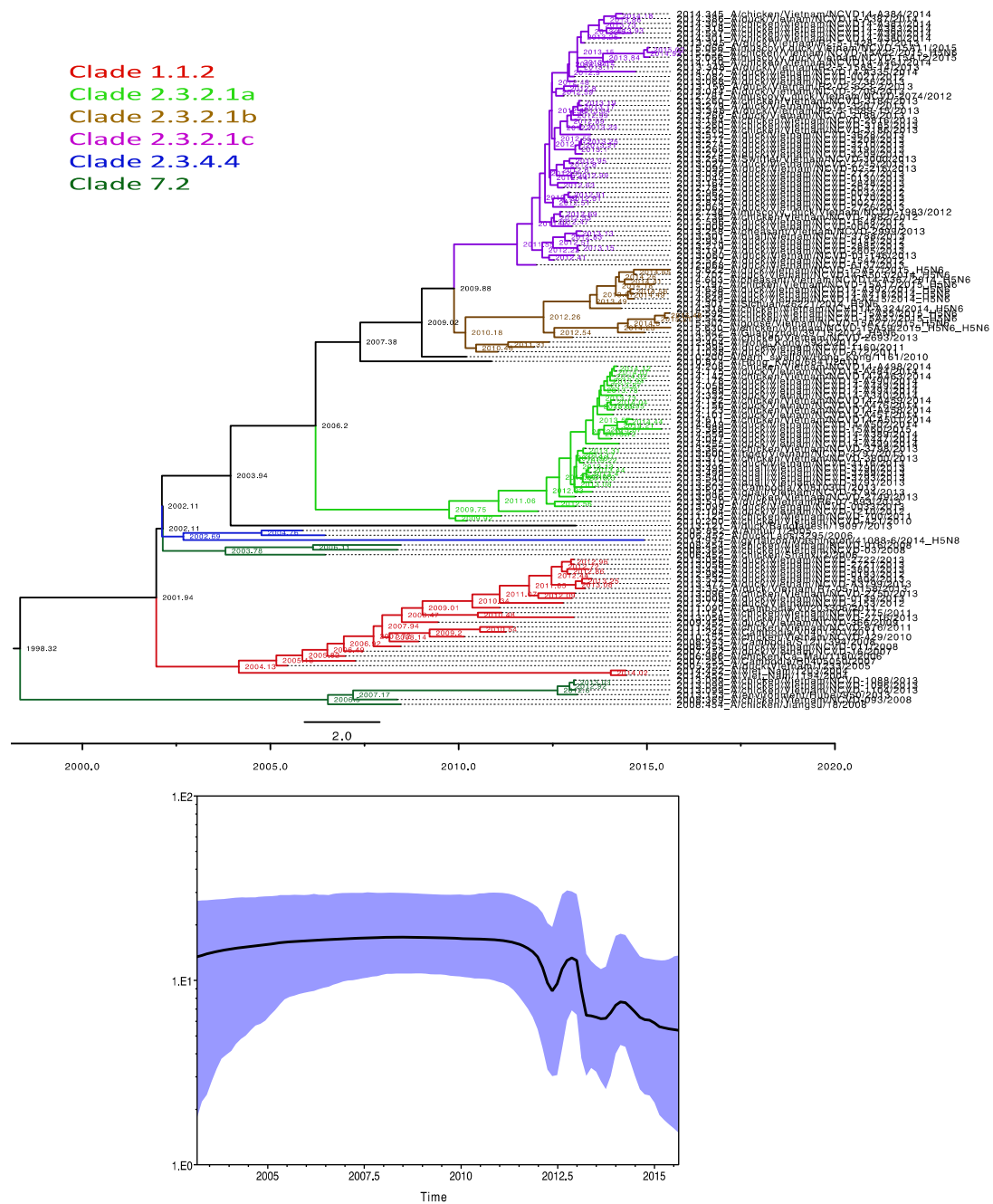
Appendix 7: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of PB2 gene segment of all clade A(H5) viruses. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. The gene segments of each virus are color-coded based on the origin of the HA gene. Tree branches for clade 1.1.2 viruses are shown in red, 2.3.2.1a in light green, 2.3.4.4 A(H5N6) in brown (except for the N1 NA gene tree), 2.3.2.1c in purple, 2.3.4 in blue, and 7.2 in dark green.



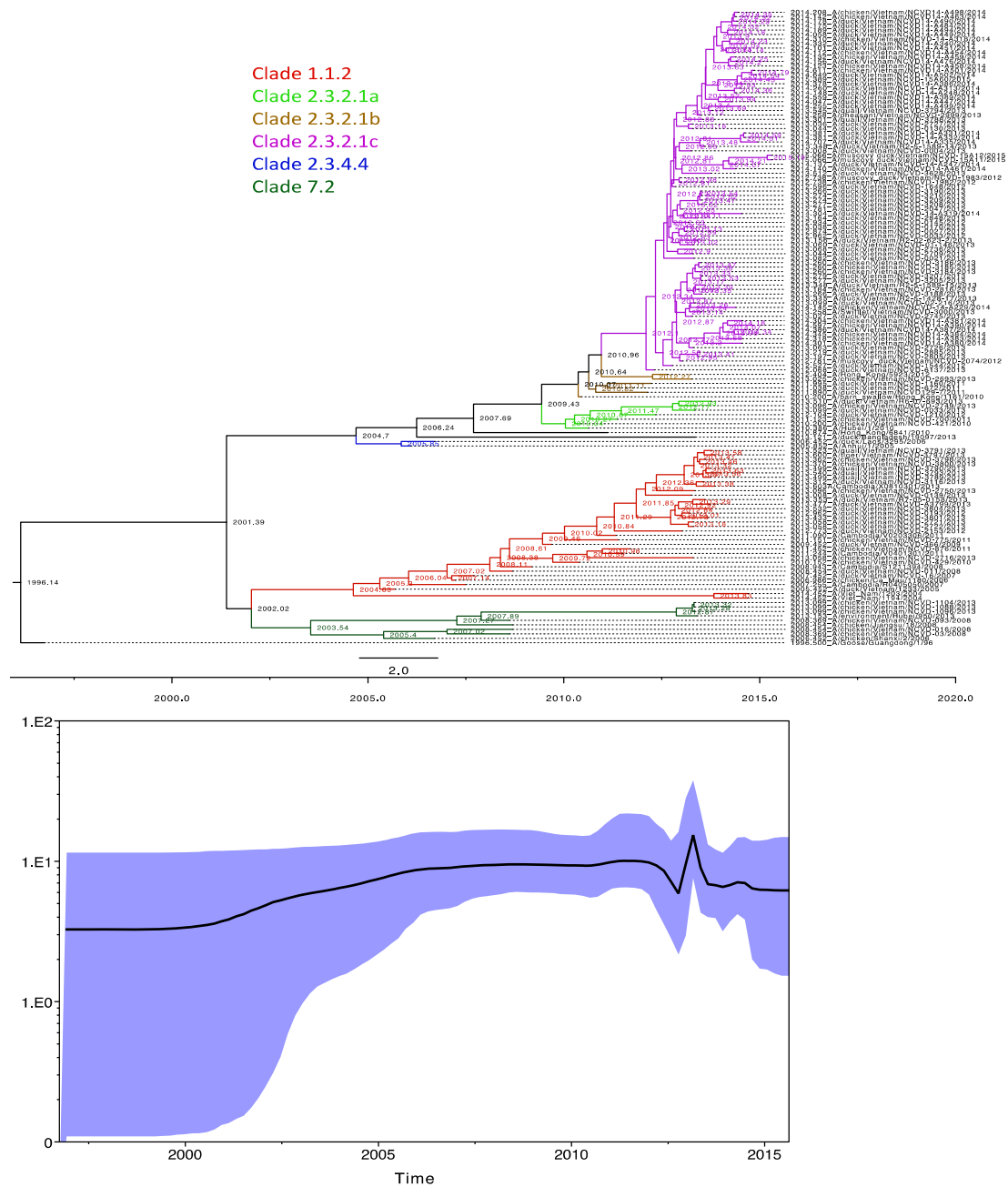
Appendix 8: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of PB1 gene segment of all clade A(H5) viruses. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. The gene segments of each virus are color-coded based on the origin of the HA gene. Tree branches for clade 1.1.2 viruses are shown in red, 2.3.2.1a in light green, 2.3.4.4 A(H5N6) in brown (except for the N1 NA gene tree), 2.3.2.1c in purple, 2.3.4 in blue, and 7.2 in dark green.



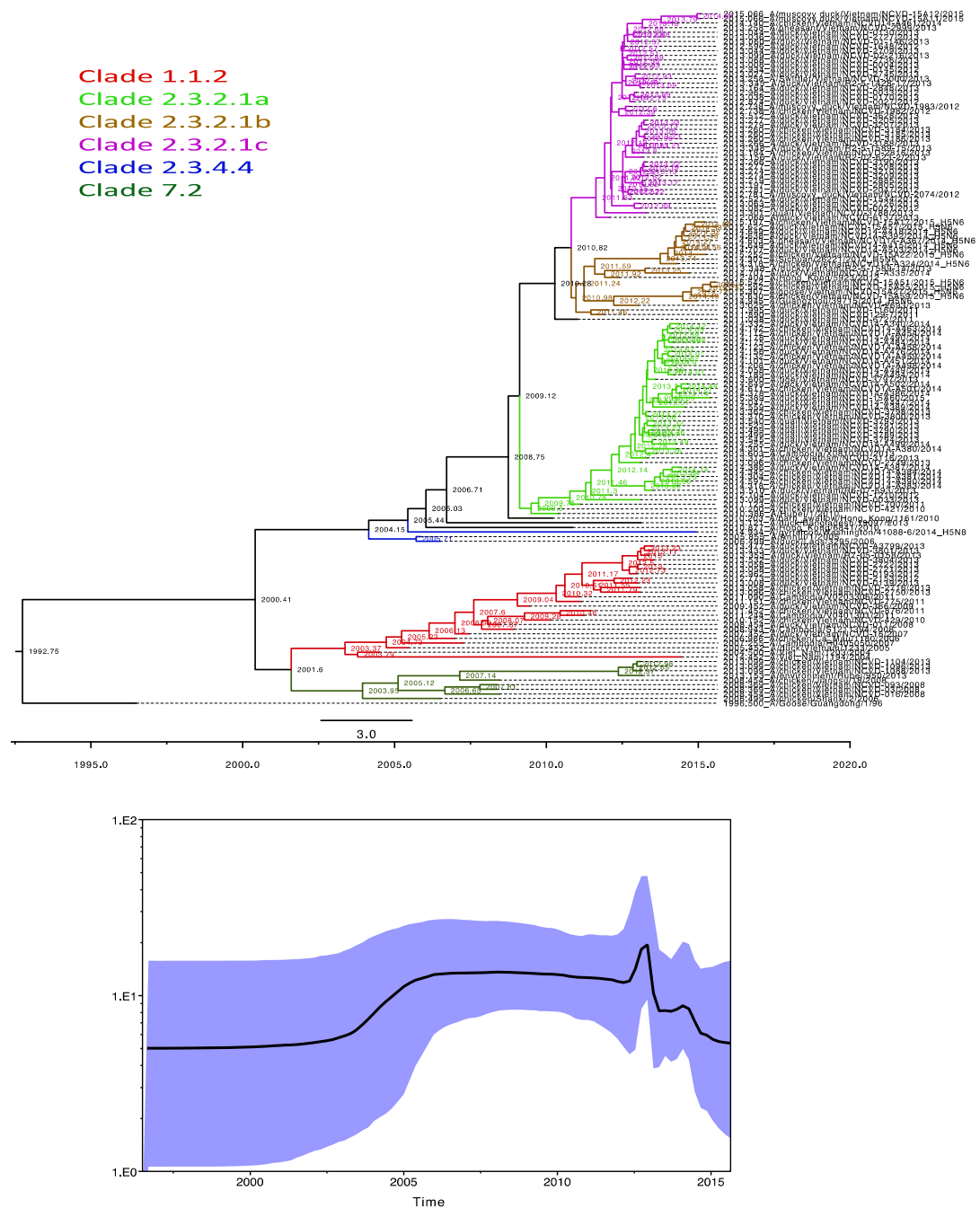
Appendix 9: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of PA gene segment of all clade A(H5) viruses. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. The gene segments of each virus are color-coded based on the origin of the HA gene. Tree branches for clade 1.1.2 viruses are shown in red, 2.3.2.1a in light green, 2.3.4.4 A(H5N6) in brown (except for the N1 NA gene tree), 2.3.2.1c in purple, 2.3.4 in blue, and 7.2 in dark green.



