The clinical and virological aspects of human influenza virus A (H5N1) infection in South Vietnam 2004-2005

Thesis

THE CLINICAL AND VIROLOGICAL ASPECTS OF HUMAN INFLUENZA VIRUS A (H5N1) INFECTION IN SOUTH VIETNAM 2004-2005

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DECLARATION

I am a member of the Virology Laboratory of the Oxford University Clinical Research Unit based in the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam, and in this capacity have been involved in the detection of avian influenza A (H5N1) in humans since 2004. Clinical data of a subset of patients (2004 outbreak) described in this thesis were published in 2005 (NEJM 350 (12):1179-88) and (NEJM 352 (7):686-91, but these data have been pooled with data from subsequent patients (2005 outbreak) and reanalyzed for my thesis (Chapter 3). Results of studies of oseltamivir resistance in H5N1 patients were published at the end of 2005 (NEJM 353 (25):2667-72). Chapter 4 describes further work carried out to investigate the kinetics of mutant virus replication in-vivo on this sub-set of samples. These studies have not yet been published.

I declare that other than the help outlined in the acknowledgments, the work described in this thesis is my own work and has not been submitted for any degree or qualification to this or other universities.

ABSTRACT

Since the re-emergence of avian influenza A H5N1 virus in 2003 among poultry, the virus has reached endemic levels in Vietnam and continues to cause human infections [4]. At present (9 February 2011), 119 infections have been reported, 59 of which were fatal [5]. The cases in 2004 and 2005 accounted for 76% (90/119) of the total number of reported cases from Vietnam [5]. This thesis retrospectively studied the clinical course of 16 cases (14%). The clinical features in most cases were typical for the human disease caused by the H5N1 virus. The prominent clinical signs and symptoms were those of a severe influenza syndrome with fever, cough, and shortness of breath. However, this thesis also shows that human H5N1 influenza may be associated with extrapulmonary disease, including gastrointestinal and CNS symptoms and multi-organ failure. The abnormalities on chest radiographs included extensive bilateral infiltration, lobar collapse, focal consolidation, and air bronchograms. The prominent laboratory factors were thrombocytopenia, neutropenia, lymphopenia, and increased levels of serum transaminases.

Most patients (15/16) were treated with standard doses of the neuraminidase inhibitor oseltamivir. This drug has antiviral efficacy against H5N1 viruses and observational studies suggest that treatment with oseltamivir reduces mortality. Drug resistance may emerge during treatment and seems associated with antiviral and clinical failure. When testing for genotypic and phenotypic resistance, we detected 2 oseltamivir resistant H5N1 variants in two patients who failed to clear the virus and died from the infection. In contrast, four other patients who showed effective suppression of viral replication during treatment all survived.

Molecular analysis of 13 H5N1 virus strains isolated from 13/16 patients showed that all viruses belonged to clade 1, genotype Z H5N1 viruses. The viruses retained the molecular characteristics of avian viruses. Some virus strains had mutations in the HA1 gene that

potentially affect receptor specificity, i.e. reduce recognition of 'avian-type' receptors, increase recognition of 'human-type' receptors, or both. A Glu-627-Lys substitution in the viral polymerase PB2, associated with adaptation and virulence of H5N1 viruses in mammals, was observed in 5 of 8 isolates from fatal cases and in 3 of 4 isolates from patients who survived. However, no association between the presence of Lys-627 and clinical outcome was observed. However, in all except one of the isolates without the Glu-627-Lys substitution, an alternative change at position 701 (Asp to Asn) was observed which has also been associated with adaptation of avian viruses to replication in mammals. Data from this study also provided evidence for the selection of mutations in HA gene during infection, supporting the notion that continuing transmission of H5N1 virus from poultry to humans may provide a greater opportunity for the virus to adapt to humans.

In addition, to support the diagnostic programme, I developed a molecular diagnostic assay that can detect both clade 1 (South-Vietnam, Cambodia, Thailand) and clade 2 (North-Vietnam, Indonesia, Egypt) H5N1 virus variants. This molecular assay may be useful for diagnosis of H5 virus infections in regions where different genetic clades co-circulate.

LIST OF ABBREVIATIONS

Ala	Alanine
ALT	Alanine aminotransferase
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
AST	Aspartate transaminase
BHQ1	Black hole quencher 1
Bp	Base pair
°C	Celsius degree
CD	Cluster of differentiation
cDNA	Complement DNA
Ck	Chicken
CNS	Central nerve system
CSF	Cerebrospinal fluid
Cys	Cysteine
CV	Coefficient of variation
Dk	Duck
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribosenucleic acid
DTPH	Dong Thap Provincial Hospital
dTTP	Deoxytreonine triphosphate
F2	Frame 2
FAM	5-carboxyfluoresceseine
Gal	Galactose

GCS	Lactose coma score
GD	Guandong
Glu	Glutamic
Gly	Glycine
Gs	Goose
GX	Guangxi
H1-H16	Haemagglutin subtypes 1-16
HA	Haemagglutin
His	Histidine
HK	Hong Kong
HN	Hunan
HPAI	Highly pathogenic avian influenza virus
HTD	Hospital for Tropical Diseases
HTML	Hyper text markup language
IFN	Interferon
IL	Interleukine
Ile	Isoleucine
IND	Indonesia
IV	Intravenous
IP-10	Interferon inducible protein 10
LPAI	Low pathogenic avian influenza virus
Leu	Leucine
Lys	Lysine
MIG	Macrophage attractant chemokines
µl	Microlitre

N1-N9	Neuraminidase subtypes 1-9
NA	Neuraminidase
NA	Not available
ND	Not determined
ng	Nanogram
LNA	Locked nucleic acid
M	Matrix
MDCK	Madin darby canine kidney
MDk	Mallard duck
Met	Methionine
NP	Nucleoprotein
NS	Non-structural protein
OUCRU	Oxford University Clinical Research Unit
PA	Acidic polymerase
PAUP*	Phylogenetic analysis using parsimony * and others
PB1	Basic polymerase 1
PB2	Basic polymerase 2
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Ph	Pheasant
Phe	Phenylalanine
PH1	Pediatric Hospital Number One
PH2	Pediatric Hospital Number Two
PPi	Pyrophosphate
PSQ	Pyrosequencing

Qa	Quail
RNA	Ribonucleic acid
Rpm	Round per minute
RT	Reverse transcription
rRT-PCR	Real-time reverse transcription polymerase chain reaction
SA	Sialic acid
Ser	Serine
SNP	Single nucleotide polymorphisms
SQA	Short sequence analysis
ST	Santose
T	Treonine
Ta	Annealing temperature
TAMRA	N'tetramethyl-6-carboxyrhodamine
TE	Tris-EDTA
THA	Thailand
Thr	Threonine
Tyr	Tyrosine
Val	Valine
VNM	Vietnam
VTM	Viral transport medium
WHO	World Health Organization
X-gal	5-bromp-4-chloro-3-indolyl-b-D galactopyranoside
YN	Yunnan

Chapter 1

INTRODUCTION

1.1 Influenza

Influenza in humans is a highly contagious respiratory disease caused by influenza viruses. It is transmitted from person-to-person via virus laden respiratory droplets secreted by infected person during symptoms of coughing and sneezing [6]. Transmission occurs through the respiratory route (up to 1-2 metres) or direct surface or skin-to-mucosa contact with respiratory secretions [7]. Influenza viruses cause a broad range of illness, from asymptomatic infection to various, mostly mild respiratory illnesses and, infrequently, fulminant viral pneumonia and/or secondary bacterial pneumonia [6]. It is estimated that annually influenza viruses cause 1 billion infections, 3-5 million cases of severe illness leading to 3-5 hundred thousands deaths worldwide [8]. Birds and mammals can also be infected by influenza viruses, and occasionally transmission to humans of these animal viruses occur, causing health problems [6, 9].

1.2 Influenza A viruses

1.2.1 Biological properties

Influenza viruses are pleomorphic, enveloped, negative single stranded RNA viruses belonging to the family of Orthomyxoviridae. Three types (A, B, and C) can be distinguished based on structural, genetic and antigenic differences. Of these three types, influenza A and B viruses cause most severe human infections. Both influenza A and B viruses cause annual epidemics but influenza A virus is the only type that poses a (continuous) pandemic threat through the circulation of various antigenic subtypes in multiple hosts, particularly in their natural reservoir of wild waterbirds, from which new subtypes can enter the human population.

Based on the antigenic properties of their two surface glycoproteins - haemagglutinin (HA) and neuraminidase (NA)- influenza A viruses can be subtyped into 16 different HA (H1-H16) and 9 different NA (N1-N9) subtypes. All influenza A subtypes have been found to circulate in the natural reservoir of wild aquatic birds [10, 11]. However, in humans, only a limited number of subtypes (H1N1, H2N2 and H3N2) have established themselves as stable lineages. These three subtypes of influenza viruses were responsible for three pandemics recorded in the past century: the 'Spanish flu' pandemic (H1N1) in 1918, 'Asian influenza' pandemic (H2N2) in 1957, and 'Hong Kong influenza' pandemic (H3N2) in 1968. The most devastating pandemic ever was the 'Spanish flu' which killed estimatedly 50-100 million people and was most likely caused by direct transmission and adaptation to humans of a purely avian influenza virus. The 'Asian influenza' and 'Hong Kong influenza' pandemics each killed estimatedly 1 million lives and were caused by reassortant viruses from mixed human and avian origin [7, 12]. Currently, we are witnessing the new Influenza virus A/H1N1/2009 pandemic that has emerged and spread globally from Mexico and the United States of America from April 2009 onward, thus far causing mostly mild seasonal flu-like disease with occasional severe illness particularly in those with underlying medical conditions and pregnant women [http://wwwwhoint/csr/don/2009_11_27a/en/indexhtml, accessed on March 12, 2009], [13]. This virus is the result of complex reassortment events between viruses of recent porcine origin and more distant porcine, human and avian origin [13].

Influenza A viruses have eight gene segments, contained within the host-derived viral envelope. The eight gene segments vary in size and each encodes one or two proteins which function differently in the life cycle of the virus (table 1.1). During the virus life cycle (figure 1.1), HA binds to receptors identified as glycans terminated by an 2,3-linked sialic acid (SA) or an 2,6-linked SA present on the epithelium of host cells [11]. Receptor-bound viruses are

then internalized through receptor- mediated endocytosis, and viruses possessing cleaved HA (by host cell proteases into HA1 and HA2) undergo fusion with the endosomal membrane in acidic pH (pH~5.0), mediated by the M2 ion channel, resulting in the release of viral ribonucleoprotein (vRNP) into the cell cytoplasm [14]. vRNPs is then translocated into the nucleus where it undergoes transcription (mRNA synthesis) and replication. The replication process includes synthesis of complete positive-sense complementary RNA (cRNA), minus-strand viral RNA (vRNA), and vRNP complex. Progeny vRNPs made inside the nucleus are then exported into the cytoplasm with the involvement of M1 and NS2. Finally, the envelope proteins (HA, NA, and M2), matrix protein (M1), and vRNP are transported to the cell membrane where progeny virus particles bud [14]. The newly budded viruses are still connected to the host cell SA receptors, and only be liberated when the terminal SA residue of the receptor is cleaved by the NA [11].

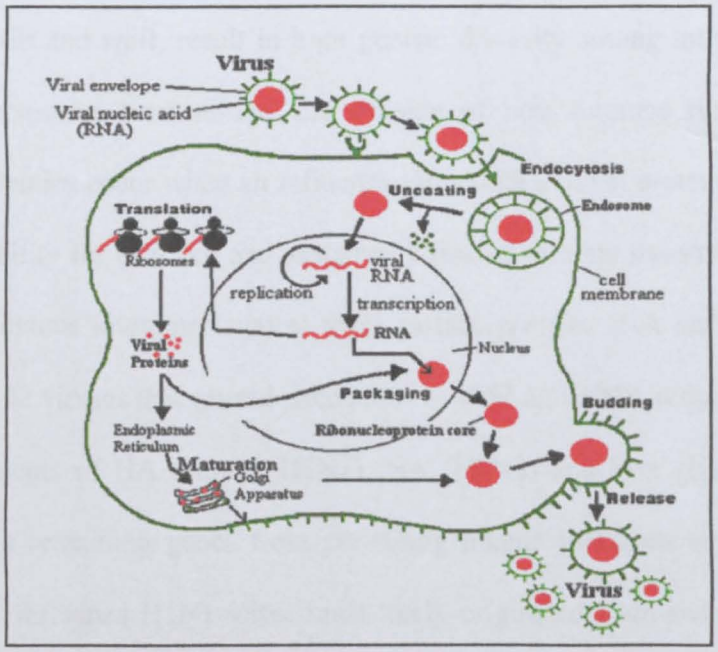

Figure1.1 Influenza A virus life cycle. Figure reproduced from http://nursingcrib.com/wp-content/uploads/image/flu%20virus.PNG, accessed on July 27, 2010.

HA and NA protrude from the viral envelope and are the major targets of host neutralizing antibodies. During replication, because of the lack of proof-reading replication mechanism of the viral RNA polymerase, mutations in these two genes occur readily and under pressure of the immune response, virus variants possessing host immune escape mutations are selected for, leading to antigenic drift over time. This continuous adaptation results in yearly (seasonal) epidemics, requiring active surveillance for timely selection of representative vaccine strains [9].