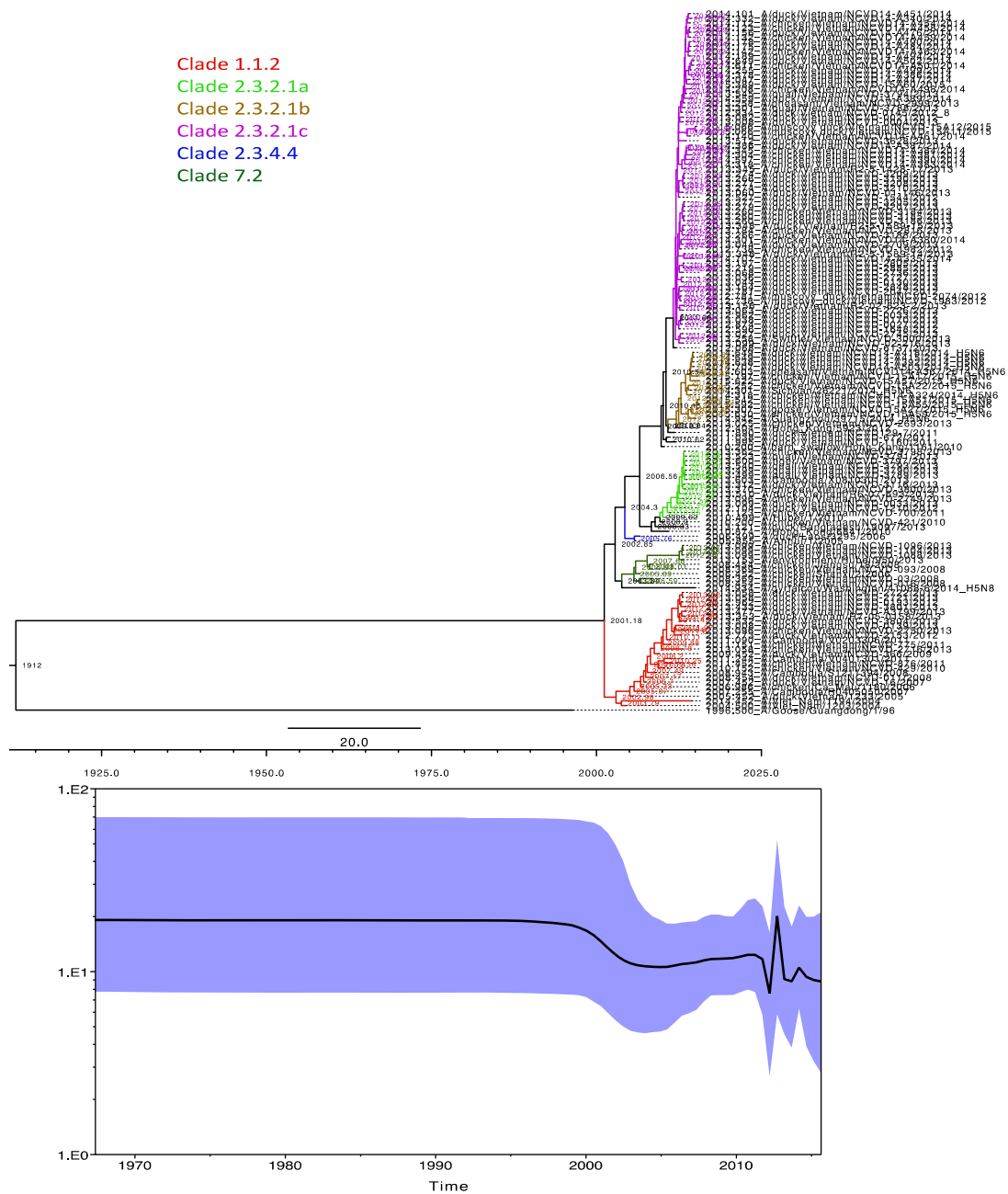
Appendix 10: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of NP gene segment of all clade A(H5) viruses. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. The gene segments of each virus are color-coded based on the origin of the HA gene. Tree branches for clade 1.1.2 viruses are shown in red, 2.3.2.1a in light green, 2.3.4.4 A(H5N6) in brown (except for the N1 NA gene tree), 2.3.2.1c in purple, 2.3.4 in blue, and 7.2 in dark green.



Appendix 11: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of NA gene segment of all clade A(H5) viruses. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. The gene segments of each virus are color-coded based on the origin of the HA gene. Tree branches for clade 1.1.2 viruses are shown in red, 2.3.2.1a in light green, 2.3.4.4 A(H5N6) in brown (except for the N1 NA gene tree), 2.3.2.1c in purple, 2.3.4 in blue, and 7.2 in dark green.



Appendix 12: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of MP gene segment of all clade A(H5) viruses. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. The gene segments of each virus are color-coded based on the origin of the HA gene. Tree branches for clade 1.1.2 viruses are shown in red, 2.3.2.1a in light green, 2.3.4.4 A(H5N6) in brown (except for the N1 NA gene tree), 2.3.2.1c in purple, 2.3.4 in blue, and 7.2 in dark green.



Appendix 13: Bayesian maximum clade credibility (MCC) phylogenetic trees based on tMRCA analysis of NS gene segment of all clade A(H5) viruses. Bayesian skyline plots are shown below each tree with the y-axis representing the effective population size. The x-axis on both the tree and skyline plot is the estimated timeline of genetic divergence among viruses (tree) and variation in population size of viruses (skyline plot) in each clade. The estimated tMRCA in years is shown to the right of each node on the tree. The gene segments of each virus are color-coded based on the origin of the HA gene. Tree branches for clade 1.1.2 viruses are shown in red, 2.3.2.1a in light green, 2.3.4.4 A(H5N6) in brown (except for the N1 NA gene tree), 2.3.2.1c in purple, 2.3.4 in blue, and 7.2 in dark green.

Appendix 14: Annual passive and active sample collection by province, region and A(H5) HA clade from September 2012 to August 2015.

No.	Province	Region	2012					2013						2014						2015						2012-2015						
			Outbreak	Surveillance	1.1.2	2.3.2.1a	2.3.2.1b	2.3.2.1c	Outbreak	Surveillance	1.1.2	2.3.2.1a	2.3.2.1b	2.3.2.1c	7.2	Out-break	Surveillance	1.1.2	2.3.2.1a	2.3.2.1b	2.3.2.1c	2.3.4.4	Outbreak	Surveillance	1.1	2.3.2.1a	2.3.2.1b	2.3.2.1c	2.3.4.4	7.2		
1	Bac Giang	Northeastern							1				1											1				1				
2	Bac Kan	Northeastern	1				1															1					1					
3	Cao Bang	Northeastern																														
4	Ha Giang	Northeastern																														
5	Lang Son	Northeastern	2				2	4	1			1	4		4					1	3		10	1			1	7	3			
6	Lao Cai	Northeastern													8					7	1	1	9				7	2				
7	Phu Tho	Northeastern													3						3		3					3				
8	Quang Ninh	Northeastern						10	2				12									10	2				12					
9	Thai Nguyen	Northeastern							1				1										1				1					
10	Tuyen Quang	Northeastern													4					4			4				4					
11	Yen Bai	Northeastern																														
12	Dien Bien	Northwestern													1					1		1					1					
13	Hoa Binh	Northwestern																			1	1						1				
14	Lai Chau	Northwestern																														
15	Son La	Northwestern	1				1	2					2		1					1			4				4					
16	Bac Ninh	Red River Delta						1		1					3					3			4		1		3					
17	Ha Nam	Red River Delta																			1	1						1				
18	Ha Noi (city)	Red River Delta							3				3								1	1	3					1	3			
19	Hai Duong	Red River Delta																														
20	Hai Phong (city)	Red River Delta						1					1									1					1					
21	Hung Yen	Red River Delta						1					1									1					1					
22	Nam Dinh	Red River Delta		2		1	1		1				1		2					2		2	3		1		4					
23	Ninh Binh	Red River Delta													2					2		2					2					
24	Thai Binh	Red River Delta																														
25	Vinh Phuc	Red River Delta																														
26	Ha Tinh	North Central Coast						1					1		2					1	1		3				2	1				
27	Nghe An	North Central Coast						1					1		3					3		4				2	2	6	2			
28	Quang Binh	North Central Coast													1					1			1				1					
29	Quang Tri	North Central Coast													7					4	3		7				4	3				
30	Thanh Hoa	North Central Coast	1				1								1					1		1	3				2	1				
31	Thua Thien-Hue	North Central Coast																														
32	Binh Dinh	South Central Coast													9					9			9				9					
33	Binh Thuan	South Central Coast													1					1			1				1					
34	Da Nang (city)	South Central Coast													1					1			1				1					

35	Khanh Hoa	South Central Coast	1		1				2				2		19				19						22		1			21							
36	Ninh Thuan	South Central Coast						1					1											1					1								
37	Phu Yen	South Central Coast																																			
38	Quang Nam	South Central Coast						1					1		8				8					8	1				9								
39	Quang Ngai	South Central Coast												19				15	4					19				15	4								
40	Dak Lak	Central Highlands																	1				1	1				1									
41	Dak Nong	Central Highlands																																			
42	Gia Lai	Central Highlands																																			
43	Kon Tum	Central Highlands												3				1	2					3				1	2								
44	Lam Dong	Central Highlands						1		1															1		1										
45	Ba Ria–Vung Tau	Southeastern						1		1														1		1											
46	Binh Duong	Southeastern																																			
47	Binh Phuoc	Southeastern																																			
48	Dong Nai	Southeastern																																			
49	Ho Chi Minh (city)	Southeastern						1		1														1		1											
50	Tay Ninh	Southeastern						2		1	1													2		1	1										
51	An Giang	Mekong Delta																																			
52	Bac Lieu	Mekong Delta						1		1				2				2						3		1		2									
53	Ben Tre	Mekong Delta																																			
54	Ca Mau	Mekong Delta		1			1	1				1		5	1		4		1			1		7		1		7									
55	Can Tho (city)	Mekong Delta						3		3				9	1		8		2			2		14		4		10									
56	Dong Thap	Mekong Delta																																			
57	Hau Giang	Mekong Delta																																			
58	Kien Giang	Mekong Delta		1	1			2		2				2	1		1		1			1		5		4		2									
59	Long An	Mekong Delta							1		1														1		1										
60	Soc Trang	Mekong Delta						1	1	1			1	2			2						3	1	1			3									
61	Tien Giang	Mekong Delta						7		4			3										7		4		0	3									
62	Tra Vinh	Mekong Delta												4	1		3						4		1		0	3									
63	Vinh Long	Mekong Delta						2		2				2			2						4		2		0	2									
Totals			6	4	2	1	0	7	45	13	17	3	1	34	3	128	0	4	0	0	107	17	14	0	0	0	0	7	7	143	15	23	4	1	155	24	3
## provinces (isolates) / year					8						27						28						10						45								

Appendix 15: Strain names, collection information, genotypes and GISAID accession numbers associated with whole genome sequences created for this study.

	Virus	Year (dd/mm/yy)	Species	Subtype	Genotype	Location	Surveil- llance	Gene segment							GenBank accession number								
								PB2	PB1	PA	HA	NP	NA	MP	NS	PB2	PB1	PA	HA	NP	NA	MP	NS
1	A/duck/Vietnam/NCVD-2153/2012	10/9/2012	duck	HSN1	VN3	Khanh Hoa	Out	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171379	KY171380	KY171381	KY171382	KY171383	KY171384	KY171385	KY171386
2	A/chicken/Vietnam/NCVD-2716/2013	1/21/2013	chicken	HSN1	VN3	Vinh Long	Out	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171395	KY171396	KY171397	KY171398	KY171399	KY171400	KY171401	KY171402
3	A/duck/Vietnam/NCVD-2721/2013	1/21/2013	duck	HSN1	VN3	Bac Lieu	Out	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171403	KY171404	KY171405	KY171406	KY171407	KY171408	KY171409	KY171410
4	A/duck/Vietnam/NCVD-2722/2013	1/21/2013	duck	HSN1	VN3	Kien Giang	Out	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171411	KY171412	KY171413	KY171414	KY171415	KY171416	KY171417	KY171418
5	A/chicken/Vietnam/NCVD-2750/2013	2/4/2013	chicken	HSN1	VN3	Tay Ninh	Out	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171483	KY171484	KY171485	KY171486	KY171487	KY171488	KY171489	KY171490
6	A/duck/Vietnam/NCVD-0139/2013	1/3/2013	duck	HSN1	VN3	Can Tho	LBM	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171541	KY171542	KY171543	KY171544	KY171545	KY171546	KY171547	KY171548
7	A/duck/Vietnam/NCVD-0193/2012	12/17/2012	duck	HSN1	VN3	Kien Giang	LBM	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171565	KY171566	KY171567	KY171568	KY171569	KY171570	KY171571	KY171572
8	A/duck/Vietnam/NCVD-3116/2013	4/24/13	duck	HSN1	VN49	Bac Ninh	Out	2.3.2.1a	2.3.2.1a	2.3.2.1a	1.1.2	2.3.2.1a	1.1.2	2.3.2.1a	2.3.2.1a	KY170924	KY170925	KY170926	KY170927	KY170928	KY170929	KY170930	KY170931
9	A/quail/Vietnam/NCVD-3789/2013	7/1/13	quail	HSN1	VN49	Tien Giang	Out	2.3.2.1a	2.3.2.1a	2.3.2.1a	1.1.2	2.3.2.1a	1.1.2	2.3.2.1a	2.3.2.1a	KY170940	KY170941	KY170942	KY170943	KY170944	KY170945	KY170946	KY170947
10	A/quail/Vietnam/NCVD-3790/2013	7/1/13	quail	HSN1	VN49	Tien Giang	Out	2.3.2.1a	2.3.2.1a	2.3.2.1a	1.1.2	2.3.2.1a	1.1.2	2.3.2.1a	2.3.2.1a	KY170948	KY170949	KY170950	KY170951	KY170952	KY170953	KY170954	KY170955
11	A/quail/Vietnam/NCVD-3791/2013	7/10/13	quail	HSN1	VN49	Tien Giang	Out	2.3.2.1a	2.3.2.1a	2.3.2.1a	1.1.2	2.3.2.1a	1.1.2	2.3.2.1a	2.3.2.1a	KY170956	KY170957	KY170958	KY170959	KY170960	KY170961	KY170962	KY170963
12	A/quail/Vietnam/NCVD-3793/2013	7/16/13	quail	HSN1	VN49	Tien Giang	Out	2.3.2.1a	2.3.2.1a	2.3.2.1a	1.1.2	2.3.2.1a	1.1.2	2.3.2.1a	2.3.2.1a	KY170980	KY170981	KY170982	KY170983	KY170984	KY170985	KY170986	KY170987
13	A/tiger/Vietnam/NCVD-3797/2013	8/7/13	tiger	HSN1	VN49	Ho Chi Minh city	Out	2.3.2.1a	2.3.2.1a	2.3.2.1a	1.1.2	2.3.2.1a	1.1.2	2.3.2.1a	2.3.2.1a	KY170988	KY170989	KY170990	KY170991	KY170992	KY170993	KY170994	KY170995
14	A/chicken/Vietnam/NCVD-3798/2013	5/12/13	chicken	HSN1	VN49	Ba Ria - Vung Tau	Out	2.3.2.1a	2.3.2.1a	2.3.2.1a	1.1.2	2.3.2.1a	1.1.2	2.3.2.1a	2.3.2.1a	KY170996	KY170997	KY170998	KY170999	KY171000	KY171001	KY171002	KY171003
15	A/duck/Vietnam/NCVD-3799/2013	6/23/13	duck	HSN1	VN3	Soc Trang	Out	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171259	KY171260	KY171261	KY171262	KY171263	KY171264	KY171265	KY171266
16	A/chicken/Vietnam/NCVD-3800/2013	5/15/13	chicken	HSN1	VN49	Kien Giang	Out	2.3.2.1a	2.3.2.1a	2.3.2.1a	1.1.2	2.3.2.1a	1.1.2	2.3.2.1a	2.3.2.1a	KY171004	KY171005	KY171006	KY171007	KY171008	KY171009	KY171010	KY171011
17	A/duck/Vietnam/NCVD-3801/2013	6/7/13	duck	HSN1	VN3	Can Tho	Out	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171012	KY171013	KY171014	KY171015	KY171016	KY171017	KY171018	KY171019
18	A/duck/Vietnam/NCVD-3804/2013	7/13/13	duck	HSN1	VN3	Vinh Long	Out	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171020	KY171021	KY171022	KY171023	KY171024	KY171025	KY171026	KY171027
19	A/duck/Vietnam/R7-05-0158/2013	5/9/13	duck	HSN1	VN3	Can Tho	LBM	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	1.1.2	KY171267	KY171268	KY171269	KY171270	KY171271	KY171272	KY171273	KY171274
20	A/duck/Vietnam/NCVD-14A248/2014	2/23/2014	duck	HSN1	ND	Tra Vinh	Out	No data	No data	No data	1.1.2	No data	2.3.2.1b	No data	No data				partial		partial		
21	A/duck/Vietnam/NCVD-14A313/2014	4/5/2014	duck	HSN1	ND	Can Tho	Out	No data	No data	No data	1.1.2	No data	2.3.2.1b	No data	No data				partial		partial		
22	A/chicken/Vietnam/NCVD-2749/2013	2/4/2013	chicken	HSN1	VN12	Tay Ninh	Out	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	KY171475	KY171476	KY171477	KY171478	KY171479	KY171480	KY171481	KY171482
23	A/duck/Vietnam/NCVD-0033/2013	2/5/2013	duck	HSN1	VN12	Lam Dong	LBM	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	KY171621	KY171622	KY171623	KY171624	KY171625	KY171626	KY171627	KY171628
24	A/chicken/Vietnam/NCVD-2693/2013	1/9/2013	chicken	HSN1	VN45	Lang Son	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171387	KY171388	KY171389	KY171390	KY171391	KY171392	KY171393	KY171394
25	A/duck/Vietnam/NCVD-0033/2012	12/17/2012	duck	HSN1	VN50	Nam Dinh	LBM	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1a	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171581	KY171582	KY171583	KY171584	KY171585	KY171586	KY171587	KY171588
26	A/duck/Vietnam/R6-07-693/2013	7/5/13	duck	HSN1	VN12	Long An	LBM	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	2.3.2.1a	KY171028	KY171029	KY171030	KY171031	KY171032	KY171033	KY171034	KY171035
27	A/duck/Vietnam/NCVD-1648/2012	8/5/2012	duck	HSN1	VN48	Bac Kan	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171339	KY171340	KY171341	KY171342	KY171343	KY171344	KY171345	KY171346
28	A/chicken/Vietnam/NCVD-1982/2012	9/26/2012	chicken	HSN1	VN48	Lang Son	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171347	KY171348	KY171349	KY171350	KY171351	KY171352	KY171353	KY171354
29	A/Muscovy duck/Vietnam/NCVD-1983/2012	9/26/2012	muscovy duck	HSN1	VN48	Lang Son	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171355	KY171356	KY171357	KY171358	KY171359	KY171360	KY171361	KY171362
30	A/duck/Vietnam/NCVD-2047/2012	10/9/2012	duck	HSN1	VN48	Thanh Hoa	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171363	KY171364	KY171365	KY171366	KY171367	KY171368	KY171369	KY171370
31	A/Muscovy duck/Vietnam/NCVD-2074/2012	10/12/2012	muscovy duck	HSN1	VN48	Son La	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171371	KY171372	KY171373	KY171374	KY171375	KY171376	KY171377	KY171378
32	A/duck/Vietnam/NCVD-2726/2013	1/23/2013	duck	HSN1	VN48	Ha Tinh	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171419	KY171420	KY171421	KY171422	KY171423	KY171424	KY171425	KY171426
33	A/duck/Vietnam/NCVD-2727/2013	1/23/2013	duck	HSN1	VN48	Nghie An	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171427	KY171428	KY171429	KY171430	KY171431	KY171432	KY171433	KY171434
34	A/duck/Vietnam/NCVD-2736/2013	1/25/2013	duck	HSN1	VN48	Lang Son	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171435	KY171436	KY171437	KY171438	KY171439	KY171440	KY171441	KY171442
35	A/duck/Vietnam/NCVD-2709/2013	1/16/2013	duck	HSN1	VN48	Hai Phong	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171443	KY171444	KY171445	KY171446	KY171447	KY171448	KY171449	KY171450
36	A/duck/Vietnam/NCVD-2805/2013	3/13/2013	duck	HSN1	VN48	Son La	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171451	KY171452	KY171453	KY171454	KY171455	KY171456	KY171457	KY171458
37	A/swiftlet/Vietnam/NCVD-3000/2013	4/4/2013	swiftlet	HSN1	VN48	Ninh Thuan	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171459	KY171460	KY171461	KY171462	KY171463	KY171464	KY171465	KY171466
38	A/duck/Vietnam/NCVD-2745/2013	1/10/2013</																					