The segmented genome facilitates easy exchange of genes between two or more viruses during co-infection of a single host cell. A sudden drastic change of antigenic properties may occur when such genetic exchange involves the HA and/or NA genes. This process is called antigenic *shift*, as opposed to the more gradual changes of antigenic *drift*. These two phenomena, drift and shift, result in high genetic diversity among influenza A viruses, that allow for interspecies transmission and evasion of host immune responses. At irregular intervals, pandemics occur when an influenza virus with a novel protein HA or NA (or both) acquires the ability for efficient and sustained human-to-human transmission in a population that has no previous immunity against these surface proteins (HA and NA) [6, 9, 12]. The H2N2 and H3N2 viruses that caused pandemics in 1957 and 1968, respectively, arose through gene reassortments of HA (H3N2, H2N2), NA (H3N2) and PB1 (H2N2, H3N2) of avian origin with the remaining genes from prevailing human influenza strains [9]. The current pandemic new influenza H1N1 virus, most likely originated from swine and has a complex reassortment history involving recent gene exchange between porcine viruses and more distant reassortment between porcine, human and avian viruses [13]. In addition to reassortment, a novel pandemic virus can also arise through direct transmission and adaptation of purely avian

influenza viruses in humans. The H1N1 virus that caused the devastating pandemic in 1918 is believed to have arisen from such adaptation [15].

Table 1.1 Genes and protein functions of influenza A virus (adapted from Wright et al., [11])

Gene ID	Segment	Length (bp)	Protein name	Protein function
1	Polymerase 2 (PB2)	2341	Polymerase 2 (PB2)	Internal proteins, RNA transcription and virus replication
2	Polymerase 1 (PB1)	2341	Polymerase 1 (PB1) PB1-F2	Internal proteins, RNA transcription and virus replication Mitochondrial targeting and apoptosis
3	Polymerase A (PA)	2233	Polymerase A (PA)	Internal proteins, RNA transcription and virus replication
4	Hemagglutin (HA)	1778	Hemagglutin (HA)	Surface glycoproteins, viral attachment, antigenic determinant, subtype specific (H1-16) determinant
5	Nucleoprotein (NP)	1565	Nucleoprotein (NP)	Interacts with viral RNA to form ribonucleoprotein (RNP), nuclear targeting, RNA transcription, type specific (A, B, and C) determinant
6	Neuraminidase (NA)	1413	Neuraminidase (NA)	Surface glycoproteins, viral release from host cells, antigenic determinant, subtype specific (N1-N9) determinant, target of neuraminidase inhibitors
7	Matrix (M)	1027	Matrix 1 Matrix 2	Membrane protein stability, type specific (A,B, and C) Membrane proteins, viral fusion, type specific (A, B, and C) determinant, target of M2 inhibitors
8	Non-structural (NS)	890	Non-structural 1 (NS1) Non-structural 2 (NS2)	Internal proteins, IFN-γ antagonism Regulation of mRNA transcription, localization of viral nucleic proteins

1.2.2 Natural reservoirs and interspecies transmissions

The nature reservoir of influenza A viruses are wild aquatic birds; mainly ducks, shorebirds, and gulls [16]. In these hosts, influenza A viruses have acquired optimal adaptation and therefore only cause asymptomatic infection or mild disease. The virus preferentially replicates in the gastrointestinal tract resulting in high-titer shedding of virus in the feces.

Therefore, the major route of transmission among aquatic birds is through ingestion of faecally contaminated water [16, 17] .

From their natural reservoirs of wild aquatic birds, influenza A viruses can be transmitted to other wild and domestic birds, including turkeys, chickens, and quails. In these new hosts, avian influenza viruses can cause a broad range of syndromes, from asymptomatic to mild upper respiratory infections to egg loss and systemic fatal disease. Among domestic birds, turkeys and chickens are more frequently involved in influenza outbreaks [17].

Occasionally, avian influenza viruses are transmitted to mammalian species, including humans, pigs, horses, whales, seals, cats and dogs [17]. Some viruses have adapted and established species-specific lineages in these hosts, e.g. H1N1, H2N2 and H3N2 in humans, H1N1 and H3N2 in pigs, H3N8 in horses.

1.2.3 Highly pathogenic avian influenza viruses

Based on the severity of disease in turkeys and chickens, avian influenza viruses are classified as low pathogenic (LPAI) or highly pathogenic avian influenza viruses (HPAI) [17]. The high pathogenicity of HPAI viruses is determined mostly by the HA glycoprotein [17]. In the viral life cycle, HA attaches to cell receptors and allows entry of the viral genome into the host cell by enabling fusion of the host endosomal membrane with the viral envelope [17]. The latter function requires post-translational cleavage of the precursor HA0 protein into HA1 and HA2 by host proteases to expose a fusogenic domain in the N-terminal of HA2 [16]. HAs of LPAI viruses are cleaved only by trypsin-like proteases, which are present in a limited range of host cells, such as respiratory and gastrointestinal epithelia. Consequently, infections are limited to these organs. In contrast, HAs of HPAI viruses are sensitive to a broad range of proteases,

such as subtilisin-like proteases, which have an ubiquitous distribution thereby permitting viral replication in organs beyond the respiratory and gastrointestinal tracts [17, 18] and resulting in systemic infections [17, 19].

This promiscuity of HPAI HAs for cellular proteases is determined by the structure of the HA cleavage site. Sequence analyses of the HA show that HPAI viruses have a stretch of multiple basic amino acid residues, usually Lys-Lys-Lys-Arg (K-K-K-R) immediately upstream from the cleavage site, whereas LPAI viruses usually only have a single arginine residue (R) at this site [20-22]. HPAI viruses can evolve from LPAI viruses by acquiring these multiple basic amino acids at the cleavage site through insertion of a short repeat sequence during replication [12, 17]. Of the 16 known HA subtypes, only H5 and H7 so far seem to have the potential to become HPAI viruses [9].

1.3 Bird-to-human transmission of avian influenza viruses

1.3.1 The species barrier

In general, avian influenza viruses rarely cause human infections. Factors that limit direct transmission and replication of avian influenza viruses in humans are not fully understood but are believed to be determined by several viral gene products, including HA, NA and the polymerase complex [17].

The HA of human influenza viruses binds to glycan receptors terminated by a sialic acid (SA) linked to a galactose residue by α2,6 (SA α-2,6 Gal) linkages that are predominantly present on human respiratory cells while HA of avian influenza viruses preferably binds glycan receptors terminated by a sialic acid linked to a galactose residue by α2,3 (SA α2,3 Gal)

linkages that are present on avian epithelial cells [23-25]. This receptor specificity is believed to be a major factor that limits avian influenza viruses to readily infect humans [17].

It is known that pigs have both human-type (SA α-2,6 Gal) and avian-type (SA α-2,3 Gal) receptors [26], and therefore pigs are thought to play a role as intermediate host for genetic exchange between avian and human viruses, thereby potentially generating new human pandemic strains [27]. Indeed, both avian and human viruses have been found to co-circulate in pigs [26], and pig-originating avian-human reassortant viruses have also been isolated from children with influenza illness [27]. Moreover, the current H1N1 pandemic virus has the genetic signature of a swine influenza virus, with segments of both human and avian origin [13].

Beside receptor specificity of HA, specificity of NA also plays a role. NA of human influenza viruses preferably catalyses cleavage of the human SA α2,6 Gal linkage rather than of the avian SA α2,3 Gal linkage, and vise versa [9]. Considering the opposing functions of HA and NA, optimal viral replication requires a balance between the activity of NA in removing sialic acids and the activity of HA in virus attachment and release [28]. The NA of H5N1 viruses that currently cause poultry outbreaks and human infections has a 20 amino acid deletion in the stalk region which is believed to reduce enzymatic activity of NA thereby restoring the balance between HA and NA when crossing the species barrier from aquatic birds to terrestrial poultry [29].

The polymerase complex, in particular PB2, also seems to play an important role in determining host range and pathogenicity in mammalian hosts. Avian influenza viruses typically have Glu (E) at position 627 in the PB2 protein, while human viruses have Lys (K) at this position [9]. This mutation (E627K) allows avian viruses to replicate more efficiently in the upper respiratory tract of mice where the temperature is lower than in the respiratory tract

[30, 31] and this may play a role in transmission and replication of avian influenza viruses in mammals and humans [32, 33, 34]. The other mutation in PB2 gene that may also play a role in efficient replication and transmission of avian influenza viruses in mammals is the substitution of Asp (D) for Asn (N) at position 701 (D701N) [35]. Studies in animals have shown that avian influenza A H5N1 viruses with the D701N can transmit as efficient as viruses with the E627K [34], confirming the compensation role of D701N for the lack of E627K observed in patients [36].

1.3.2 Transmission and disease caused by avian non-H5N1 viruses

Before the last decade of the previous century, direct transmission of avian influenza viruses to humans was thought to occur only under experimental conditions [37]. However, more recently, direct transmission of avian influenza viruses to humans during outbreaks in birds has been reported with increasing frequency. Besides H5N1 influenza virus, several incidents of direct transmission of avian influenza viruses of H7 and H9 subtypes to humans have been reported. Since 1996, more than 100 cases of human infections with these two virus subtypes have been documented (table 1.2) [http://www.cdc.gov/flu/avian/gen-info/avian-flu-humans.htm, accessed on October 1, 2010] [38]. Most reported patients with H7 or H9 influenza infection had symptoms of mild influenza or conjunctivitis alone or mild influenza with conjunctivitis [39-41]. So far, only one patient died of H7N7 infection, reported in the Netherlands in 2003. This patient had severe pneumonia, renal failure and respiratory manifestations of acute respiratory distress syndrome [41].

Table 1.2 Confirmed instances of avian non-H5N1 influenza virus infections of humans since 1996.

Year	Avian influenza subtype	Country/area	Source of infection	No of cases	Symptoms	Outcome	Ref.
1996	H7N7	England	Ducks	1	Conjunctivitis	Recovered	[39]
1999	H9N2	Hong Kong, Special Administrative Region	Unknown	2	Uncomplicated influenza-like illness	Both recovered	[38]*
2002	H7N2	Virginia, USA	Poultry	1	Uncomplicated influenza-like illness	Recovered	[38]*
2003	H7N7	The Netherlands	Poultry	89	78 cases with conjunctivitis, 5 with conjunctivitis and influenza-like illness, 2 with influenza-like illness, and 4 with other symptoms	1 died and 88 recovered	[41]
2003	H9N2	Hong Kong, Special Administrative Region	Unknown	1	Influenza-like illness	Recovered	[42]
2003	H7N2	New York, USA	Unknown	1	Influenza-like illness	Recovered	[38]*
2004	H7N3	Canada	Poultry	2	Conjunctivitis and coryza	Recovered	[43]
2007	H7N2	United Kingdom	Poultry	4	Conjunctivitis and mild influenza-like illness	All recovered	[44]
2007	H9N2	Hong Kong, Special Administrative Region	Unknown	1	Influenza-like illnes	Recovered	[38]*

Note: * indicates data were obtained from http://www.cdc.gov/flu/avian/gen-info/avian-flu-humans.htm (accessed on October 1, 2010) [38].

1.4 Avian H5N1 influenza

1.4.1 Emergence and spread of the virus

Avian influenza H5N1 viruses are classified as HPAI viruses [9]. Current circulating H5N1 virus lineages that cause worldwide epidemics among domestic birds are descendants of A/Goose/Guangdong/1/96 (H5N1: A/GS/GD/1/96-like virus) which was detected in sick geese in Guangdong province, People's Republic of China in 1996 [45, 46, 47]. In 1997, H5N1 viruses caused poultry outbreaks and associated human infections in Hong Kong. The virus responsible for the Hong Kong outbreak (HK97) possessed an HA gene that was derived from A/GS/GD/1/96-like viruses and the remaining seven viral genes were derived from other viruses, possibly H9N2 (A/Quail/HongKong/G1/97(H9N2)) and H6N1 (A/Teal/HongKong/W312/97(H6N1)) viruses prevalent in quails and teals [1, 48].

After the Hong Kong outbreak, as a result of strict quarantine and infection control measures, HK97 H5N1 virus has been eliminated [49, 50]. However, descendants from its progenitor, A/GS/GD/1/96-like viruses, continued to circulate in geese in Southern China – the region that has been considered as the epicenter of influenza viruses. In 1999/2000, A/GS/GD/1/96-like viruses were isolated from healthy geese and ducks imported to Hong Kong [51]. During this period of time (1999/2000), A/GS/GD/1/96-like viruses underwent multiple reassortment events in domestic ducks leading to a rapid increase in genetic diversity [52, 53]. Subsequently, a series of reassortants or genotypes (a genotype is a designation that reflects the constellation of eight gene segments of the virus which is given in capital letters) bearing the HA gene of A/GS/GD/1/96-like viruses (figure 1.2) were detected in chicken, ducks, and other terrestrial poultry in following years (2001/2002) [9, 45, 46, 51]. The detection of multiple genotypes in various domestic bird species suggests that the selection for virus

variants that are well adapted to multiple hosts and easy to transmit from species to species have occurred in these years.