56	A/duck/Vietnam/R2-5-1589-15/2013	5/7/13	duck	H5N1	VN48	Quang Ninh	LBM	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171283	KY171284	KY171285	KY171286	KY171287	KY171288	KY171289	KY171290
57	A/duck/Vietnam/R2-02-623-2/2013	2/26/13	duck	H5N1	VN48	Thai Nguyen	LBM	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY170900	KY170901	KY170902	KY170903	KY170904	KY170905	KY170906	KY170907
58	A/duck/Vietnam/NCVD-2885/2013	3/21/13	duck	H5N1	VN48	Lang Son	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171291	KY171292	KY171293	KY171294	KY171295	KY171296	KY171297	KY171298
59	A/chicken/Vietnam/NCVD-2816/2013	3/8/13	chicken	H5N1	VN48	Hung Yen	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY170908	KY170909	KY170910	KY170911	KY170912	KY170913	KY170914	KY170915
60	A/pheasant/Vietnam/NCVD-2999/2013	4/4/13	pheasant	H5N1	VN48	Tien Giang	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY170916	KY170917	KY170918	KY170919	KY170920	KY170921	KY170922	KY170923
61	A/duck/Vietnam/NCVD-3628/2013	7/6/13	duck	H5N1	VN48	Son La	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171299	KY171300	KY171301	KY171302	KY171303	KY171304	KY171305	KY171306
62	A/chicken/Vietnam/NCVD-3184/2013	4/5/13	chicken	H5N1	VN48	Quang Ninh	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171307	KY171308	KY171309	KY171310	KY171311	KY171312	KY171313	KY171314
63	A/chicken/Vietnam/NCVD-3185/2013	4/5/13	chicken	H5N1	VN48	Quang Ninh	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY170932	KY170933	KY170934	KY170935	KY170936	KY170937	KY170938	KY170939
64	A/chicken/Vietnam/NCVD-3186/2013	4/5/13	chicken	H5N1	VN48	Quang Ninh	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171315	KY171316	KY171317	KY171318	KY171319	KY171320	KY171321	KY171322
65	A/duck/Vietnam/NCVD-3188/2013	4/7/13	duck	H5N1	VN48	Quang Ninh	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171323	KY171324	KY171325	KY171326	KY171327	KY171328	KY171329	KY171330
66	A/duck/Vietnam/NCVD-3190/2013	4/7/13	duck	H5N1	VN48	Quang Ninh	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171331	KY171332	KY171333	KY171334	KY171335	KY171336	KY171337	KY171338
67	A/quail/Vietnam/NCVD-3794/2013	7/18/13	quail	H5N1	VN52	Tien Giang	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY170964	KY170965	KY170966	KY170967	KY170968	KY170969	KY170970	KY170971
68	A/quail/Vietnam/NCVD-3788/2013	4/20/13	quail	H5N1	VN48	Tien Giang	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY170972	KY170973	KY170974	KY170975	KY170976	KY170977	KY170978	KY170979
69	A/chicken/Vietnam/NCVD-14A229/2014	2/22/2014	chicken	H5N1	ND	Tra Vinh	Out	No data	No data	No data	2.3.2.1c	No data	2.3.2.1b	No data	No data				partial		partial		
70	A/duck/Vietnam/NCVD-14A247/2014	2/19/2014	duck	H5N1	ND	Can Tho	Out	No data	No data	No data	2.3.2.1c	No data	2.3.2.1b	No data	No data				partial		partial		
71	A/chicken/Vietnam/NCVD-14A318/2014	4/23/2014	chicken	H5N1	ND	Tuyen Quang	Out	No data	No data	No data	2.3.2.1c	No data	2.3.2.1b	No data	No data				partial		partial		
72	A/duck/Vietnam/NCVD-14A319/2014	4/21/2014	duck	H5N1	ND	Dien Bien	Out	No data	No data	No data	2.3.2.1c	No data	2.3.2.1b	No data	No data				partial		partial		
73	A/duck/Vietnam/NCVD-14A331/2014	5/19/2014	duck	H5N1	ND	Bac Ninh	Out	No data	No data	No data	2.3.2.1c	No data	2.3.2.1b	No data	No data				partial		partial		
74	A/duck/Vietnam/NCVD-14A332/2014	5/19/2014	duck	H5N1	ND	Bac Ninh	Out	No data	No data	No data	2.3.2.1c	No data	2.3.2.1b	No data	No data				partial		partial		
75	A/duck/Vietnam/NCVD-14A335/2014	4/1/2014	duck	H5N1	VN51	Bac Ninh	Out	2.3.2.1b	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171060	KY171061	KY171062	KY171063	KY171064	KY171065	KY171066	KY171067
76	A/duck/Vietnam/NCVD-14A340/2014	5/1/2014	duck	H5N1	VN52	Ninh Binh	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY170880	KY170879	KY170881	KY170876	KY170877	KY170878	KY170882	KY170883
77	A/chicken/Vietnam/NCVD-14A380/2014	4/20/2014	Chicken	H5N1	VN53	Can Tho	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171068	KY171069	KY171070	KY171071	KY171072	KY171073	KY171074	KY171075
78	A/chicken/Vietnam/NCVD-14A381/2014	4/21/2014	Chicken	H5N1	VN53	Can Tho	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171076	KY171077	KY171078	KY171079	KY171080	KY171081	KY171082	KY171083
79	A/chicken/Vietnam/NCVD-14A383/2014	4/26/2014	Chicken	H5N1	VN53	Can Tho	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171084	KY171085	KY171086	KY171087	KY171088	KY171089	KY171090	KY171091
80	A/chicken/Vietnam/NCVD-14A384/2014	5/6/2014	Chicken	H5N1	VN53	Kien Giang	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171092	KY171093	KY171094	KY171095	KY171096	KY171097	KY171098	KY171099
82	A/duck/Vietnam/NCVD-14A387/2014	5/21/2014	duck	H5N1	VN53	Soc Trang	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171100	KY171101	KY171102	KY171103	KY171104	KY171105	KY171106	KY171107
83	A/duck/Vietnam/NCVD-14A389/2014	7/23/2014	duck	H5N1	VN53	Soc Trang	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171108	KY171109	KY171110	KY171111	KY171112	KY171113	KY171114	KY171115
84	A/chicken/Vietnam/NCVD-14A390/2014	8/6/2014	chicken	H5N1	VN53	Can Tho	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171116	KY171117	KY171118	KY171119	KY171120	KY171121	KY171122	KY171123
85	A/duck/Vietnam/NCVD-14A447/2014	1/17/2014	duck	H5N1	VN52	Quang Ngai	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171124	KY171125	KY171126	KY171127	KY171128	KY171129	KY171130	KY171131
87	A/duck/Vietnam/NCVD-14A451/2014	2/6/2014	duck	H5N1	VN52	Quang Ngai	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171132	KY171133	KY171134	KY171135	KY171136	KY171137	KY171138	KY171139
88	A/chicken/Vietnam/NCVD-14A454/2014	2/10/2014	chicken	H5N1	ND	Kon Tum	Out	2.3.2.1a	No data	No data	2.3.2.1c	No data	2.3.2.1b	2.3.2.1a	2.3.2.1b	partial		partial		partial	partial	partial	partial
89	A/chicken/Vietnam/NCVD-14A458/2014	2/14/2014	chicken	H5N1	VN52	Quang Ngai	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171140	KY171141	KY171142	KY171143	KY171144	KY171145	KY171146	KY171147
90	A/chicken/Vietnam/NCVD-14A459/2014	2/17/2014	chicken	H5N1	VN52	Quang Ngai	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171148	KY171149	KY171150	KY171151	KY171152	KY171153	KY171154	KY171155
91	A/chicken/Vietnam/NCVD-14A461/2014	2/20/2014	chicken	H5N1	VN48	Binh Dinh	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171156	KY171157	KY171158	KY171159	KY171160	KY171161	KY171162	KY171163
92	A/chicken/Vietnam/NCVD-14A463/2014	2/21/2014	chicken	H5N1	VN52	Quang Nam	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171164	KY171165	KY171166	KY171167	KY171168	KY171169	KY171170	KY171171
93	A/duck/Vietnam/NCVD-14A476/2014	2/26/2014	duck	H5N1	VN52	Quang Ngai	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171172	KY171173	KY171174	KY171175	KY171176	KY171177	KY171178	KY171179
95	A/duck/Vietnam/NCVD-14A490/2014	3/6/2014	duck	H5N1	VN52	Quang Nam	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171180	KY171181	KY171182	KY171183	KY171184	KY171185	KY171186	KY171187
96	A/duck/Vietnam/NCVD-14A494/2014	3/10/2014	Duck	H5N1	VN52	Quang Nam	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171188	KY171189	KY171190	KY171191	KY171192	KY171193	KY171194	KY171195
97	A/chicken/Vietnam/NCVD-14A498/2014	3/17/2014	chicken	H5N1	VN52	Da Nang	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171196	KY171197	KY171198	KY171199	KY171200	KY171201	KY171202	KY171203
98	A/duck/Vietnam/NCVD-14A499/2014	4/3/2014	duck	H5N1	VN52	Quang Ngai	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171204	KY171205	KY171206	KY171207	KY171208	KY171209	KY171210	KY171211
99	A/chicken/Vietnam/NCVD-14A501/2014	8/11/2014	chicken	H5N1	VN52	Quang Ngai	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171212	KY171213	KY171214	KY171215	KY171216	KY171217	KY171218	KY171219
100	A/duck/Vietnam/NCVD-14A502/2014	8/25/2014	duck	H5N1	VN52	Quang Ngai	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171220	KY171221	KY171222	KY171223	KY171224	KY171225	KY171226	KY171227
101	A/duck/Vietnam/NCVD-15A60/2015	5/22/2015	duck	H5N1	VN52	Dak Lak	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1b	KY171733	KY171734	KY171735	KY171736	KY171737	KY171738	KY171739	KY171740
102	A/Muscovy duck/Vietnam/NCVD-15A11/2015	1/24/2015	Muscovy duck	H5N1	VN48	Nghe An	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171228	KY171229	KY171230	KY171231	KY171232	KY171233	KY171234	KY171235

103	A/Muscovy duck/Vietnam/NCVD-15A12/2015	1/24/2015	Muscovy duck	H5N1	VN48	Nghe An	Out	2.3.2.1a	2.3.2.1b	2.3.2.1a	2.3.2.1c	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.2.1b	KY171236	KY171237	KY171238	KY171239	KY171240	KY171241	KY171242	KY171243
104	A/chicken/Vietnam/NCVD-14-A324/2014	4/26/2014	chicken	H5N6	VN54	Lang Son	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171244	KY171245	KY171246	KY171247	KY171248	KY171249	KY171250	KY171251
105	A/pheasant/Vietnam/NCVD14-A367/2014	8/8/2014	Pheasant	H5N6	VN54	Lao Cai	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171637	KY171638	KY171639	KY171640	KY171641	KY171642	KY171643	KY171644
106	A/duck/Vietnam/NCVD14-A392/2014	8/21/2014	duck	H5N6	VN54	Quang Tri	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171647	KY171648	KY171649	KY171650	KY171651	KY171652	KY171653	KY171654
107	A/duck/Vietnam/NCVD14-A415/2014	8/25/2014	duck	H5N6	VN54	Quang Tri	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171657	KY171658	KY171659	KY171660	KY171661	KY171662	KY171663	KY171664
108	A/duck/Vietnam/NCVD14-A418/2014	8/25/2014	duck	H5N6	VN54	Quang Tri	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171665	KY171666	KY171667	KY171668	KY171669	KY171670	KY171671	KY171672
109	A/duck/Vietnam/NCVD14-A503/2014	9/15/2014	duck	H5N6	VN54	Quang Ngai	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171673	KY171674	KY171675	KY171676	KY171677	KY171678	KY171679	KY171680
110	A/chicken/Vietnam/NCVD-15A59/2015	8/18/2015	chicken	H5N6	VN54	Lao Cai	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171722	KY171723	KY171724	KY171725	KY171726	KY171727	KY171728	KY171729
111	A/goose/Vietnam/NCVD-15A27/2015	4/22/2015	Goose	H5N6	VN55	Ha Nam	Out	H6N6*	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171691	partial	KY171692	KY171693	KY171694	KY171695	KY171696	KY171697
112	A/chicken/Vietnam/NCVD-15A55/2015	4/8/2015	chicken	H5N6	VN55	Hoa Binh	Out	H6N6	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171706	partial	KY171707	KY171708	KY171709	KY171710	KY171711	KY171712
113	A/chicken/Vietnam/NCVD-15A51/2015	7/17/2015	chicken	H5N6	VN55	Nghe An	Out	H6N6	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171698	partial	KY171699	KY171700	KY171701	KY171702	KY171703	KY171704
114	A/chicken/Vietnam/NCVD-15A22/2015	4/2/2015	chicken	H5N6	VN56	Nghe An	Out	H7N7*	2.3.2.1b	2.3.2.1a	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171252	partial	KY171253	KY171254	KY171255	KY171256	KY171257	KY171258
115	A/duck/Vietnam/NCVD-15A57/2015	8/15/2015	duck	H5N6	VN54	Ha Noi	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171713	KY171714	KY171715	KY171716	KY171717	KY171718	KY171719	KY171720
116	A/chicken/Vietnam/NCVD-15A17/2015	3/13/2015	chicken	H5N6	VN54	Thanh Hoa	Out	2.3.2.1b	2.3.2.1b	2.3.2.1b	2.3.4.4	2.3.2.1b	N6	2.3.2.1b	2.3.2.1b	KY171683	KY171684	KY171685	KY171686	KY171687	KY171688	KY171689	KY171690
117	A/chicken/Vietnam/NCVD-1096/2013	2/5/2013	chicken	H5N1	VN43	Ha Noi	LBM	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	KY171491	KY171492	KY171493	KY171494	KY171495	KY171496	KY171497	KY171498
118	A/chicken/Vietnam/NCVD-1088/2013	2/5/2013	chicken	H5N1	VN43	Ha Noi	LBM	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	KY171501	KY171502	KY171503	KY171504	KY171505	KY171506	KY171507	KY171508
119	A/chicken/Vietnam/NCVD-1104/2013	2/5/2013	chicken	H5N1	VN43	Ha Noi	LBM	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	KY171511	KY171512	KY171513	KY171514	KY171515	KY171516	KY171517	KY171518

Appendix 16: Amino acid differences identified in each virus compared to the nearest vaccine strains (in red) for clade 1.1.2.
Amino acid differences at putative antigenic sites are annotated on the right side of the table to indicate the antigenic site of the residue or other molecular marker.

Mature HA1	A/Vietnam/1203/2004	A/Vietnam/1194/2004	A/Cambodia/R0405050/2007	A/chicken/Vietnam/NCVD-2716/2013	A/duck/Vietnam/NCVD-2153/2012	A/duck/Vietnam/NCVD-2721/2013	A/duck/Vietnam/NCVD-2722/2013	A/duck/Vietnam/NCVD-A3799/2013	A/duck/Vietnam/R7-05-0158/2013	A/duck/Vietnam/NCVD-3804/2013	A/duck/Vietnam/NCVD-0193/2012	A/duck/Vietnam/NCVD-3801/2013	A/duck/Vietnam/NCVD-0139/2013	A/chicken/Vietnam/NCVD-2750/2013	A/duck/Vietnam/NCVD-14-A248/2014	A/duck/Vietnam/NCVD-14-A313/2014	A/quail/Vietnam/NCVD-3793/2013	A/quail/Vietnam/NCVD-3789/2013	A/quail/Vietnam/NCVD-3790/2013	A/duck/Vietnam/NCVD-3116/2013	A/tiger/Vietnam/NCVD-3797/2013	A/chicken/Vietnam/NCVD-3798/2013	A/quail/Vietnam/NCVD-3791/2013	A/chicken/Vietnam/NCVD-3800/2013	Annotation	
10	N				S																				A/duck/Vietnam/NCVD-2153/2012-lacks glycosylation site	
14	E			K	K																				antibody accessible	
35	K			R																						
36	K	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T		
43	D			A																					Antigenic site C	
47	V			I																						
48	K			R	R	R	R	R	R	R		R	R	R	R	R	R	R	R	R	R	R	R	R	R	Antigenic site E
94	D			V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	
117	I					F	F	F	F	F	F	F														
120	S				N																			I	antibody accessible	
123	S		P	P	P			P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	Antigenic site B, antibody accessible
133	S		A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	Antigenic site A; Receptor binding
138	Q													L	L	L	L	L	L	L	L	L	L	L	L	Antigenic site A, antibody accessible
140	K			N		Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Antigenic site A, antibody accessible; Egg adaptive site reported
141	S			P																						Antigenic site A; Egg adaptive site reported;
155	S		N												N	N	N	N	N	N	N	N	N	N	N	Antigenic site B, antibody accessible
175	L		M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	
189	K		N																							Antigenic site B; Role in sialic binding, antibody accessible
195	T			I											N	N										Antigenic site B Strict conservation flu A, Egg adaptive site reported
200	V																I									
210	V		T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	Antigenic site D
219	V			L																						
232	I										V															
266	K				R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
272	G					S																				
273	N												D													Antigenic site C, antibody accessible
292	H																	Q								
309	N			T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	antibody accessible
324	E										K															
325	R		G	G	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	
326	R											K														
No. Subst.		1	8	16	14	11	12	12	12	12	13	12	13	12	13	13	14	14	13	12	12	12	12	13	13	vs A/Vietnam/1203/2004
No. Subst.			7	15	13	10	11	11	11	11	12	11	12	11	12	13	13	12	11	11	11	11	11	12	12	vs A/Vietnam/1194/2004
No. Subst.				12	11	10	11	9	9	9	10	9	10	9	8	8	9	9	8	7	7	7	7	8	8	vs A/Cambodia/R0405050/2007

Appendix 17: Amino acid differences identified in each virus compared to the nearest vaccine strains (in red) for clade 2.3.2.1a. Amino acid differences at putative antigenic sites are annotated on the right side of the table to indicate the antigenic site of the residue or other molecular marker.

Mature HA1	A/duck/Guangdong/S1322/2010 (Re-6)	A/Hubei/1/2010	A/duck/Vietnam/NCVD-0033/2012	A/duck/Vietnam/R6-07-693/2013	A/chicken/Vietnam/NCVD-2749/2013	A/duck/Vietnam/NCVD-0033/2013	Annotation
66	M		L				
71	I		T				
72	D	N	N	N	N	N	
89	L					I	
115	R	Q	Q	Q	Q	Q	
120	D	N				N	
123	P	S	S	S	S	S	Antigenic site B
124	D			N			
136	S	P		P	P	P	Antigenic site A
140	N	K					Antigenic site A
151	L	I	I	V	V	I	
154	D				G		
156	A				E		
159	I	T	T	T	T	T	
163	S	G	G	G	G	G	
169	K	Q	R	Q	Q	Q	
174	V		I		I		
175	L			M			
189	R			K	K	K	Antigenic site B
198	V	I	I	I	I	I	
212	R	K	K	K	K	K	
219	V	I	I	I	I	I	
240	N	H	H	H	H	H	
266	R	K			K	K	
275	S	N	N	N	N	N	Antigenic site C
282	M	I	I	I	I	I	
323	I	R	R	R	R	R	
325	R	del	del	del	del	del	
No. Subst.		19	18	19	21	20	vs A/duck/Guangdong/S1322/2010 (Re-6)
No. Subst.			8	7	7	3	vs A/Hubei/1/2010

Appendix 18: Amino acid differences identified in each virus compared to the nearest vaccine strains (in red) for clade 2.3.2.1b. Amino acid differences at putative antigenic sites are annotated on the right side of the table to indicate the antigenic site of the residue or other molecular marker.

Mature HA1	A/duck/Guangdong/S1322/2010 (Re-6)	A/barn swallow/Hong Kong/1161/2010	A/chicken/Vietnam/NCVD-2693/2013	Annotation
83	A		T	
94	N		S	
124	D		G	
155	N		D	Antigenic site B
161	K		T	
188	T		I	Antigenic site B
192	Q		K	Antigenic site B
210	V		I	Antigenic site D
218	K		R	
223	S		R	Antigenic site D
No. Subst.			10	vs A/duck/Guangdong/S1322/2010 (Re-6)
No. Subst.				vs A/barn swallow/HongKong/1161/2010

Antigen-binding site		CD4		CD8		CD16		CD32		CD64		CD128		CD136		CD152		CD160		CD164		CD168		CD180		CD184		CD188		CD192		CD200		CD220		CD230		CD234		CD240		CD244		CD248		CD252		CD256		CD260		CD264		CD268		CD272		CD276		CD280		CD284		CD288		CD292		CD296		CD300		CD304		CD308		CD312		CD316		CD320		CD324		CD328		CD332		CD336		CD340		CD344		CD348		CD352		CD356		CD360		CD364		CD368		CD372		CD376		CD380		CD384		CD388		CD392		CD396		CD400		CD404		CD408		CD412		CD416		CD420		CD424		CD428		CD432		CD436		CD440		CD444		CD448		CD452		CD456		CD460		CD464		CD468		CD472		CD476		CD480		CD484		CD488		CD492		CD496		CD500		CD504		CD508		CD512		CD516		CD520		CD524		CD528		CD532		CD536		CD540		CD544		CD548		CD552		CD556		CD560		CD564		CD568		CD572		CD576		CD580		CD584		CD588		CD592		CD596		CD600		CD604		CD608		CD612		CD616		CD620		CD624		CD628		CD632		CD636		CD640		CD644		CD648		CD652		CD656		CD660		CD664		CD668		CD672		CD676		CD680		CD684		CD688		CD692		CD696		CD700		CD704		CD708		CD712		CD716		CD720		CD724		CD728		CD732		CD736		CD740		CD744		CD748		CD752		CD756		CD760		CD764		CD768		CD772		CD776		CD780		CD784		CD788		CD792		CD796		CD800		CD804		CD808		CD812		CD816		CD820		CD824		CD828		CD832		CD836		CD840		CD844		CD848		CD852		CD856		CD860		CD864		CD868		CD872		CD876		CD880		CD884		CD888		CD892		CD896		CD900		CD904		CD908		CD912		CD916		CD920		CD924		CD928		CD932		CD936		CD940		CD944		CD948		CD952		CD956		CD960		CD964		CD968		CD972		CD976		CD980		CD984		CD988		CD992		CD996		CD1000		CD1004		CD1008		CD1012		CD1016		CD1020		CD1024		CD1028		CD1032		CD1036		CD1040		CD1044		CD1048		CD1052		CD1056		CD1060		CD1064		CD1068		CD1072		CD1076		CD1080		CD1084		CD1088		CD1092		CD1096		CD1100		CD1104		CD1108		CD1112		CD1116		CD1120		CD1124		CD1128		CD1132		CD1136		CD1140		CD1144		CD1148		CD1152		CD1156		CD1160		CD1164		CD1168		CD1172		CD1176		CD1180		CD1184		CD1188		CD1192		CD1196		CD1200		CD1204		CD1208		CD1212		CD1216		CD1220		CD1224		CD1228		CD1232		CD1236		CD1240		CD1244		CD1248		CD1252		CD1256		CD1260		CD1264		CD1268		CD1272		CD1276		CD1280		CD1284		CD1288		CD1292		CD1296		CD1300		CD1304		CD1308		CD1312		CD1316		CD1320		CD1324		CD1328		CD1332		CD1336		CD1340		CD1344		CD1348		CD1352		CD1356		CD1360		CD1364		CD1368		CD1372		CD1376		CD1380		CD1384	
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Appendix 20: Amino acid differences identified in each virus compared to the nearest vaccine strains (in red) for clade 2.3.4.4. Amino acid differences at putative antigenic sites are annotated on the right side of the table to indicate the antigenic site of the residue or other molecular marker.