In 2003, genotype Z and V emerged as the predominant genotypes and spread to numerous countries in East and South East Asia [45]. Notably, these genotypes have acquired a 20 amino acid deletion in the NA gene that allows them to transmit efficiently among terrestrial poultry [9]. While genotype V viruses spread to Japan and South Korea [45], genotype Z viruses spread to Vietnam, Laos, Cambodia, Thailand, Malaysia, and Indonesia causing extensive poultry outbreaks associated with human infections [45]. The introduction route of viruses into these countries was likely through the movement of poultry across borders [46, 54]. However, since these countries are in parts of the East Asia - Australian flyway of migratory birds [55], it is also possible (but has yet proven) that H5N1 viruses being brought into these countries by bird migration [56].

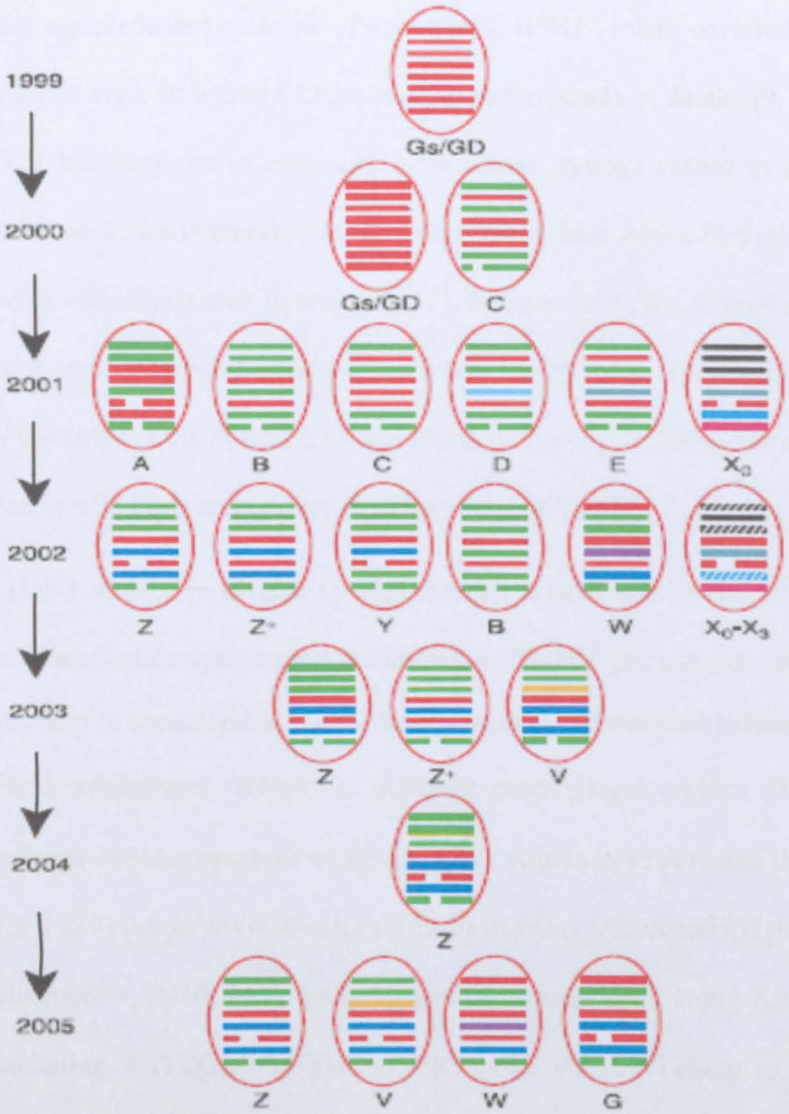

Figure 1.2 Genesis of avian influenza A H5N1 viruses in Asia from 1999-2005. The eight gene segments (represented by horizontal bars) starting from top downwards are PB2, PB1, PA, HA, NP, NA, M, and NS. Each color represents a distinct virus lineage. Red represents the A/Goose/Guandong/1/96-like lineage. Distinct genotypes (gene constellations) are denoted by a capital letter. Figure reproduced from Peiris et al., [9].

In May 2005, an unprecedented outbreak of genotype Z H5N1 viruses occurred in migratory waterfowl in Qinghai Lake in western China leading to thousands of deaths [9, 57]. Notably, Qinghai Lake is a breeding site of migratory birds whose flyways extend to India, Siberia, Middle East, and East Africa (Central Asia and West Asia – East Africa flyways) and overlap with the Black Sea - Mediterranean flyway [55, 57]. Subsequently, the viruses rapidly spread westward causing poultry outbreaks and human infections in India, Turkey, Egypt, Nigeria, and many other countries in the Middle East and North Africa [5]. In 2006, this virus genotype was also reported in wild birds and poultry in European countries [9].

The spread of H5N1 viruses in diverse species through a large part of the world combined with high rates of nucleotide substitution (estimated 4.77×10^{-3} per site per year) in the HA gene [53] has resulted in considerable genetic/antigenic drift and the establishment of multiple genetically related sublineages (clades) in different geographical regions [46]. Currently, phylogenetic analyses of HA sequences of HPAI H5N1 viruses have revealed the presence of 10 clades (clades 0 to 9) [http://www.who.int/csr/disease/avian_influenza/H5Trimmedtree.pdf, accessed on November 4, 2010] [58]. Early progenitor viruses from Hong Kong and China (1996-2002), including A/GS/GS/1/96-likeand HK97-like viruses, belong to clade 0. The genotype Z viruses introduced to Vietnam, Thailand, Cambodia, and Malaysia in 2003-2004 belong to clade 1. Clade 2 viruses are more divergent and have been divided into 5 further subclades (clades 2.1-2.5). The viruses introduced to Indonesia belong to clade 2.1. Those that caused outbreaks in wild birds in Qinghai Lake and subsequently spread to the Middle East, Europe, and Africa belong to clade 2.2. The genotype V viruses introduced to South Korea and Japan belong to clade 2.5 [45, 46]. In 2005-06, clade 2.3 (subclade 4) viruses emerged predominantly in Southern China and spread to Hong Kong, Laos, Malaysia, Thailand, and North-Vietnam [54, 59] causing poultry outbreaks and sporadic human infections [4, 59].

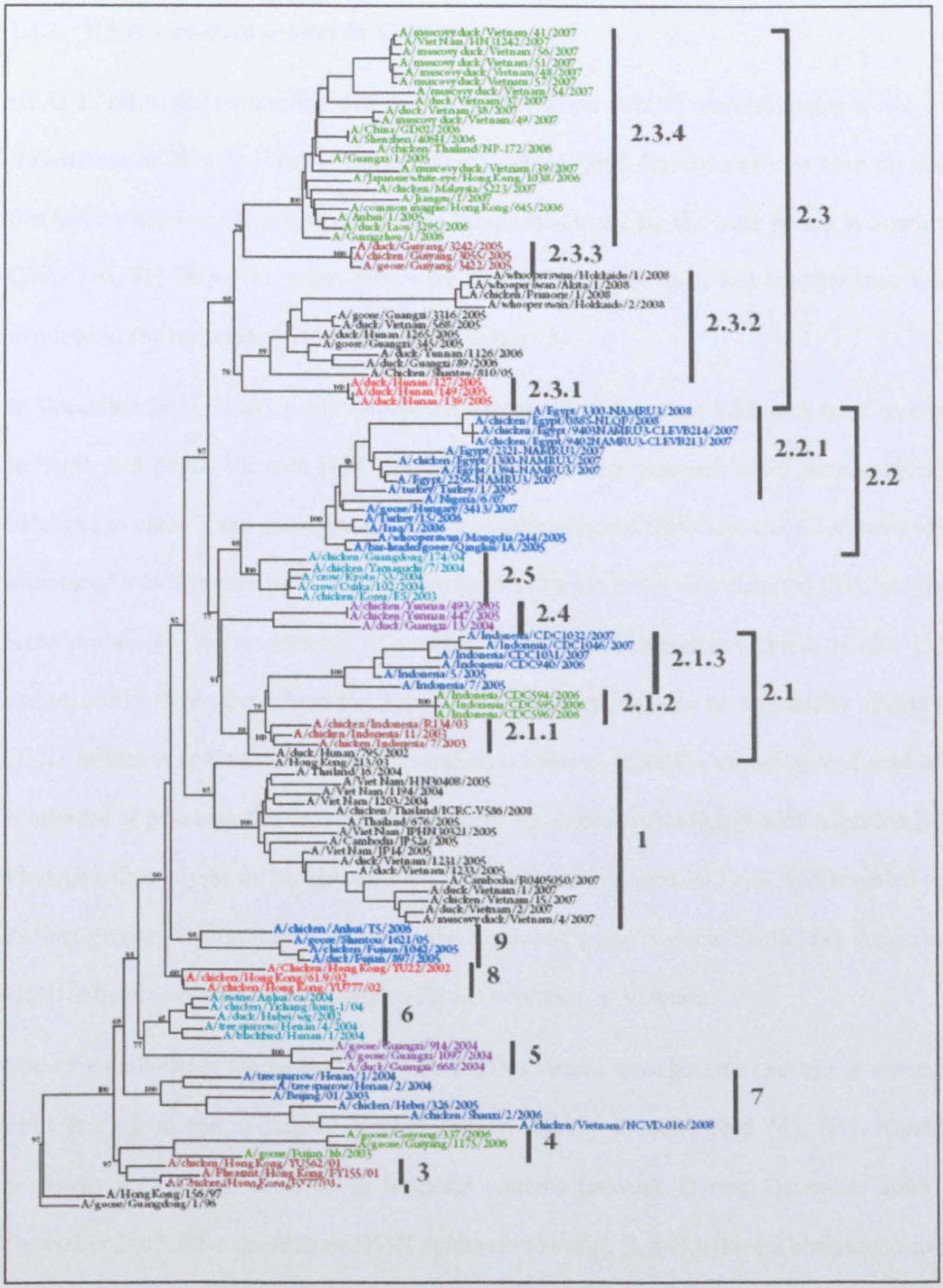

Figure 1.3 Neighbor-joining tree of 121 reference H5N1 HA sequences. Numbers following vertical bars indicate virus clades. Tree was reproduced from http://www.who.int/csr/disease/avian_influenza/H5Trimmedtree.pdf [58]

1.4.2 H5N1 viruses in poultry in Vietnam

HPAI H5N1 viruses were first detected in healthy geese sold in markets during a two-day surveillance in 2001 in Hanoi – the capital of Vietnam [60]. Genetic analyses have revealed that these viruses are genetically similar to those isolated during the same period in Southern China [60, 61]. However, when they were introduced to Vietnam, and whether they were involved in any undetected outbreaks remain unknown.

In December 2003, H5N1 poultry outbreaks associated with human infections were reported in North and South Vietnam [62]. Influenza H5N1 viruses responsible for these outbreaks belonged to clade 1 and genotype Z. Recent studies revealed that these clade 1 viruses were introduced into Vietnam 3-6 months before the first human cases were detected [53], and they were introduced by movements of poultry across the Vietnamese-Chinese border [54]. Subsequently, they spread from North to South [63]. However, due to the scarcity of data on H5N1 influenza in Central Vietnam, it remains unclear whether the viruses spread gradually southward or pass over this region through North-South poultry trading or bird migration [63]. Phylogenetic analyses of the HA gene of H5N1 viruses insolated in 2003-2005 revealed two distinct geographical groups: group N in the North and group S in the South [54], suggesting H5N1 influenza viruses have undergone further evolution in Vietnam.

Since the outbreak of December 2003, HPAI H5N1 viruses have become endemic in domestic birds leading to the culling of tens of million poultry in 2004-2005 [63, 64]. Notably, outbreaks in Vietnam occurred in temporal patterns (waves). During December 2003 to December 2005, three continuous H5N1 epidemic waves (I, II, and III) were observed: wave I began in December 2003 and lasted to June 2004, wave II started from June 2004 to June 2005, and wave III continued from June 2005 to December 2005 [64, 65] (figure 1.4). The peak of these waves was in the months of high demand on poultry consumption: traditional

Tet festival (Chinese newyear; January-February: waves I and II), and November-December (wave III: weeding season). This leads to an increase of poultry rearing and trading activities that may contribute to enhanced infection transmission. In addition, the transmission in these months could be further enhanced by cool weather – as average temperature in these months are the lowest of the year and range from 15 - 20 °C (25-30 °C year round) in the north and 25 - 28 °C (30-35 °C year round) in the south– that allow viruses to persist longer in the environment [64]. Indeed, these months have been identified as high-risk period for spread of H5N1 viruses in Vietnam [66] and other South East Asian countries [67].

In an attempt to contain H5N1 outbreaks, a nationwide poultry vaccination campaign targeting chicken and ducks (≥ 14 day old) was implemented in September 2005 [64]. Possibly, as a result of this mass-vaccination campaign, H5N1 outbreaks did not occur in 2006. However, in the following year, outbreaks occurred in both northern (the Red River Delta) and southern (the Mekong Delta) Vietnam. While the outbreak peak in this year was still in the months of the Tet festival in the South, the outbreak peak in the North occurred in months of summer (May to June 2007) when the weather temperature is high (range from 27-30 °C) [64]. This change could be due to the introduction of new H5N1 variants, genotype V clade 2.3.4 viruses, and replacement of genotype Z clade 1 viruses in northern Vietnam in 2006/2007 [68]. Clade 2.3.4 genotype V viruses have also been detected in southern Vietnam recently, but this genotype has yet replaced genotype Z in this region. Our most recent data collected in February 2010 show that clade 1 viruses remain predominant (paper in preparation).

In addition to genotypes Z (clade 1) and V (clade 2.3.4), genotype G viruses have also been detected in Vietnam. This virus genotype was introduced to Vietnam in late 2005 and was detected in a portal province with China where illegal poultry trading activities are high [46].

Genotype G viruses were then detected in some other provinces in northern Vietnam. However, they have not yet been implicated in any large poultry outbreak in Vietnam [68].

It is likely that all identified H5N1 variants were introduced to Vietnam through the movement of poultry across Vietnam-China border. Therefore, surveillance for H5N1 viruses in imported poultry would be crucial, and in fact has been implemented at ports of entry in North Vietnam. As a result of this activity, early detection of new variants of H5N1 viruses (e.g genotype G and clade 7 viruses) have been reported [46, 69]. However, besides that, surveillance for H5N1 viruses should extend to migratory birds since Vietnam is in part of the East Asia-Australian flyway [55] and is at risk for viruses being brought into by bird migration.

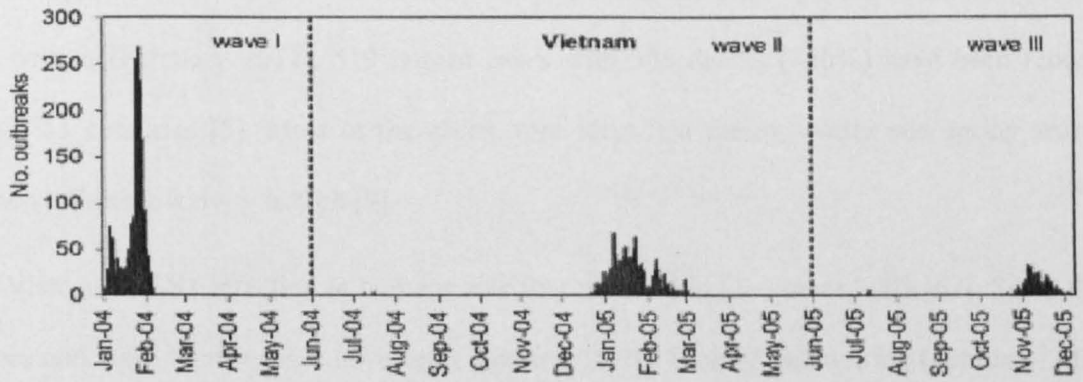

Figure 1.4 Temporal distribution of daily HPAI H5N1 reports recorded in Vietnam between January 2004 to December 2005. Figure reproduced from Gilbert et al [65].

1.4.3 Transmission to humans and epidemiology of human cases

Direct transmission of HPAI H5N1 viruses to humans was first reported during a large poultry outbreak in Hong Kong in 1997. During this outbreak, 18 human infections were laboratory confirmed and 6 of them were fatal [2]. The source of infections was live poultry sold in markets [70]. After this outbreak, human H5N1 infections were not reported until February 2003 when influenza H5N1 virus infections were confirmed in two Hong Kong residents: a father and his child who had just returned from a holiday in mainland China. The father succumbed to the infection [71]. Another case of H5N1 infection occurred in November 2003 in China and was retrospectively confirmed [72]. Subsequently, with the increasing spread of H5N1 in poultry and migratory birds across large regions in the world, further human cases were reported from many countries in Asia, the Middle East, Europe and Africa. At the time of writing (February 2011), 519 human cases with 306 deaths (~60%) have been reported from 15 countries [5]. Most of the cases were identified during winter and spring seasons when influenza activity is high [9].

In Vietnam, H5N1 infection in humans was first reported in December 2003 [62]. Since then, more and more human cases have been reported. At the time of this writing (February 2011), 119 human cases have been confirmed, 59 of which were fatal [5]. The temporal relationship between H5N1 outbreaks in poultry and human infections in Vietnam from December 2003 to August 2007 have been studied by Minh et al., [73]. However, a clear seasonal pattern of human H5N1 cases has not been observed [73]. Nevertheless, in their studies, they found that human cases were detected at the same time or slightly earlier than the reported start of poultry outbreaks (figure 1.5) [73]. This indicates that in Vietnam, poultry outbreaks may have gone undetectable until human cases were confirmed.

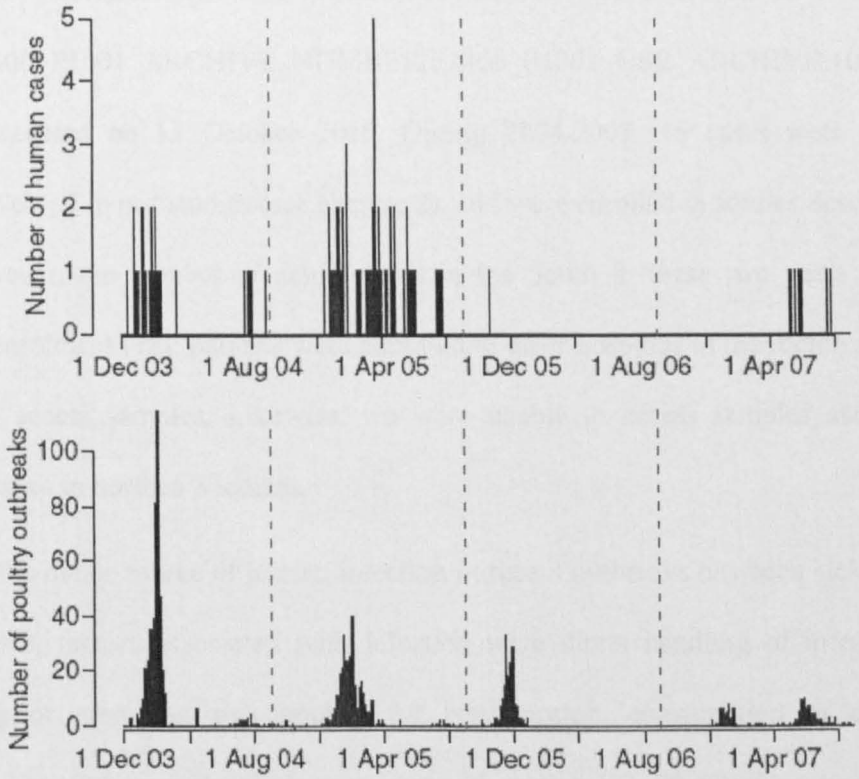

Figure 1.5. Epidemic curves showing the daily number of H5N1 cases and poultry outbreaks recorded in Vietnam between December 2003 to August 2007. Vertical lines mark four periods, one year each, started on 1 September and ending on 31 August in the following year. Figure reproduced from Minh et al., [73].

Most of human cases in Vietnam were identified in 2004 and 2005 with the majority in the North [74, 75]. The total number of cases in Vietnam in these two years was 90, accounting for 76% of the total reported cases from Vietnam [5]. 2005 was the year with the highest number of confirmed cases (61 cases) followed by 2004 (29 cases). Notably, in the South, human cases were identified mostly in these two years [36, 62, 76, 77]. After that, no further human cases have been reported in this region until March 2009 when H5N1 virus was

confirmed in a 3 year-old boy in Dong Thap province http://wwwpromedmailorg/pls/otn/f?p=2400:1001:2609219060520617:F2400_P1001_BACK_PAGE,F2400_P1001_ARCHIVE_NUMBER,F2400_P1001_USE_ARCHIVE:1001,200903201118,Y, accessed on 13 October 2010. During 2004-2005, 16 cases were admitted to hospitals involved in our studies (see chapter 2), and were enrolled in studies described in this thesis. However, the number of actual cases in the South in these two years exceeds the number of enrolment [78]: patients were admitted to other hospitals in the region and we were not able to access samples. Likewise, we were unable to access samples and data from confirmed cases in northen Vietnam.

In general, the major source of human infection in recent outbreaks has been sick poultry [9]. In most cases, factors associated with infection were direct handling of infected poultry, slaughtering or preparing sick poultry for consumption, consumption of uncooked or undercooked poultry products, or close contact with poultry [62, 79, 80]. Contamination of the environment with fecal material from infected birds is an alternative source of infection [36, 81]. However, current data suggest that the transmission of H5N1 virus from poultry to humans and from human-to-human remains inefficient [62, 80, 82, 83].

Since the emergence of avian influenza H5N1 in humans, clusters of cases have been identified in most of affected countries [81, 84, 78, 85]. Up to 25 November 2009, a total of 52 clusters, involving 103 cases and accounting for 22% of reported cases, have been documented (table 1.3)[78]. Interestingly clusters occurred predominantly within families and within those families almost exclusively among direct blood relatives (48/52 clusters) [78]. These data, coupled with the inefficient transmission of H5N1 viruses among humans, suggest that genetic factors may be involved in human susceptibility to infection.

Table 1.3 Number of confirmed H5N1 cases (as of 25 November 2009) and clusters by country. Table reproduced from Horby et al., [78].

Country	Total laboratory-confirmed cases	No. of clusters	*n*/*N* (%) of confirmed cases occurring in clusters
Azerbaijan	8	2	6/8 (75)
Bangladesh	1	0	0/1 (0)
Cambodia	8	1	1/8 (12)
China, mainland	38	4	4/38 (10)
Djibouti	1	0	0/1 (0)
Egypt	88	4	9/88 (10)
China, Hong Kong	20	2	4/20 (20)
Indonesia	141	18	36/141 (25)
Iraq	3	1	2/3 (67)
Laos PDR	2	0	0/2 (0)
Myanmar	1	0	0/1 (0)
Nigeria	1	1	1/1 (100)
Pakistan	3	1	3/3 (100)
Thailand	25	3	5/25 (20)
Turkey	12	2	6/12 (50)
Vietnam	111	13	26/111 (23)
Total (all countries)	463	52	103/463 (22)

Convincing evidence for possible human-to-human transmission remains scarce. Presently, only two clusters of human-to-human transmission have been documented: one in Thailand and another in China [86, 87]. The family cluster in Thailand was identified in 2005 included three family members: a child, her mother, and her aunt. In this family cluster, H5N1 virus was possibly transmitted from the child to her mother and her aunt through prolonged close contact during care of the child [86]. Another family cluster was found in China in 2007

comprised two family members, a young man and his father. H5N1 virus was believed to transmit from the young man to his father [87]. In these two cluster families, both patients from whom H5N1 viruses were transmitted to others died.

Nosocomial person-to-person transmission possibly occurred during the outbreak in Hong Kong in 1997 [88, 89] but this was not observed in recent outbreak in Vietnam and Thailand [90-92].

1.4.4 H5N1 disease in humans

Human infections with avian influenza H5N1 virus mostly present as influenza-like illness with symptoms of fever, shortness of breath and cough with rapid progression to more severe respiratory disease, requiring mechanical ventilation, and in a substantial proportion to multi-organ failure and death. Based on reported cases, the mortality of human H5N1 infections is approximately 60%. At presentation, most patients have radiological evidence of pneumonia, characterized by extensive bilateral infiltration, lobar collapse, focal consolidation, and air bronchograms [62, 80]. Moreover, patients with H5N1 influenza may present with abdominal pains, bleeding from nose and gums, vomiting, and diarrhea [2, 62, 80]. The latter can be the only symptom at presentation [76, 93]. In severe cases, lower respiratory manifestations develop shortly after admission. Complications associated with fatal outcome include acute respiratory distress syndrome, and multi-organ dysfunction [62, 80]. During hospitalization, some patients may develop symptoms of acute encephalitis leading to fatal outcome [76]. In recovered patients, radiological evidence of lung damage may still be observed after several months [12].

Patients with H5N1 influenza disease have relatively long lasting high pharyngeal viral loads. High pharyngeal viral loads were found to be associated with fatal outcome [36]. Pathological studies revealed evidence of active viral replication in trachea and lungs [94-96] and the presence of H5N1 viruses in type II pneumocytes of the lower respiratory tract [96]. Beside the respiratory tract, the presence of viral RNA was demonstrated in multiple bodily fluids and organs including blood, cerebrospinal fluid, rectum, intestines, liver, spleen and brain [94-96]. In addition, in a pregnant woman, viral RNA was detected in the placenta and fetus [94].

Patients with severe H5N1 disease usually have lymphocytopaenia, often with conversion of the CD4:CD8 T-lymphocyte ratio, thrombocytopaenia, and increased levels of serum aminotransferases. Some have hyperglycemia, and elevated levels of lactate dehydrogenase and creatine kinase [2, 62, 80]. Most of examined patients have increased levels of plasma pro-inflammatory cytokines including IP10 (interferon-inducible protein 10), MIG (macrophage attractant chemokines), IL-8, IL-6 and IL-10, and the levels of these cytokines were positively correlated with pharyngeal virus loads and fatal outcome [36].