Mature HA1	A/Anhui/1/2005_ (Re-5)	A/duck/Guangdong/S1322/2010 (Re-6)	A/chicken/Vietnam/NCVD14-A324/2014_H5N6	A/duck/Vietnam/NCVD14-A503/2014_H5N6	A/pheasant/Vietnam/NCVD14-A367/2014_H5N6	A/duck/Vietnam/NCVD14-A392/2014_H5N6	A/duck/Vietnam/NCVD14-A415/2014_H5N6	A/duck/Vietnam/NCVD14-A418/2014_H5N6	A/chicken/Vietnam/NCVD-15A22/2015_H5N6	A/chicken/Vietnam/NCVD-15A17/2015_H5N6	A/duck/Vietnam/NCVD-15A57/2015_H5N6	A/chicken/Vietnam/NCVD-15A59/2015_H5N6	A/goose/Vietnam/NCVD-15A27/2015_H5N6	A/chicken/Vietnam/NCVD-15A55/2015_H5N6	A/chicken/Vietnam/NCVD-15A51/2015_H5N6	Annotation
45	D	N	N	N	N	N			N	N	N	N	N	N	N	
53	R	K	K	K	K	K	K	K	K	K	K	K	K	K	K	
72	N	D	R	R	R	R	R	R	R	R	R	R	R	R	R	
115	Q	R	L	L	L	L	L	L	L	L	L	L	L	L	L	
120	S	D														
123	S	P	T	T	T	T	T	T	T	T	T	P	P	P	P	Antigenic site B
124	D		N	N	N	N	N	N	N	N	N	N	N	N	N	
129	S	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
133	S	A	A	A	A	A	A	A	A	A	A	A	A	A	A	Antigenic site A
141	P	S								S						Antigenic site A
151	I	L										T	T	T	T	Antigenic site B
156	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	Antigenic site B
159	T	I														
169	Q	K										R	R	R	R	
181	S	P											P	P	P	Antigenic site B
183	D		N	N					N		N	N	N	N	N	Antigenic site B
184	A	E											E	E	E	Antigenic site B
192	Q		K	K	K	K	K	K	K	K	K	K	K	K	K	Antigenic site B
218	K		Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	
223	S		R	R	R	R	R	R	R	R	R	R	R	R	R	Antigenic site D

Appendix 21: Amino acid differences identified in each virus compared to the nearest vaccine strains (in red) for clade 7. Amino acid differences at putative antigenic sites are annotated on the right side of the table to indicate the antigenic site of the residue or other molecular marker.

Mature HA1	A/chicken/Shanxi/2/2006 (Re-4)	A/chicken/Vietnam/NCVD-1088/2013	A/chicken/Vietnam/NCVD-1096/2013	A/chicken/Vietnam/NCVD-1104/2013	Annotation
84	S	G	G	G	Antigenic site E
126	E	N	N	N	Antigenic site A
138	G	E	E	E	Antigenic site A
182	D	N	N	N	Antigenic site B
184	T	A	A	A	Antigenic site B
187	V	K	K	K	Antigenic site B
188	K	M	M	M	Antigenic site B
193	P	S	S	S	Antigenic site B
225	M	I	I	I	Antigenic site D

Appendix 22: HI fold difference using ferret antisera

REFERENCE ANTIGENS		Clade	GD/1	VN/ 1203	CB/ R0405050	IN5/2005	TK1/2005	EG/ 321	N03072/2010 RG29	W5/MG/244	HB/1/2010 RG30	BS/HK/1161/10	NCVD-672/ 2011	HK/ 6841/ 2010	NCVD-1544/ 2012	NCVD-1584/2012	AH/1/2005	NCVD-293/ 2009	NCVD-3/2008
1	A/goose/Guangdong/1/1996	0	1	1	2	32	8	8	8	1	4	4	2	8	1	2	2	4	32
2	A/Vietnam/1203/2004	1	8	1	2	64	32	32	64	4	4	16	32	64	4	16	8	16	128
3	A/Cambodia/R0405050/2007	1.1	16	8	1	128	64	64	128	8	16	32	32	16	8	16	64	16	128
4	A/Indonesia/5/2005	2.1.3.2	64	4	32	1	16	1	64	16	32	4	4	4	1	2	2	32	128
5	A/turkey/Turkey/1/2005	2.2.1	4	4	4	4	1	1	4	1	2	4	2	2	0.25	0.5	4	16	128
6	A/Egypt/321-Namru3/2007	2.2.1	4	4	64	8	4	1	8	1	8	16	8	4	2	2	4	16	128
7	A/Egypt/N03072/2010 (IDCDC-RG29)	2.2.1	1	2	1	8	2	2	1	1	1	0.5	2	1	0.25	0.5	4	4	128
8	A/Hubei/1/2010 (IDCDC-RG30)	2.3.2.1a	16	8	8	16	2	4	32	1	1	2	2	1	1	1	16	4	64
9	A/barn-Swallow/Hong Kong/D10-1161/2010	2.3.2.1b	64	64	32	32	4	8	64	2	2	1	1	2	2	8	32	8	128
10	A/duck/Vietnam/NCVD-672/2011	2.3.2.1c	64	32	8	32	8	4	128	4	1	4	1	8	2	2	8	32	64
11	A/Hong Kong/6841/2010	2.3.2.1c	64	32	32	16	2	8	32	2	1	2	2	1	1	2	16	32	128
12	A/duck/Vietnam/NCVD-1544/2012	2.3.2.1c	64	16	32	16	2	8	32	2	2	4	8	0.5	1	2	32	32	64
13	A/duck/Vietnam/NCVD-1584/2012	2.3.2.1c	64	32	32	16	2	8	64	2	2	4	4	1	1	1	16	32	128
14	A/Anhui/1/2005	2.3.4	8	4	2	4	16	8	8	2	64	32	16	8	8	16	1	1	64
15	A/duck/Vietnam/NCVD-293/2009	2.3.4.1	2	32	1	4	4	64	8	2	4	1	8	1	0.5	1	4	1	64
16	A/chicken/Vietnam/NCVD-3/2008 (IDCDC-RG25A)	7.1	16	32	16	256	32	64	64	16	64	4	8	32	4	8	128	16	1
17	A/Whooper Swan/Mongolia/244/2005 (Ag8)	2.2	4	8	4	16	2	8	4	1	1	4	4	2	0.25	4	4	32	64
TEST ANTIGENS																			
18	A/duck/Vietnam/NCVD-016/2007	1.1	8	8	2	64	32	64	128	8	4	16	32	8	4	16	64	16	128
19	A/chicken/Vietnam/NCVD-1192/2012	1.1	64	4	2	64	32	64	128	16	64	16	32	8	8	16	16	16	128
20	A/duck/Vietnam/NCVD-19/2003	1	8	16	2	16	64	4	16	4	2	8	32	4	2	4	2	8	64
21	A/Indonesia/5/2005 (CDC-RG2)	2.1.3.2	64	4	4	1	4	0.5	64	8	32	4	32	2	1	4	2	16	32
22	A/Egypt/321-Namru3/2007 (IDCDC-RG11)	2.2.1	4	2	4	16	2	1	8	0.5	4	4	4	4	1	1	4	16	128
23	A/duck/Vietnam/10/2005	2.3.2	4	4	4	16	8	8	32	2	2	4	2	4	0.25	2	1	8	32
24	A/Common magpie/Hongkong/5052/2007	2.3.2.1	32	16	32	4	4	8	64	2	1	2	4	1	0.5	1	8	32	128
25	A/chicken/Vietnam/NCVD-675/2011	2.3.2.1a	32	2	8	64	2	4	64	1	1	1	1	4	0.25	1	8	32	32
26	A/duck/Vietnam/NCVD-1207/2012	2.3.2.1a	0	32	32	16	8	8	64	4	5.33	2	8	2	0.5	0.5	64	32	128
27	A/duck/Vietnam/NCVD-1171/2012	2.3.2.1a	4	32	32	32	4	8	64	4	2	4	2	2	1	4	64	32	128
28	A/duck/Vietnam/NCVD-1163/2012	2.3.2.1b	32	16	32	8	4	4	256	4	1	0.5	2	2	0.5	0.5	16	32	128
29	A/duck/Vietnam/NCVD129-7/2011	2.3.2.1b	32	64	16	32	4	8	64	2	8	2	2	2	2	1	16	32	64
30	A/Barheaded goose/Mongolia/X53/2009	2.3.2.1 c	64	32	32	8	2	8	32	1	1	2	2	1	0.5	1	32	32	128
31	A/duck/Vietnam/NCVD-1648/2012	2.3.2.1C	64	16	16	16	1	8	32	1	2	4	2	1	0.5	1	16	32	64
32	A/duck/Vietnam/NCVD-471/2010	2.3.4	8	16	8	128	8	128	32	4	8	8	4	4	1	2	0.5	8	16
33	A/duck/Vietnam/NCVD-003/2008	2.3.4	16	4	4	16	16	32	32	4	128	16	4	8	4	8	2	4	64
34	A/duck/Vietnam/NCVD-391/2009	2.3.4	4	2	1	4	4	32	16	2	4	4	4	2	1	2	2	1	64
35	A/Japanese-white-eye/Hongkong/1038/06	2.3.4	2	2	2	4	8	32	16	2	8	16	2	2	4	2	4	1	64
36	A/chicken/India/NIV33487/2006 (IBCDC-RG7)	2.3.4	2	4	4	4	1	32	2	0.5	2	2	2	2	0.5	1	4	16	64
37	A/chicken/Vietnam/NCVD-35/2008	2.3.4.2	8	16	4	16	64	128	128	8	128	16	8	4	2	4	8	2	32
38	A/chicken/Vietnam/NCVD-279/2009	2.3.4.3	8	16	2	16	64	64	32	4	128	8	2	4	1	2	8	2	64
39	A/goose/Vietnam/113/2001	3	2	16	2	64	128	64	16	4	4	16	2	8	4	4	128	32	32