1.4.5 Specific molecular determinants of pathogenicity

1.4.5.1 The hemaggutinin (HA)

Similar to the pathogenicity in birds, the promiscuity of HPAI HAs for cleavage by a range cellular proteases may explain the propensity for viral dissemination in mammals, including humans (see section 1.2.3) In addition, the presence of avian-type receptors in the human lower respiratory tract may explain the high risk of severe pneumonia during human H5N1 infection [83]. In contrast to the idea that human cells only express SA α2,6-Gal receptors, recent studies revealed the expression of SA α2,3-Gal receptors in human non-ciliated

respiratory epithelial cells [25]. However, different SA expression profiles were observed in different parts of the human respiratory tract: cells of the upper respiratory tract (nasal mucosa, paranasal sinuses, pharynx, and trachea) express mainly SA α2,6-Gal, whereas cells in the lower respiratory tract (type II pneumocytes and non-ciliated cuboidal epithelial cells) express both SA α2,6- and α2,3-Gal [97]. Since SA α2,3-Gal bearing cells are believed to act as primary target cells for H5N1 virus infections, their presence in the lower respiratory tract may explain the propensity of H5N1 viruses to cause pneumonia in humans [83]. Compared to adults, cells in the upper respiratory tract of children express more SA α2,3-Gal, suggesting – in accordance with epidemiological data - that children are more susceptible to H5N1 infection [98]. More recent studies have shown binding and replication of H5N1 viruses in upper respiratory tract epithelia as well, suggesting that other factors besides SA α2,3-Gal and SA α2,6-Gal linkage of receptors, potentially including other linkages and the spatial shape of the receptor, may be involved in infection and host specificity [99, 100].

1.4.5.2 The polymerases

Three viral polymerase proteins, the Acid polymerase (PA), Basic polymerase 1 (PB1) and 2 (PB2), and the nucleoprotein (NP) together form the viral replication complex. Certain amino acid changes in avian influenza viruses may provide a replication advantage, particularly in mammalian hosts. The presence of a lysine (Lys, K) instead of a glutamic acid (Glu, E) at position 627 in PB2 has been identified as a major determinant of efficient replication, virulence and host range of influenza A viruses in mammals [32]. Interestingly, Lys627 was also found in an H7N7 virus isolated from a man who died of severe pneumonia in The Netherlands in 2003, but was not found in viruses isolated from patients with mild disease or from chickens [41]. While Lys627 has not been identified in PB2 of avian H5N1 viruses

isolated in Vietnam so far, some H5N1 viruses isolated from Vietnamese patients possessed the mutation [36, 54]. Studies in mice showed that the presence of Lys627 in PB2 is essential for virulence and systemic infections of H5N1 viruses responsible for human diseases in Hong Kong in 1997 [33], but does not increase the tissue tropisms [101]. However, recent studies showed that Lys627 is important for efficient transmission of avian influenza A H5N1 viruses in animal models [34].

A substitution of aspartic acid (Asp, D) to asparagine (Asn, N) at position 701 in PB2 is also associated with high pathogenicity and efficient transmission of avian viruses in mammals [102, 34]. The virulence of H5N1 viruses in mammals is attenuated by the absence of an Asn residue at position 701, resulting in an inability to replicate in mice [103]. In contrast, H5N1 viruses with D701N exhibits enhanced transmission efficiency in guinea pigs [34]. In H7N7, an Asn residue at position 701 has been shown to be involved in binding to mammalian importin 1α, thereby enabling more efficient transport of the replication machinery to the nucleus of the host cell. The identification of these single amino acid changes that abolish pathogenicity are important steps in our understanding of virulence. However, introducing these two mammalian virulence associated amino acids in any PB2 gene will not necessarily result in a hypervirulent virus [104]. Recent studies showed that viral virulence is possibly determined by the whole polymerase complex (PB2, PB1, and PA) rather than PB2 alone [105].

1.4.5.3 The NS1 protein

The NS1 protein is the only nonstructural protein of influenza viruses. This protein seems to be playing important roles in the increased viral virulence of influenza viruses, mainly by protecting the virus from the antiviral effects of host interferon (IFN) responses [106]. NS1

exerts its IFN antagonism by binding dsRNA and by binding to the cellular RNA helicase retinoic acid inducible gene 1 (RIG-1), an upstream regulatory component of the IFN production cascade [107-109]. Furthermore, partial down regulation of NF-kappaB pathways by NS1 has been shown [107, 110].

Compared to human H1N1 and H3N2 viruses, the NS1 gene of H5N1 viruses seems responsible for hyperinduction or dysregulation the cytokine response, particularly TNF-α, as shown in in-vitro studies using human-derived macrophages [111]. H1N1 virus bearing the NS1 of H5N1 virus induced high pulmonary concentrations of IL1α, IL1β, IL6, IFN-γ and IL8-like chemokines, and decreased concentrations of the anti-inflammatory cytokine IL10 in mice [112], and prolonged viral shedding in pigs [113]. The latter was associated with the presence of glutamic acid (Glu, E) instead of aspartic acid (Asp, D) at position 92 of the H5N1 NS gene [113]. However, Glu92 has not been found in recent human H5N1 isolates [9, 29]. In addition, amino acid changes at positions 149 and 42 appear to be critical for virulence, in chickens and mice, respectively [114, 115]. Furthermore, recent studies have revealed the presence of a 'postsynaptic density protein-95, disclarge tumor suppressor protein, zonula occludes-1' (PDZ) ligand motif in the carboxy terminus of the NS1 of avian influenza virus, including H5N1, as a potential virulence determinant [116].

1.4.5.4 Other proteins

The M2 protein is a target of the adamantane drugs (amantadine and rimantadine). Recently, an increase in amantadine resistance, caused by mutations in the M2 gene, has been observed in both seasonal human H3N2 and H1N1 viruses, seemingly without antiviral pressure. Viruses from some H5N1 clades invariably carry amantadine resistance conferring mutations [79, 117]. Resistance in these avian H5N1 clades may be explained by the massive use of amantadine in Chinese poultry farming [118], but the fact that resistance increases in human

viruses in the absence of selective pressure by drug use, and the finding that mortality is higher in mice infected with amantadine resistant H1N1 strains suggest that M2 may also be important for virulence [119].

The recently discovered PB1-F2 protein, which is translated from an alternate reading frame of the PB1 gene segment, causes apoptosis in macrophages, reducing their ability to induce an immune response and therefore delaying viral clearance [120]. This reading frame is also present in H5N1, and is believed to contribute to the viral virulence [121, 122].

1.4.6 Laboratory diagnosis

Preferred clinical specimens for laboratory diagnosis of suspected cases of H5N1 influenza include throat swabs or lower respiratory tract specimens in case of pneumonia [62]. Compared to nasal swabs, throat swabs show higher viral loads and therefore higher yields for the detection of H5N1 virus [12], [This thesis]. For critically ill patients who require mechanical ventilation, endotracheal aspirate specimens can also be used as diagnostic specimens [12, 123]. Although H5N1 is a systemic disease, and clinical specimens collected outside the respiratory tract such as blood, stools, CSF, may contain detectable levels of viral RNA, these are not recommended for diagnostic use [124].

Current options for diagnosis of human infection with influenza A H5N1 virus at the time of disease include antigen detection, virus culture and detection of viral RNA by reverse transcription polymerase chain reaction (RT-PCR) [9]. Although current methods for detection of viral antigen (e.g., direct immunofluorescence, enzyme immunoassay and rapid tests) are rapid and easy to perform, these methods are not useful for routine diagnosis of H5N1 influenza because currently available tests are unable to differentiate infection caused by

H5N1 from circulating seasonal influenza virus subtypes [9, 12]. In addition, low sensitivity limits the use of rapid antigen tests for diagnosis of H5N1 infection [79]. Virus isolation in embryonated chicken eggs or cell culture remains the 'gold standard' for influenza diagnosis, including avian influenza H5N1 [12]. However, factors that limit its usefulness include time to diagnosis (culture is relatively slow and requires 2 to 3 days) and biosafety level 3 (BSL3) laboratory requirements [12]. Therefore, RT-PCR rather than virus isolation is the method of choice for diagnosis of clinical specimens from suspected cases [9]. RT-PCR is sensitive, specific and only requires 6 to 8 hours to produce a result [9]. When using real-time RT-PCR (rRT-PCR), the per-sample turn around time can be shortened to 4-6 hours. Besides its sensitivity and specificity, the use of rRT-PCR allows for minimizing the risk of carryover contamination as it requires no post-amplification manipulation of generated nucleic acid amplicons [9, 12]. In addition, rRT-PCR also allows for (semi-) quantitative analyses of the virus load in clinical specimens important for studying viral factor in pathogenesis and possibly for monitoring treatment response [36, 77], [This thesis].

Detection of H5 specific antibodies by hemagglutinin-inhibition or (micro)neutralization assays are not useful for timely diagnosis because antibodies are usually not yet detectable during the acute stage, serological analysis ideally requires paired sera, and the most sensitive method, i.e. (micro)neutralization, requires virus culture in BSL3-facilities. However, serological assays are very important for retrospective diagnosis (in the case of absence of acute respiratory specimens) and sero-epidemiology studies [79].

1.4.7 Treatment

At present, two classes of drugs are available for the treatment of influenza virus infections: the adamantanes (amantadine and rimantadine) and the neuraminidase inhibitors (zanamivir

and oseltamivir). Adamantanes target the M2 ion channel of influenza A viruses, preventing replication [9]. During the Hong Kong H5N1 outbreak in 1997, amantadine was used to treat several patients [2]. However, amantadine was not used in recent outbreaks due to a high rate of adamantane resistance [79]. Patients with H5N1 disease have mostly been treated with the neuraminidase inhibitor oseltamivir [62, 79].

Oseltamivir inhibits viral replication through binding to the active site of the neuraminidase enzyme, thereby interfering with its function to hydrolyse host cell receptors and releasing progeny viruses from infected cells [125]. In uncomplicated influenza, treatment with oseltamivir is only beneficial when initiated early (<48 hours) in the course of illness [79]. When given later, it may not affect the course of mild seasonal influenza since the host' immune system is clearing the infection by then. In more severe and prolonged illness, it may still be of benefit to give oseltamivir after 48 hours of disease. For most of H5N1 infected patients studied in this thesis treatment was started relatively late when symptoms had already progressed, possibly explaining its limited effect [79].

Compared to H5N1 viruses from the 1997 outbreak, clade 1 H5N1 viruses are more susceptible to oseltamivir carboxylate *in vitro* but animal models suggest that higher doses and longer treatment durations may be required for antiviral efficacy [126, 127]. Compared to clade 1 viruses, clade 2 viruses are 20 to 30-fold less susceptible to oseltamivir *in vitro* [79]. Viruses with oseltamivir resistance conferring mutations have been shown to evolve during treatment [77, 128], and may be associated with fatal outcome [77]. Oseltamivir resistant viruses remain susceptible to zanamivir [128], which is at this time only available as a locally active inhaled drug. Intravenous formulations of zanamivir and the neuraminidase inhibitor peramivir are under development, possibly providing more options for treatment of influenza illness, including H5N1 influenza [79].

1.5 The aims of this thesis

Since the re-emergence of human H5N1 infections in 2003, the clinical understanding of H5N1 disease and its pathogenesis has increased but remains limited due to the relatively small number of cases, therefore additional clinical studies of human H5N1 disease remain important.

The neuraminidase inhibitor oseltamivir is the only readily available treatment option for human H5N1 infection, but little is known about the effectiveness and optimal dosage of the drug in H5N1 disease. Furthermore, we have reported the emergence of oseltamivir resistant variants in two patients during therapy resulting in treatment failure [77].

To contribute to the current knowledge, in the work that led to this thesis it was aimed 1) to study the clinical and virological features of 16 human H5N1 infections identified in 2004-05 in southern Vietnam, and 2) to study the antiviral efficacy of treatment with oseltamivir and the emergence of drug resistance in 8 patients who were followed during treatment.

Furthermore, it was shown that H5N1 viruses prevalent in Vietnam during 2004-05 belonged to clade 1 of genotype Z [54]. Sequence analyses revealed that, compared to strains isolated from birds, some human H5N1 strains have amino acid changes, mostly in the HA and PB2 genes. However, little is known about the correlation between amino acid changes, particularly in the PB2 gene, and disease severity as well as the role of amino acid changes in HA in adaptation to human receptor recognition. Against this background, I aimed 3) to understand the molecular characteristics of isolated human virus strains, 4) to identify possible virulence determinants and, if possible, their correlation with clinical outcome, and 5) to identify amino acid changes in HA that potentially affect receptor specificity.

In recent years, clade 2.3.4 H5N1 viruses have been found to co-circulate with clade 1 H5N1 viruses in some southeast Asian countries, including Vietnam. The co-circulation of two distinct genetic clades provides a challenge to laboratory diagnosis of H5N1 infection since assays for H5N1 detection are usually designed based on clade-specific amplification of the HA gene. To improve H5N1 diagnostics in Vietnam, I aimed 6) to develop a highly sensitive real-time PCR for generic detection of currently circulating H5N1 influenza viruses important for timely diagnosis and outbreak control.

Chapter 2

PATIENTS AND METHODS

2.1 Patients and clinical samples

Between January 2004 and February 2005, 16 patients with influenza virus A H5N1 disease were confirmed in 4 hospitals in southern Vietnam. Three of these hospitals are tertiary hospitals for southern Vietnam based in Ho Chi Minh City: the Hospital for Tropical Diseases (HTD), Paediatric Hospital One (PH1), and Paediatric Hospital Two (PH2). At the start of avian influenza A H5N1 outbreaks, these three hospitals were designated as hospitals for receiving and treating patients with suspected infection (patient who has fever, respiratory symptoms and a history of poultry exposure or exposure to a H5N1-confirmed patient). Suspected patients identified in other hospitals in southern Vietnam were referred to one of these three hospitals. The remaining hospital of this study was a provincial hospital, the Dong Thap Provincial Hospital (DTPH), Cao Lanh district, Dong Thap province. DTPH is a primary level hospital situated in the Mekong Delta approximately 250 km southwest of Ho Chi Minh City.

The Virology Laboratory of the Oxford University Clinical Research Unit (OUCRU) based in HTD was responsible for diagnosis of avian influenza A H5N1 infection.

The 16 patients were renamed as Patients 1-16 (table 2.1). Informed consent was obtained from all patients or their guardians during hospitalization. From these 16 patients, epidemiological data were obtained by interviewing them or their relatives who cared them at home or during their hospital stay. Clinical data and laboratory test results were obtained by means of patient files. These clinical data were collected by clinicians at the Hospital for Tropical Diseases, Peadiatric Hospital Number 1 and 2, and Dong Thap Provincial Hospital as part of a study on clinical characteristics of human H5N1 influenza that I was involved in. This study was directed by Professors Tran Tinh Hien, Menno de Jong, and Jeremy Farrar. The clinical data of most of these patients have been published previously as case series or

case reports (appendix 1). Permission for use of these clinical case notes and database was obtained from Professor Tran Tinh Hien, vice-director of the Hospital for Tropical Diseases and Prof Jeremy Farrar, director of OUCRU.

All respiratory clinical specimens obtained from these 16 patients were used in studies described in this thesis. These specimens included diagnostic nasal and throat swabs obtained from 15/16 patients and sequential throat swabs obtained during and after oseltamivir treatment from 8/16 patients (table 2.1 and chapter 4). In addition, during admission, other non-respiratory clinical specimens including blood specimens from all 16 patients, rectal swabs from 7 patients, and CSF from one patient (table 2.1) were also obtained and used.

Table 2.1 Clinical samples available for analysis from 16 patients.

Patient	*Patient group*	*Diagnostic respiratory swab*	*Follow-up respiratory swab*	*Extrapulmonary specimens*		
				Rectal swab	**Blood**	**Cerebrospinal fluid (CSF)**
1	II	No	Yes	No	Yes	No
2	II	Yes	Yes	No	Yes	No
3	II	Yes	Yes	Yes	Yes	No
4	II	Yes	Yes	Yes	Yes	No
5	I	Yes	Yes	No	Yes	No
6	I	Yes	Yes	No	Yes	No
7	I	Yes	Yes	No	Yes	No
8	II	Yes	No	No	Yes	No
9	I	Yes	No	No	Yes	No
10	I	Yes	No	Yes	Yes	No
11	I	Yes	No	Yes	Yes	No
12	I	Yes	No	No	Yes	No
13	II	Yes	No	Yes	Yes	No
14	II	Yes	No	Yes	Yes	No
15	II	Yes	Yes	No	Yes	No
16	II	Yes	No	Yes	Yes	Yes

For studies described in chapter 6, beside 10 respiratory clinical specimens obtained from 10 of the 16 index patients with clade 1 H5N1 virus infection (see chapter 5), 48 additional

clinical samples from 29 patients with confirmed clade 2.1 and 2.3.4 H5N1 influenza viruses were used (table 2.2). All H5N1 clinical sample use in this chapter included 14 nose swabs, 31 throat swabs, 8 pharyngeal aspirates, 1 rectal swab, 2 plasma, and 2 pleural fluids. The clade 2.1 clinical samples were collected from patients with H5N1 disease in Indonesia and the clade 2.3.4 samples were obtained from Vietnamese patients in northern Vietnam, between 2004 and 2008 (table 2.2).

To validate the specificity of the assay described in chapter 6, throat swab samples from 19 patients with seasonal influenza (H1N1: n=10; H3N2: n=9), confirmed by conventional RT-PCRs and/or virus isolation as described previously [62], and 29 throat swab samples from 29 patients with non-influenza respiratory illness admitted to the Hospital for Tropical Diseases, Ho Chi Minh City during the H5N1 outbreaks in 2004 – 2005 were also used.

Ethical approval for use of these specimens has been received from institutional review boards in Vietnam, Indonesia and the Oxford Tropical Research Ethical Committee.

Table 2.2 Additional H5N1 samples used

Clinical samples / virus clade	Nose swab	Throat swab	Tracheal aspirate	Plasma	Pleural fluid	Stool	Total	**No of patient**
Clade 1*	2	7	1	0	0	0	10	**10**
Clade 2.1	7	17	1	0	0	0	25	**25**
Clade 2.3	5	7	6	2	2	1	23	**4**
Total	**14**	**31**	**8**	**2**	**2**	**1**	**58**	**39**

Note: * indicates samples were obtained from 10 of the16 patients described in chapter 3.

2.2 Methods

2.2.1 Sample collection

On admission and during hospitalization, nose and throat, and if possible, rectal swab specimens were collected using rayon swabs with plastic shafts. Nose swabs were collected by inserting a swab stick into the nostril with most secretions. The swab was left in place for 5-10 seconds to absorb secretions. Throat swabs were collected by wiping the swab stick over the tonsils and posterior pharyngeal wall without touching the buccal mucosa or tongue [http://wwwwhoint/csr/resources/publications/surveillance/CDS_EPR_ARO_2006_1pdf, accessed on October 12, 2009]. Rectal swabs were collected by spreading the patient's buttocks, inserting the swab stick into the anus and further into the rectum, and rotating the swab gently before taking it out. During specimen collection, personal protective equipments including N95 mask, non-sterile latex gloves, face shield, gown and head-cover were used according to WHO guidelines [http://www.searo.who.int/LinkFiles/CDS_CDS-Guidelines-Laboratory.pdf, accessed on May 13, 2008] [129].

After swabbing, swabs were immediately placed into a sterile vial containing 2 ml of Viral Transport Medium (VTM) (Minimum Essential Medium Eagle with Hanks' salts, supplemented with 0.5 percent gelatin and antibiotics [Sigma-Aldrich, Singapore, Singapore]) or PBS (phosphate-buffered saline) [62].

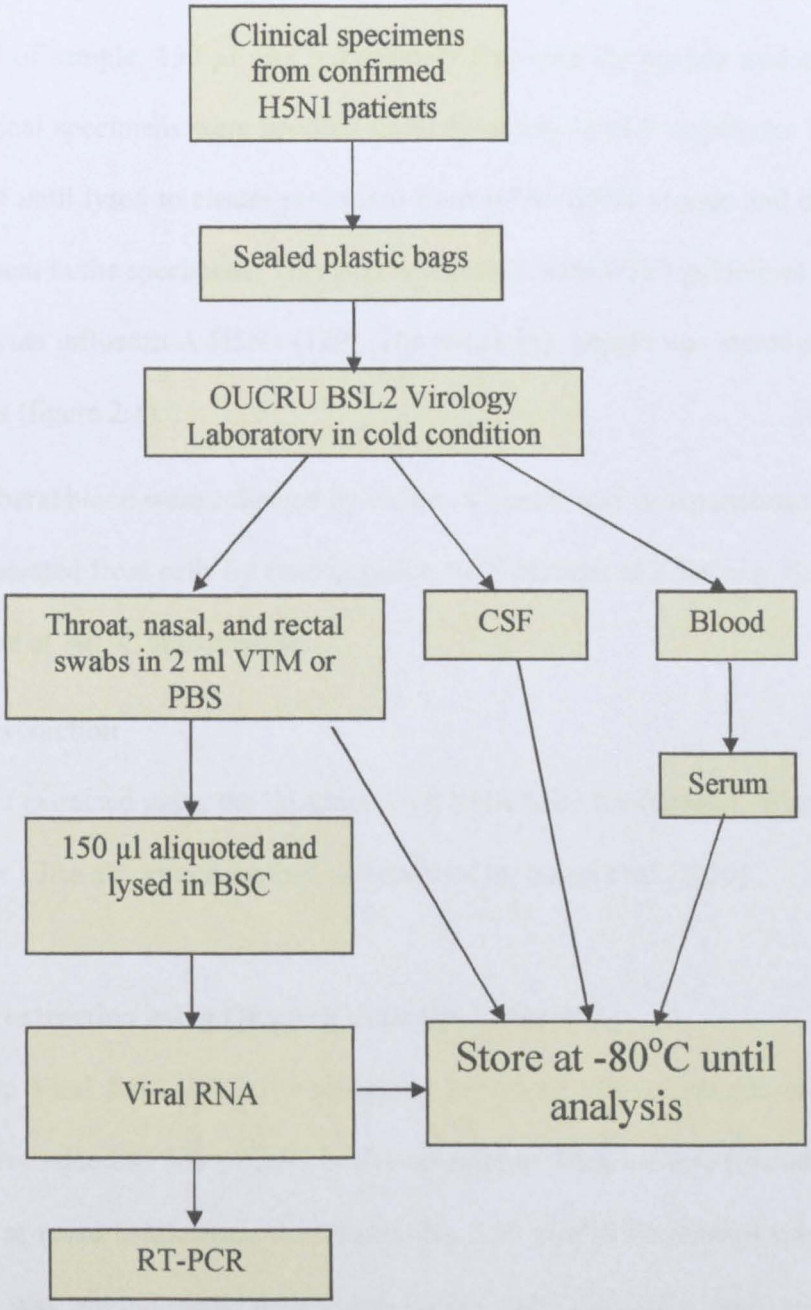

Figure 2.1 Flow chart illustrating how clinical specimens were handled

All swab specimens were immediately transported to the OUCRU Virology Laboratory in individual sealed bags and under cold conditions (4°C). Upon receiving clinical specimens, from each vial of sample, 150 µl was immediately aliquoted for nucleic acid extraction and RT-PCR. Clinical specimens were handled under biosafety level 2 conditions in a biosafety cabinet class II until lysed to ensure protection from HPAI H5N1 viruses and other potential pathogens present in the specimens. This is in accordance with WHO guidelines on laboratory diagnosis of avian influenza A H5N1 [129]. The remaining sample was stored at -80 °C until further analysis (figure 2.1).

CSF and peripheral blood were collected by means of lumbar and vein punctures, respectively. Serum was separated from cells by centrifugation for 5 minutes at 2.500 x g. Serum and CSF were then stored at -80 °C until analysis.

2.2.2 RNA extraction

Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, West Sussex, UK) and/or in-house silica absorption method as described by Boom et al., [130].

2.2.2.1 RNA extraction using QIAamp Viral RNA Mini Kit

In the QIAamp Viral RNA Mini Kit (Qiagen), 140 µl of clinical sample or virus culture supernatant were added to 560 µl lysis buffer containing RNA carriers (included in the kit), and incubated at room temperature for 10 minutes. 560 µl of 95% ethanol was added to the lysate, which was subsequently transferred into a collection tube with silica filter and centrifuged at 16363 x g for 1 minute. The flow-through was discarded and the collection tube was washed with 500 µl AW1 buffer (included in the kit) and then with 500 µl AW2 buffer (included in the kit). The collection tube was spun at 16363 x g for 3 minutes to remove any

trace of extraction buffers which may inhibit the RT-PCR. Viral RNA bound to the silica filter was eluted in 60 µl of elution buffer (included in the kit) and was used in cDNA synthesis.

2.2.2.2 RNA extraction using Boom assay

In the silica absorption method, all extraction reagents including lysis buffer L6 containing 4M guanidinium thiocyanate (Promega Corporation, Wisconsin, USA), activated silica (Promega Corporation, Madison, WI 53711 USA) in 1X TE (Tris-HCl, EDTA) pH 8.0 for nucleic acid absorption, washing buffer L2, 70% ethanol (DBH Prolabo, Singapore), acetone (DBH Prolabo, Singapore) and 1X TE pH 8.0 buffer) were prepared at the Virology Laboratory of OUCRU according to Boom et al., [130]. Briefly, 100 µl of clinical sample or virus culture supernatant was mixed with 1 ml lysis buffer L6 on a vortex mixer. Twenty µl of activated silica were added to absorb nucleic acids and the mixture was incubated at room temperature for 10 minutes. The silica with absorbed nucleic acids was pelleted by centrifugation and the liquid phase was discarded. The pellet was treated twice with 1 ml L2 washing buffer, and twice with 1 ml 70% ethanol. Finally, the pellet was washed with 1 ml of acetone and air dried at 56 °C for 10 minutes. Silica-bound nucleic acids were eluted in 100 µl of 1X TE buffer pH 8.0 at 56 °C for 10 minutes. The aqueous phase containing viral RNA was collected and was used for making cDNA.

2.2.3 Primer and probe design

PCR primers were designed using Primer Express software version 2.0 (Applied Biosystems). This software package includes specific applications including PCR primer design, sequencing primer design, and TaqMan probe design. The software generates a set of possible primers and

probes based on a reference or consensus sequence of the gene of interest. Parameters that can be set include primer position and length, GC content, melting temperature, etc. Generated primer sets are scored between 1-1000 points; the lower the score, the better the primer set (e.g. minimal risk of aspecific hybridization and formation of secondary structures due to self-complementarity or primer dimer formation).