40	A/duck/Vietnam/NCVD-1/2002	5	2	1	4	8	4	2	4	1	2	8	2	4	1	2	1	4	32
41	A/chicken/Vietnam/NCVD-016/2008	7.1	32	32	16	256	64	64	128	16	128	8	8	32	8	8	128	64	16
42	A/chicken/Vietnam/NCVD-016/2008(IDCDC-RG12)	7.1	16	16	16	256	32	64	64	16	64	8	8	32	8	8	128	32	8

Appendix 23: HI fold difference using chicken antisera

REFERENCE ANTIGENS		Clade	VN/1203	VN/DK/016	CB/1005050	DK/VN/1192	IND/5/RG2	TK/1	EG/321	EG/321/RG11	NO3072/2010 RG29	CW/HK/5052	HB/1/RG30	CK/VN/675	DK/VN/1207	BS/HK/1161	DK/VN/672	DK/VN/1163	BHG/MG/553	HK/1841	CK/VN/1648	DK/VN/1544	DK/VN/003	DK/VN/391	JWE/HK/1038	CK/IND/33487/RG7	ANH/1	DK/VN/293	CK/VN/35	CK/VN/279	CK/VN/016	CK/VN/016/RG12	CK/VN/3/RG25A	
1	A/goose/Guangdong/1/1996	0	1	4	4	1	4	2	4	2	4	2	2	4	1	2	16	4	64	32	16	1	4	2	4	4	2	0.25	4	0.5	1	32	32	
2	A/Vietnam/1203/2004	1	1	0.5	2	0.5	4	16	16	4	4	8	2	4	8	8	128	16	64	128	64	4	8	8	64	16	2	0.25	32	4	16	64	128	
3	A/duck/Vietnam/NCVD-016/2007	1.1	2	1	1	0.5	8	8	16	4	4	4	2	2	2	4	64	16	64	64	32	4	8	4	16	16	8	0.13	8	2	8	64	128	
4	A/Cambodia/R0405050/2007	1.1	4	1	1	16	16	16	16	8	8	4	2	16	8	16	64	16	128	128	32	32	8	8	8	16	8	2	8	8	16	64	128	
5	A/chicken/Vietnam/NCVD-1192/2012	1.1	4	1	1	1	8	8	16	4	8	4	2	2	4	4	64	16	64	64	32	8	4	4	16	8	1	0.5	8	4	16	64	128	
6	A/Indonesia/5/2005	2.1.3.2	8	4	1	8	1	8	8	2	2	1	2	16	4	2	4	8	32	16	32	4	8	8	16	8	1	8	8	1	8	64	128	
7	A/Indonesia/5/2005 (IDCC-RG2)	2.1.3.2	8	4	2	16	1	8	8	2	2	1	2	32	8	2	4	16	32	8	32	4	4	2	16	8	4	8	4	0.5	16	64	128	
8	A/turkey/Turkey/1/2005	2.2.1	2	2	8	4	4	1	1	1	1	1	1	8	0.25	2	8	4	64	64	4	2	8	4	16	2	4	0.25	16	2	8	64	64	
9	A/Egypt/321-Namru3/2007	2.2.1	4	2	1	4	2	2	1	1	2	1	2	16	2	4	4	8	128	16	16	8	8	4	8	4	4	0.5	16	2	8	64	64	
10	A/Egypt/321-Namru3/2007 (IDCC-RG11)	2.2.1	2	2	1	1	2	1	0.5	1	2	1	2	16	4	4	8	8	128	64	8	2	4	1	16	1	2	0.13	8	2	8	32	128	
11	A/Egypt/NO3072/2010 (IDCC-RG29)	2.2.1	2	4	4	0.5	1	1	1	2	1	0.5	1	16	0.25	4	4	2	32	64	4	1	1	8	8	1	1	0.13	32	1	8	64	128	
12	A/Common magpie/Hongkong/5052/2007	2.3.2.1	16	4	8	16	2	32	2	1	2	1	1	2	2	1	8	8	64	16	8	4	8	8	16	16	8	0.25	8	4	16	64	128	
13	A/Hubei/1/2010 (IDCC-RG30)	2.3.2.1a	4	2	0.5	8	2	16	4	1	4	1	1	2	0.5	2	4	4	8	2	2	2	8	8	64	8	2	0.25	2	2	16	64	128	
14	A/chicken/Vietnam/NCVD-675/2011	2.3.2.1a	4	8	4	2	2	32	16	8	4	1	1	1	1	2	8	4	16	16	8	8	4	4	32	8	4	0.5	4	1	16	64	128	
15	A/duck/Vietnam/NCVD-1207/2012	2.3.2.1a	32	8	8	16	4	32	32	8	4	1	2	2	1	2	32	4	4	4	2	4	8	8	64	8	4	2	4	2	16	64	128	
16	A/barn Swallow/Hong Kong/D10-1161/2010	2.3.2.1b	64	8	8	16	2	16	4	2	8	1	1	4	1	1	2	2	16	16	4	4	8	8	64	16	16	2	16	16	16	64	128	
17	A/duck/Vietnam/NCVD-672/2011	2.3.2.1b	32	8	8	16	4	32	4	2	16	1	1	4	1	1	1	2	16	16	4	4	8	8	64	16	16	1	32	16	16	64	128	
18	A/duck/Vietnam/NCVD-1163/2012	2.3.2.1b	32	8	8	16	4	32	4	1	16	1	2	4	0.5	2	8	1	32	16	8	4	8	16	64	16	16	2	32	16	16	64	128	
19	A/Barheaded goose/Mongolia/K53/2009	2.3.2.1c	32	8	8	16	2	32	8	2	2	2	1	4	0.5	2	8	4	1	1	0.5	0.5	8	8	16	16	4	0.5	4	2	16	64	128	
20	A/Hongkong/5841/2010	2.3.2.1c	32	8	8	32	2	32	4	8	2	1	1	2	1	2	16	4	4	4	1	1	0.5	8	8	64	16	4	0.5	4	4	16	64	128
21	A/duck/Vietnam/NCVD-1648/2012	2.3.2.1c	32	8	8	16	1	32	4	8	2	1	1	2	1	2	16	4	4	4	1	1	2	8	8	64	16	4	1	8	2	16	64	128
22	A/duck/Vietnam/NCVD-1544/2012	2.3.2.1c	16	8	8	8	1	32	4	4	2	2	1	2	0.5	1	16	2	2	1	1	1	1	8	8	64	16	4	0.5	8	2	16	64	128
23	A/duck/Vietnam/NCVD-003/2008	2.3.4	4	8	8	8	8	8	8	4	8	4	8	8	8	8	32	16	128	64	64	16	1	2	16	4	4	2	4	1	16	64	128	
24	A/duck/Vietnam/NCVD-391/2009	2.3.4	8	2	4	4	4	4	8	2	8	4	8	2	4	4	32	8	64	32	16	8	4	1	8	4	2	0.5	2	0.5	16	64	128	
25	A/Japanese-white-eye/Hong Kong/1038/06	2.3.4	2	0.5	2	0.5	2	2	4	1	8	1	4	1	1	4	32	8	64	64	32	2	2	2	1	1	1	0.25	2	0.13	8	64	64	
26	A/chicken/India/NIV33487/2006 (IBCCD-RG7)	2.3.4	4	8	8	2	4	2	2	2	2	2	2	16	8	8	32	16	64	64	64	8	4	2	8	1	4	0.25	8	1	16	64	128	
27	A/Anhui/1/2005	2.3.4	2	1	1	4	1	4	4	8	1	2	4	2	4	16	128	16	128	128	64	4	2	2	1	4	1	0.13	8	0.13	16	64	128	
28	A/duck/Vietnam/NCVD-293/2009	2.3.4.1	32	8	8	8	8	16	16	8	16	4	8	32	8	16	128	32	256	128	64	2	4	4	4	4	4	1	8	2	16	64	128	
29	A/chicken/Vietnam/NCVD-35/2008	2.3.4.2	8	4	8	0.5	8	32	16	4	16	4	8	2	8	4	32	4	32	64	64	4	4	2	1	4	4	0.25	1	1	16	64	128	
30	A/chicken/Vietnam/NCVD-279/2009	2.3.4.3	16	8	8	4	16	32	16	4	2	2	4	4	8	8	64	16	64	64	32	4	8	2	4	4	4	0.25	4	1	16	64	128	
31	A/chicken/Vietnam/NCVD-016/2008	7.1	64	8	8	32	16	64	32	8	32	8	8	32	16	8	128	32	256	128	64	16	16	16	64	16	8	16	32	16	1	8	8	
32	A/chicken/Vietnam/NCVD-016/2008 (IDCCD-RG12)	7.1	64	8	4	16	16	16	16	2	16	4	8	16	8	4	64	16	256	128	32	32	8	8	32	8	8	4	4	4	1	1	8	
33	A/chicken/Vietnam/NCVD-3/2008 (IDCCD-RG25A)	7.1	64	8	8	8	16	16	8	4	16	2	8	16	4	2	32	16	64	64	32	16	4	8	32	8	16	8	8	4	1	4	1	
34	A/Goose/Vietnam/113/2001	3	8	4	2	8	2	1	4	4	8	4	8	32	8	8	128	32	128	64	32	32	4	8	64	2	8	4	2	1	16	32	128	
TEST ANTIGENS																																		
35	A/duck/Vietnam/NCVD-1/2002	5	1	4	2	1	4	1	4	1	1	1	1	2	0.5	4	1	16	8	32	64	16	4	2	2	8	2	2	0.13	16	0.25	4	32	64
36	A/Whooper Swan/Mongolia/244/2005	2.2	4	4	1	16	2	2	2	2	1	2	2	16	4	2	64	16	64	16	4	8	8	4	32	2	4	1	32	2	8	64	128	
37	A/duck/Vietnam/10/2005	2.3.2	2	0.5	1	0.5	0.5	1	4	1	2	2	1	0.5	4	0.5	2	2	32	32	16	4	2	1	2	1	2	0.5	4	0.5	4	16	32	
38	A/duck/Vietnam/NCVD-471/2010	2.3.4.1	8	0.5	4	4	4	4	16	4	2	4	8	0.5	4	4	32	8	64	32	16	4	4	0.5	4	8	2	0.25	1	0.25	4	16	64	
39	A/duck/Vietnam/NCVD-472/2010	2.3.4.2	8	0.5	4	2	4	4	16	4	16	16	8	8	4	4	32	16	256	32	16	8	8	2	4	8	2	1	1	0.5	16	64	128	
40	A/duck/Vietnam/NCVD-19/2003	1	2	4	4	1	8	2	16	4	4	4	8	1	8	8	64	16	64	64	32	4	8	2	32	8	8	0.5	32	2	4	64	64	
41	A/chicken/Vietnam/NCVD-426/2009	1.1	4	0.5	2	2	16	8	16	4	8	16	8	2	2	4	64	32	128	64	16	4	4	8	32	8	4	1						