The NA1-609F/NA1-811R shown in table 2.3 is the one of the primer sets designed by this software. The reference sequence used in designing this primer set was a consensus nucleotide sequence of NA gene generated from NA sequences of a large selection of avian influenza A H5N1 strains available in GeneBank [131].

The design of TaqMan probes is similar to that of PCR primers except TaqMan probes should have a T_m of at least 5 °C greater than the T_m of primers.

2.2.4 Reverse transcription – Polymerase chain reaction (RT-PCR)

In this thesis, RT-PCR reactions were performed either in one step (chapter 6) or two step (chapters 3-5). One step RT-PCR combines RT and PCR reactions in one tube and primers used in one step RT-PCR reactions are sequence specific primers. Two step RT-PCR reactions separate the RT from the PCR. In two step RT-PCR, viral RNA was first converted into cDNA using either random hexamers (Roche Diagnostics, Indianapolis, IN, USA) or a universal primer – the Uni12 primer (Sigma-Aldrich). Uni12 primer is a universal primer that anneals to the 3' UTR of all eight gene segments of influenza A virus (regardless of its subtype) allowing full length gene segment cDNA synthesis [132]. The synthesized cDNA then served as template in subsequent PCR reactions (chapters 3-5).

2.2.4.1 Two step RT-PCR

2.2.4.1.a Reverse transcription (RT)

RT was performed in total volume of 20 µl containing 5 µl of viral RNA, 4 µl of 5X first-strand buffer (Invitrogen, Carlsbad, CA, USA), 500 mM of each deoxynucleoside triphosphate (dNTP; dATP, dTTP, dCTP, and dGTP) (Roche Diagnostics), 40 units RNA inhibitor (RNase OUT, Invitrogen), 0.2 µl (40 units) of reverse transcriptase (Superscript III, Invitrogen), and 0.4 µM of Uni12 primer or 2 µg of random hexamers. Reverse transcription (RT) was carried out at 25 °C for 10 minutes (only for random hexamers), 50 °C for 60 minutes, followed by enzyme inactivation at 70 °C for 5 minutes. The RT reaction was performed in an Eppendorf Mastercycler (Eppendorf, Cambridge, UK).

A negative control using H_2O instead of RNA template was included in each experiment to ensure that there was no viral RNA contamination of the reagents. cDNA was stored at -20 °C until used.

2.2.4.1.b PCR

PCR was used extensively in this thesis. Several types of PCR amplification including standard PCR, touchdown PCR, hot-start PCR, qualitative and quantitative real-time PCR, were all used depending on the aim of experiment.

Standard PCR: Standard PCR was conducted using 2 µl of cDNA in total volume of 25 µl containing 2.5 µl of 10x PCR buffer, 1.5 mM magnesium chloride, 0.4 mM of each dNTP, 0.4 µM of each primer, and 1 unit of High Fidelity Taq Polymerase of the Expand High Fidelity System (Roche Diagnostics). The initial cycle of the PCR program was 3 minutes at 95°C, followed by 40 cycles of 20 seconds at 94°C, 30 seconds at 50 °C, 30 seconds at 72 °C, and a

final extension step at 72 °C for 7 minutes. The PCR was performed using Eppendorf Mastercycler (Eppendorf) or DNA Tetrad Thermal Cycler (MJ Research, Waltham, MA, USA). PCR products from amplification using normal primers were analyzed using agarose gel (1.5%) electrophoresis as described by Ausubel et al., [133]. Gels were analyzed using Gel Doc XR system (Bio-Rad, Hercules, CA, USA). Products from amplification using bionitylated primers were analyzed in pyrosequencing machine, the PyroMark ID (BioTage, Uppsala, Sweden) (table 2.3).

***Touch-down PCR*:** Touch down RT-PCR was used to diagnose influenza H5N1 infection in group I patients (see table 2.1). Primer sequences for diagnosis using touch-down PCR were obtained from Yuen et al., and Bright et al., [2, 3], or kindly made available by Dr Takehiko Saito, The National of Infectious Diseases, Tokyo, Japan (table 2.4).

The purpose of touch down RT-PCR is to reduce nonspecific PCR amplification, thus providing more reliable diagnostic results [62]. The increased specificity is due to higher annealing temperatures in the initial cycles of the reaction. Unlike standard RT-PCR, the annealing temperature of initial cycles is slowly decreased during each subsequent amplification cycle. Higher annealing temperatures only permit highly specific primer binding, which allows generation of higher quality amplicons for subsequent amplification cycles.

Table 2.3 Primers for standard PCR and their application.

Primer set	Primer name	Source	Nucleotide sequence (5'-3')	Target gene (position)	Application
1	NA1-609F	Designed using Primer Express software version 2.0	GCTTTACTGTAATGACTGACGG	NA (609-630)	Direct amplification of a portion of the NA gene that contains the H274Y mutation
	NA1-811R		AGATACCCATGGCCGATT	NA (811-794)	
2	PCR-274F		GGGAAAGTGGTTAAATCAGTCGA	NA (721-743)	Direct amplification of a portion of the NA gene that contains the H274Y mutation. PCR products were then analyzed in pyrosequencing
	PCR-274R		Biotin-CCATGCCAATTATCCCTGC	NA (812-833)	
3	PCR-HA134F	Designed using BioTage primer design software version 1.0.6,	GCCAATCCAGTCAATGACC	HA (295-313)	Direct amplification of a portion of the HA gene that contains the A134V mutation. PCR products were then analyzed in pyrosequencing
	PCR-HA134R		Biotin-AAGGAGGACTTTCCCTGGTA	HA (476-457)	
4	PCR-HA151-186F		CTCAGCATGTCCATACCAGGG	HA (444-464)	Direct amplification of a portion of the HA gene that contains the I151F/L and E186D mutations. PCR products were then analyzed in pyrosequencing
	PCR-HA151-186R		Biotin-GAAATATAGGTGGTTGGGTTTTG	HA (622-644)	

Note: Positioning is based on the 1st nucleotide of the starting codon.

Table 2.4 Primer set for diagnosis of influenza A H5N1 infection in group I patients.

Primer set	Primer name	Nucleotide sequence (5' to 3')	Target gene (position)	Size of PCR product (bp)	Primer source
H5a	H5-1	GCCATTCCACAACATACACCC	HA (914-934)	358	Yuen et a., [2].
	H5-2	TAAATTCTCTATCCTCCTTTCCAA	HA(1242-1265)		
H5b	H5-515f	CATACCCAACAATAAAGAGG	HA (515-534)	708 bp	Dr Takehiko Saito, The National of Infectious Diseases, Tokyo, Japan.
	H5-1220r	GTGTTCATTTTGTTAATGAT	HA (1201-1220)		
N1	N1-1*	TTGCTTGGTCAGCAAGTGCA	NA(482-501)	615bp	Bright et al., [3].
	N1-2	TCTGTCCATCCATTAGGATCC	NA(1077-1097)		

Note: Asterisk (*) indicates a T to A modification at the 3' end to enhance specificity was performed. Positioning is based on the 1st nucleotide of the starting codon.

In this thesis, touch down PCR reactions were carried out in a total volume of 25 µl, containing 2 µl of cDNA, 2.5 µl of 10x PCR Gold buffer (Applied Biosystems Inc., Foster City, CA, USA), 2.5 mM magnesium chloride, 0.4 mM of each dNTP (Roche Diagnostics), 0.8 µM of each primer, and 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems). Thermocycling conditions were 95 °C for 10 minutes (pre-amplification hot start); 10 cycles of 95 °C for 30 seconds, annealing at 60 °C (for the N1 primer set) or 50 °C (for the H5 primer sets) for 30 seconds (the annealing temperature was decreased with 1°C per cycle), and 72 °C for 1 minute) and 40 cycles of 95 °C for 30 seconds, 55 °C (for the N1 primer set) or 45 °C (for the H5 primer sets) for 30 seconds, and 72 °C for 1 minute. Seven microliter of PCR product was analyzed by agarose gel (1-2 % (w/v)) electrophoresis.

Hot-start PCR: Hot-start PCR is more specific than conventional PCR [134]. In this thesis, hot-start PCR was used to amplify the whole genome of MDCK cultured H5N1 viruses and part of the HA1 of 'egg-adapted' H5N1 viruses. The nucleotide sequences of PCR primers (table 2.5) for whole genome amplification were adapted from Guan et al., [1]. These allowed amplification for the entire genome of influenza A H5N1 virus in 15 fragments (table 2.5). PCR reactions for amplification using these primers were performed at the Department of Microbiology, State Key Laboratory of Emerging Infectious Diseases, Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China in cooperation with Dr Gavin Smith. The reaction was conducted in a total volume of 25 µl, containing 2 µl of cDNA, 2.5 µl of 10x PCR Gold buffer, 1.5 mM magnesium chloride, 0.4 mM of each dNTP (Roche Diagnostics), 0.4 µM of each primer, and 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems). Thermocycling conditions were 95 °C for 10 minutes (pre-amplification hot start); 40 cycles of 95 °C for 20 seconds, 55 °C for 30 seconds, and 72°C for 2 minutes, and final extension at 72 °C for 10 minutes. Three microliter of PCR product was analyzed on agarose gel (1 % (w/v)).

The single primer set (H5-1028F/H5-1028R) (table 2.5) for amplification of a portion of HA1 was designed using Primer Express software version 2.0 (Applied Biosystems). PCR reactions for this primer set were carried out in a total volume of 25 µl, containing 2 µl of cDNA, 2.5 µl of 10x Hotstart PCR buffer (Qiagen), 1.5 mM magnesium chloride, 0.4 mM of each dNTP (Roche Diagnostics), 0.4 µM of each primer, and 1 unit of Hotstart Taq DNA polymerase (Qiagen). Thermocycling conditions were 95°C for 10 minutes (pre-amplification hot start); 40 cycles of 95°C for 20 seconds, 50 °C for 30 seconds, and 72°C for 2 minutes, and final extension at 72 °C for 10 minutes. Seven microliter of PCR products was analyzed on agarose gel (1 % (w/v)).

Table 2.5 Primers for whole genome and part of the HA1 amplification.

Fragment	Primer name	Nucleotide sequence (5' to 3')	Target gene	Size of PCR product (bp)	Source
1	H5-1	AGCAAAAGCAGGGGTMTAAT	HA	1120	Adapted from Guan et al., [1].
	H5-1120R	CCCTGCCATCCTCCCTCTAT			
2	H5-830	TATGCMTAYAAAATTGTCAAG		911	
	H5-1741R	GTGTTTTTAAYTAMAATCTG			
3	N1-3	CAAAAGCAGGAGATTAAAATG	NA	1136	
	N1-1139R	CATCCGTTTGGATCCCAAAT			
4	N1-561	AAGTGCTTGTCATGATGGCA		896	
	N1-1457R	GTAGAAACAAGGAGTTTTTKGAAC			
5	NS-1	AGCAAAAGCAGGGTGACAAA	NS	888	
	NS-888R	AGAAACAAGGGTGTTTTTTA			
6	NP-8	GCAGGGTAGATAATCACTCAC	NP	1020	
	NP-1028R	GAATGGCATGCCATCCATACC			
7	NP-701	ATGAGAGAATGTGCAACA		864	
	NP-1565R	AGTAGAAACAAGGGTATTTTTC			
8	M-8	GCAGGTAGATATTGAAAGATG	M	1015	
	M-1023R	GAAACAAGGTAGTTTTTTACTC			
9	PA-1	AGCAAAAGCAGGTACTGAT	PA	940	
	PA-940R	TTCATGCATTTGATCGCATC			
10	PA-717	CTATGTGGATGGATTCGAAC		1001	
	PA-1718R	ACATACAGGAACATGGGCCT			
11	PA-1386	GGCTACTGAGTACATAATGAAGGG		865	
	PA-2233R	AGTAGAAACAAGGTACCTTTTTGGAC			
12	PB1-1	AGCAAAAGCAGGCAAACCAYTTG	PB1	1511	
	PB1-511R	AAAAAGCTTGTGAATTCAAA			
13	PB1-1118	CGGACACAAATACCAGCAGA		1219	
	PB1-337R	GAAACAAGGCATTTTTTCATGAAGG			
14	PB2-1	AGCAAAAGCAGGTCAAWTATATTCA ATATG	PB2	1566	
	PB2-566R	CCCACATCATTGATGATG			
15	PB2-949	GTGGATATATGCAAGGC		1380	
	PB2-329R	GTCGTTTTTAAACAATTCGAC			
H5-1028	H5-1028F	CGACAGAGCAGGTTGACACAATA	HA	1028	Designed
	H5-1028R	GTACCCATACCAACCATCTACCATTC			

Note: M=A+C ; Y= T+C ; K=G+T ; W= A+T.