47	A/chicken/Vietnam/NCVD-876/2011	1.1.1	2	0.25	1	2	8	8	16	4	8	8	4	2	1	8	64	16	128	64	32	4	8	8	16	8	4	2	8	8	16	64	128	
48	A/duck/Vietnam/NCVD-827/2011	1.1.1	2	0.25	1	2	16	16	16	4	8	4	8	4	1	4	64	16	128	64	32	8	8	8	32	8	4	2	16	8	16	64	128	
49	A/duck/Vietnam/NCVD-0193/2012	1.1.2	8	1	2	2	16	16	32	4	16	8	8	2	2	8	64	16	256	64	32	4	4	8	32	8	2	0.5	4	4	16	64	128	
50	A/duck/Vietnam/NCVD-2153/2012	1.1.2	4	1	2	1	32	8	32	4	16	8	8	2	1	8	64	16	128	64	32	4	8	8	16	4	8	2	8	8	16	64	128	
51	A/duck/Vietnam/NCVD-0139/2013	1.1.2	8	2	2	1	32	16	32	2	8	4	8	2	2	4	32	16	256	64	32	4	8	4	16	8	8	1	8	8	16	64	128	
52	A/duck/Vietnam/NCVD-2722/2013	1.1.2	16	4	4	2	16	32	32	4	16	8	8	4	2	8	64	16	256	64	32	8	4	8	32	8	4	2	16	4	16	64	128	
53	A/duck/Vietnam/NCVD-2721/2013	1.1.2	8	1	4	1	32	16	32	4	8	8	8	4	2	8	64	16	128	64	32	8	4	8	32	8	4	2	16	4	16	64	128	
54	A/chicken/Vietnam/NCVD-2750/2013	1.1.2	8	1	2	2	16	8	32	4	16	8	8	2	1	4	64	4	128	64	32	4	8	8	32	8	8	4	16	8	16	64	128	
55	A/chicken/Vietnam/NCVD-2716/2013	1.1.2	8	2	2	1	8	8	32	4	16	8	8	1	1	8	64	16	128	64	32	8	4	8	32	4	4	1	8	4	16	64	128	
56	A/chicken/Vietnam/NCVD-1096/2013	7.2	64	8	8	32	32	32	32	8	16	16	16	16	16	16	128	32	128	128	64	32	16	16	64	16	32	16	32	16	16	64	128	
57	A/chicken/Vietnam/NCVD-93/2008	7.2	64	8	8	32	16	16	32	8	16	16	16	32	16	16	128	32	256	128	64	64	16	16	32	8	16	8	16	16	16	64	128	
58	A/chicken/Vietnam/NCVD-1088/2013	7.2	64	8	8	32	32	32	32	8	16	16	16	8	32	16	16	64	32	128	128	64	64	16	16	64	16	32	16	32	16	16	64	128
59	A/chicken/Vietnam/NCVD-1104/2013	7.2	64	8	4	16	32	32	32	8	32	16	16	32	16	16	128	32	256	128	64	64	16	16	64	16	16	8	32	16	16	64	128	
60	A/chicken/Vietnam/NCVD-449/2010	2.3.2.1	16	1	2	8	4	32	16	2	16	8	4	4	1	1	4	2	16	16	8	8	1	4	32	8	8	2	2	1	16	64	128	
61	A/chicken/Vietnam/NCVD-460/2010	2.3.2.1	32	2	2	8	2	32	16	4	32	8	8	4	2	1	8	1	16	8	8	16	2	4	16	8	8	2	2	2	16	64	128	
62	A/duck/Vietnam/NCVD-1171/2012	2.3.2.1a	16	1	4	8	4	4	16	4	2	1	1	4	2	2	16	4	8	2	2	2	8	4	32	8	4	0.5	16	2	8	32	64	
63	A/duck/Vietnam/NCVD-1207/2012	2.3.2.1a	16	1	4	2	1	32	4	8	8	1	1	2	0.5	4	16	2	4	2	2	2	8	4	32	4	16	0.5	4	2	16	64	128	
64	A/chicken/Vietnam/NCVD-1223/2012	2.3.2.1a	16	1	2	4	2	32	8	2	4	2	1	2	1	16	8	2	16	4	4	4	8	4	32	8	8	1	8	1	16	64	128	
65	A/chicken/Vietnam/NCVD-1247/2012	2.3.2.1a	8	0.5	2	4	2	64	4	2	4	2	1	2	1	16	16	2	8	4	2	4	4	8	16	8	4	1	8	2	16	64	128	
66	A/chicken/Vietnam/NCVD-1255/2012	2.3.2.1a	8	1	4	8	2	32	4	4	8	1	1	1	1	16	32	4	8	8	4	4	8	8	32	8	4	0.5	8	4	16	64	128	
67	A/chicken/Vietnam/NCVD-1488/2012	2.3.2.1a	16	2	4	4	4	32	4	4	8	2	2	1	0.5	16	8	2	16	8	4	16	4	8	32	8	8	1	4	1	16	64	128	
68	A/duck/Vietnam/NCVD-1494/2012	2.3.2.1a	8	1	4	2	4	32	8	2	8	1	2	4	1	16	8	4	32	8	8	8	8	8	32	8	8	0.5	4	1	16	64	128	
69	A/chicken/Vietnam/NCVD-1963/2012	2.3.2.1a	16	1	4	4	4	32	8	2	4	4	1	2	1	16	16	4	16	8	4	4	8	8	32	8	4	0.5	8	2	16	64	128	
70	A/chicken/Vietnam/NCVD-2749/2013	2.3.2.1a	16	1	4	4	4	32	16	8	2	2	2	4	2	16	16	2	16	16	4	4	8	8	32	16	8	1	8	2	16	64	128	
71	A/duck/Vietnam/NCVD-1161/2011	2.3.2.1b	32	8	8	32	1	32	8	2	8	0.5	2	4	1	2	8	1	16	2	2	4	4	4	32	8	8	0.5	8	8	16	64	128	
72	A/duck/Vietnam/NCVD-129-7/2011	2.3.2.1b	32	8	4	8	2	8	4	1	2	2	4	4	2	0.5	2	2	128	8	2	4	8	8	64	8	8	1	32	8	4	32	64	
73	A/duck/Vietnam/NCVD-1283/2012	2.3.2.1b	64	8	8	32	8	32	8	2	16	4	4	4	1	2	2	1	32	32	16	8	16	16	64	16	32	8	16	16	16	64	128	
74	A/chicken/Vietnam/NCVD-1437/2012	2.3.2.1b	64	8	8	32	32	32	8	2	16	4	4	8	2	2	4	1	32	16	8	16	8	16	32	8	32	8	32	8	16	64	128	
75	A/duck/Vietnam/NCVD-1463/2012	2.3.2.1b	64	8	8	32	2	32	4	2	16	1	1	4	1	0.5	4	1	16	4	4	2	16	16	32	16	4	16	32	16	16	64	128	
76	A/duck/Vietnam/NCVD-1497/2012	2.3.2.1b	32	8	8	32	2	32	8	2	16	2	1	2	0.5	2	4	2	16	8	4	4	8	8	32	8	8	16	16	16	16	64	128	
77	A/duck/Vietnam/NCVD-1940/2012	2.3.2.1b	64	8	8	32	1	32	8	2	16	1	1	2	1	1	8	1	4	8	2	2	8	8	64	8	32	8	32	8	16	64	128	
78	A/chicken/Vietnam/NCVD-2693/2013	2.3.2.1b	16	8	8	32	4	32	8	4	16	1	1	4	2	2	8	2	16	4	4	4	16	16	64	16	32	16	16	16	16	64	128	
79	A/duck/Vietnam/NCVD-1547/2012	2.3.2.1c	16	4	8	16	1	32	8	4	2	1	0.5	2	4	2	4	4	2	1	1	1	4	4	16	4	4	2	8	4	16	64	128	
80	A/duck/Vietnam/NCVD-1584/2012	2.3.2.1c	32	8	4	16	2	8	8	4	2	0.5	0.5	4	2	1	16	4	2	1	1	1	8	4	32	8	8	1	8	4	8	64	128	
81	A/duck/Vietnam/NCVD-1869/2012	2.3.2.1c	64	4	8	32	4	32	4	4	8	0.5	2	2	0.5	2	16	4	8	2	2	4	4	4	16	4	8	1	4	4	16	64	128	
82	A/duck/Vietnam/NCVD-1901/2012	2.3.2.1c	64	4	8	32	1	32	4	2	4	0.5	1	2	1	1	8	4	4	2	2	2	4	4	16	4	8	1	8	4	16	64	128	
83	A/duck/Vietnam/NCVD-1905/2012	2.3.2.1c	64	4	8	32	1	32	4	2	2	1	1	4	1	1	8	2	4	1	1	2	4	4	16	4	8	2	8	4	16	64	128	
84	A/duck/Vietnam/NCVD-1928/2012	2.3.2.1c	64	4	8	32	2	64	2	4	4	1	0.5	4	2	4	8	2	4	2	1	1	4	8	16	8	8	2	8	4	16	64	128	
85	A/duck/Vietnam/NCVD-1933/2012	2.3.2.1c	64	4	4	32	1	64	8	2	4	1	1	2	2	1	8	2	4	2	0.5	2	4	4	16	4	8	1	8	8	16	64	128	
86	A/duck/Vietnam/NCVD-1936/2012	2.3.2.1c	64	2	4	32	1	64	8	4	4	1	1	2	1	2	8	4	4	2	2	2	2	8	8	4	8	1	8	4	16	64	128	
87	A/chicken/Vietnam/NCVD-1944/2012	2.3.2.1c	64	4	8	32	2	64	4	4	4	1	1	2	0.5	2	16	2	4	2	1	2	4	8	16	4	8	1	8	4	16	64	128	
88	A/duck/Vietnam/NCVD-1968/2012	2.3.2.1c	64	4	8	32	2	32	4	4	4	1	1	4	4	2	16	2	4	2	2	4	2	4	32	8	8	2	16	8	16	64	128	
89	A/duck/Vietnam/NCVD-2047/2012	2.3.2.1c	64	4	8	32	2	32	4	2	4	1	0.5	2	2	2	16	4	8	4	2	4	4	4	16	4	4	1	8	4	16	64	128	
90	A/duck/Vietnam/NCVD-01-145/2013	2.3.2.1c	64	4	8	16	1	64	4	2	8	1	0.5	4	1	1	8	2	2	2	1	2	4	4	16	4	8	2	4	4	16	64	128	
91	A/duck/Vietnam/NCVD-0170/2013	2.3.2.1c	64	8	8	16	4	32	2	2	8	1	1	1	1	2	16	4	2	2	1	1	4	4	16	4	8	1	8	4	16	64	128	
92	A/Swiftlet/Vietnam/NCVD-3000/2013	2.3.2.1c	64	8	8	32	2	32	4	2	4	2	2	1	1	2	16	4	8	2	2	4	8	4	8	4	8	1	8	4	16	64	128	
93	A/duck/Vietnam/NCVD-2709/2013	2.3.2.1c	32	8	8	32	4	64	4	1	4	1	1	2	1	2	16	4	16	4	4	4	4	2	8	2	4	1	8	4	16	64	128	
94	A/duck/Vietnam/NCVD-2726/2013	2.3.2.1c	32	8	8	32	2	64	8	1	4	1	0.5	2	2	2	16	4	4	4	2	4	2	16	4	4	1	8	4	16	64	128		
95	A/duck/Vietnam/NCVD-2727/2013	2.3.2.1c	32	4	8	32	2	64	4	2	8	0.5	2	2	2	2	16	4	8	4	2	2	4	4	16	4	4	1	16	4	16	64	128	
96	A/duck/Vietnam/NCVD-2745/2013	2.3.2.1c	32	4	8	16	2	64	4	1	4	0.5	1	4	2	2	8	2	4	4	2	4	4	2	16	2	4	2	16	4	16	64	128	
97	A/duck/Vietnam/NCVD-0177/20																																	