Real-time PCR: Real-time PCR is more sensitive and specific than standard PCR because an additional oligonucleotide probe is used for detection of specific PCR product allowing thermocycling conditions to be more flexible. Real-time PCR results are expressed as Ct (Cycle threshold) value, which is defined as the cycle number at which the fluorescent signal generated by hybridization of probe to product passes the threshold value in the early (exponential) phase of amplification [135]. Fluorescent reporter oligonucleotides used in this thesis were TaqMan probes. These probes have a fluorophore and a quencher at either end and normally all fluorescence is quenched. When the probes hybridize to their PCR target templates and are subsequently hydrolyzed by the 5'- exonuclease activity of the DNA polymerase, the distance between quencher and fluorophore becomes sufficient for the fluorophore to emit a detectable signal. During amplification, emitted fluorescence will be detected by a charge coupled device (CCD) and is displayed as fluorophore curves in the real-time PCR diagram.

In this thesis, real-time PCR was used in a two-step format to diagnose influenza A infections and to subtype H5 and N1 subtypes in group II patients (see table 2.1). The sequences of the FluA-specific primer-probe set were kindly provided by Dr Marcel Beld, Department of Medical Microbiology, Academic Medical Centre, Amsterdam, The Netherlands [77]. The sequences of the H5 and N1 primer-probe sets were designed using Primer Express (see section 2.2.3). PCR reactions were conducted in a total volume of 25 µl containing 2.5 µl of 10x Hotstart PCR buffer (Qiagen), 0.4 mM of each dNTP (Roche Diagnostics), 5 mM magnesium chloride, 400 nM of each primer, 40 nM of Taqman probe, and 1 unit of Hotstart Taq (Qiagen). Real-time PCRs were performed using the Chromo 4 (MJ Research) or the iCycler (Bio-Rad) real-time PCR machines. The thermocycling conditions were as follows: 95 °C for 15 minutes (pre-amplification hotstart); 45 cycles of 95 °C (denaturation) for 20

seconds, 50-60 °C (annealing) for 30 seconds, and 72 °C (extension) for 30 seconds. Fluorescent signals were measured in each cycle at the end the extension step. The nucleotide sequence of primers and TaqMan probes as well as their annealing temperature (Ta) are shown in table 2.6.

Table 2.6 Primer and TaqMan probe sequences with annealing temperatures for diagnosis of group II patients.

Primer-probe set	Name	Target gene (position)	Nucleotide sequence (5'-3')	Ta (°C)
FluA-specific	INFA-165s	M (166-190)	GACAAGACCAATCCTGTCACYTCTG	60
	INFA-216r	M (242-258)	AAGCGTCT ACGCTGCAGTCC	
	INFA-probe	M (214-238)	FAM-TTCACGCTCACCGTGCCCAGTGAGC-TAMRA	
H5-specific*	AvH5-F	HA (296-318)	CCGGAATGGTCTTACATAGTGGA	50
	AvH5-R	HA (347-367)	GTCGTTGAAATCCCCTGGGTA	
	AvH5-probe	HA (293-317)	FAM-AGGCCAATCCAGTCAATGACCTCTG-TAMRA	
N1-specific*	AvN1-F	NA (1012-1029)	TGGATCGGGAGAACCAAA	55
	AvN1-R	NA (1052-1071)	TGGATCCCAAATCATTTCAA	
	AvN1-probe	NA (1030-1050)	FAM-CACTAATTCCAGGAGCGGC-TAMRA	

Note: Asterisk (*) indicates oligonucleotide sequences were designed using Primer Express software version 2.0. FAM = 5' – carboxyfluorescein; TAMRA = tetramethyl-6-carboxyrhodamine. Positioning is based on the 1st nucleotide of the starting codon.

2.2.4.2 One step RT-PCR

A one-step real-time RT-PCR format was used to develop a molecular method for generic detection of the HA gene of avian influenza A H5N1 viruses of both clade 1 and 2 (see chapter 6 for more information). The sequences of primers and probes (table 2.7) for this

application were designed using Primer Express (Applied Biosystems). To enhance the thermal stability and specificity, the TaqMan probe was further modified by incorporation of locked nucleic acid (LNA) nucleotides. LNA nucleotides contain common nucleobases (A, T, G, and C) in which the ribose ring is 'blocked' by a methylene bridge connecting the 2'-O atom and the 4'-C atom which forces the probe-target hybrid in a linear rather than the normal helical confirmation of double stranded DNA, thereby significantly increasing base pairing specificity and thermal stability of the bond [http://wwwexiqoncom/lna-technology, accessed on October 12, 2009][136].

The rRT-PCR was performed using iScript™ One-Step RT-PCR Kit Probes (Bio-Rad) in Chromo 4 real time PCR machines (Bio-Rad). The reaction was conducted in a total volume of 25 µl containing 12.5 µl of 2X RT-PCR Reaction Mix (supplied with the iScript Kit), 400 nM of each primer, 120 nM of probe, 0.5 µl of iScript Reverse Transcriptase (supplied with the Kit), and 5 µl of template. rRT-PCR conditions were as follows: one cycle of 50 °C for 15 minutes, followed by 5 minutes at 95 °C, and 45 cycles of 15 seconds at 95 °C and 1 minute at 53 °C.

Table 2.7 Primers and LNA and normal TaqMan probe designed for this study

Name	Sequence[a] (5'-3')	Tm (°C)	Nucleotide[b]
GenH5-F	TTGGTTACCATGCAAACAAYT	61	61-81
GenH5-R	TRTCTTGGGCRTGTGTAACA	61	122-141
GenH5-Probe	FAM-CAGGTTGACACAATAATGGAAAAG-BHQ1	61	91-114
GenH5-LNA Probe*	FAM-CAGGTTGACACAATAATGGAAAAG-BHQ1	66	91-114

Note: [a] Y = T or C, R = A or G. LNA residues in the probe are indicated in bold. 5' - FAM = 5' – carboxyfluorescein, BHQ = Black Hole Quencher. [b] The position in the HA gene is indicated.

2.2.4.3 Quantitative PCR

In real-time PCR with TaqMan probes, the fluorescence emitted after hydrolysis of the fluorescent reporter dye during amplification is proportional to the amount of PCR product and thus allows quantitative assessment of the amount of initial target [135]. This can only be done when a standard curve control consisting of serial dilution of known concentrations of target sequence is included in each run. The control standard can be RT-PCR product, synthetic DNA, genomic DNA or recombinant plasmid DNA [137].

In this thesis, quantitative PCR was employed to determine the viral RNA load in clinical specimens obtained before, during, and after oseltamivir treatment (see chapter 4). The real-time PCR assay for this application was the FluA-specific real-time PCR, described in section 2.2.3.1. Standard curve control used in quantitative analysis was a pCR2.1 plasmid in which FluA-specifc was cloned as described in section 2.2.7. In the preparation for standard controls, white colonies were analyzed by PCR using M13 forward and reverse primers (included in the TOPO TA Cloning kit). PCR products were analyzed by agarose gel (1.5%) electrophoresis and were then purified using the QIAquick PCR purification kit (Qiagen) as described in section 2.2.4.1.b. Presence of the correct insert was confirmed by DNA sequencing using the CEQ8000 sequencing platform (Beckman Coulter). A single bacterial clone containing the insert of interest was selected for propagation in liquid LB medium at 37°C for 16 hours. Cells were harvested by spinning down at 2500 x g for 5 minutes. Constructed plasmid was purified using the QIAprep® Miniprep Kit (Qiagen) following the manufacturer's instructions [http://wwwbiochemarizonaedu/AZ-START/graphics/QIAprep_Miniprep_Handbookpdf, accessed on July 27, 2007]. The plasmid was linearized using restriction enzymes *EcoR*I (New England Biolabs, Ipswich, UK) and DNA concentration was determined by measuring UV absorption at 260 nm using the NanoDrop ND1000 (Thermo Fisher Scientific, Wilmington,

DE, USA). The number of plasmid copies was calculated using an online calculator [http://www.uri.edu/research/gsc/resources/cndna.html, accessed on May 28, 2006]. Finally, the plasmid was diluted in TE to 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 copies per microliter and served as external standard curve control. Quantitation of viral RNA load was performed in batch-wise fashion to avoid intra assay variation [135].

2.2.5 DNA sequencing

2.2.5.1 PCR product purification

PCR product was purified prior to sequencing using the QIAquick® PCR purification kit (Qiagen). The use of this kit was done following the manufacturer's instructions. Briefly, PCR product was added with 100 µl of buffer PB (provided in the kit) and the mixture was transferred to a QIAquick spin column (provided in the kit) and was centrifuged for 1 minute at 16363 x g. The flow-through was discarded and the column was washed with 0.75 ml buffer PE (provided in the kit) and centrifuged for 1 minute at 16363 x g. The flow-through was discarded and residual PE buffer was removed by an additional centrifugation at 16363 x g for 2 minutes. The DNA product was then eluted in 25 µl buffer EB (included in the kit). The purity of DNA product was assessed by 2% agarose gel electrophoresis and the concentration of DNA product was estimated based on band brightness of DNA fragments included in a molecular mass ruler (Bio-Rad). Approximately 10-50 ng of PCR product was used for each sequencing reaction.

2.2.5.2 Whole genome sequencing

Whole genome sequencing of MDCK-cell cultured influenza H5N1 viruses was performed in collaboration with Dr Gavin Smith, Department of Microbiology, State Key Laboratory of

Emerging Infectious Diseases, Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China. PCR products of 15 reactions that cover the entire genome of avian H5N1 influenza virus were purified, assessed for concentration and subjected to dye-termination reactions (to incorporate fluorescent labeled nucleotides). To sequence single PCR fragments, beside 3' and 5' end PCR primers, additional primers to amplify internal regions of the fragment were usually needed. For example, to sequence the H5-1/H5-1120R amplicons, in addition to the PCR primers (H5-1 and H5-1120R) two sequencing primers: H5-370 and H5-540R (table 2.8) were used to allow reliable sequencing of the entire fragment. The nucleotide sequences of additional sequencing primers are shown in table 2.8.

In dye-termination, approximately 20-50 ng of PCR product was used for each reaction containing 4 µl of 2.5x BigDye® Terminator v3.1 Cycle Sequencing premix (Applied Biosystems), 2 µl of v3.1 sequencing buffer (supplied with the kit), 5 pg of single primer and 20-50 ng of PCR product in a total volume of 20 µl. The thermocycling conditions were 96 °C for 1 minute followed by 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes.

Dye-terminated sequences were purified using Centri-Sep™ 96-Well Plates according to the manufacturer's instruction (Applied Biosystems). The use of these plates allows simultaneous purification of 96 samples in approximately 15 minutes. Purified products were analyzed in the 3730x DNA Analyzer (Applied Biosystems) using the standard separation method. DNA sequences were analyzed using Sequencing analysis software v5.3.1 (Applied Biosystems).

Table 2.8 Additional primers used in whole genome sequencing

PCR fragment*	Additional sequencing primer	Primer sequence (5'-3')	Source
1	H5-370	CCC AGG GGA TTT CAA CGA CTA T	Dr Gavin Smith, Department of Microbiology, State Key Laboratory of Emerging Infectious Diseases, Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China.
	H5-540R	GCT CCT CTT TAT TGT TGG G	
3	N1-412R	GTT CTG CAT TCC AAG TGG GA	
	N1-561	AAG TGC TTG TCA TGA TGG CA	
5	NS-540	GAG GAT GTC AAA AAT GCA AT	
	NS-640R	CTT CTC CAA GCG AAT CTC TG	
6	NP-227	TAA CAA TAG AGA GAA TGG T	
	NP493R	GTT GAT CCT TGC ATC AGA G	
8	M-408R	ATA CAA CTG GCA AGT GCA CC	
	M-445	GCC ACT TGT GAG CAG ATT GC	
9	PA-622R	TCA AAT CTT TCT TCA ATT GT	
10	PA-1028	TGG CAG AAC TCC AAG ATA ATT G	
11	PA-1620	GGA GCC ACA CAA GTG GG	
12	PB1-331	TTT GAA AAC TCA TGT CTT GAA AC	
	PB1-435R	CTG GTT TCT ATT CAA TGT CCA G	
	PB1-689	TGA CCC TGA ACA CAA TGA C	
13	PB1-863R	GCC TTC TTC TCA TTC CCT CC	
	PB1-2000R	TTG CAA CAG CAT CAT ATT CC	
14	PB2-298	GGT GTC TCC CCT AGC TGT AA	
	PB2-544R	GAT GTC AAT ATT CTA GCT CCC	
15	PB2-1566R	CCC ACA TCA TTG ATG ATG	
	PB2-1850	CTA CCA TTT GCR GCA GC	

Note: Asterisk (*) indicates PCR fragment numbering is based on table 2.3. R= A+G

2.2.5.3 Partial HA1 sequencing

Sequencing of a portion of the HA1 gene of 'egg-adapted' H5N1 viruses was performed at OUCRU using an 8-capillary electrophoresis sequencer: the CEQ8000 (Beckman Coulter Inc, Fullerton, CA, USA). In addition to PCR primers (H5-1028F and H5-1028R), two primers (H5-514F: ACGCTGCAGACAAAGAATCCAC and H5-570R: TCCCAACGGAAATATAGGTGG) to target the internal region of the PCR fragment were used. Approximately 10–30ng of purified PCR product was used for each sequencing reaction containing 4 µl of CEQ Dye Terminator Cycle Sequencing Quick Start premix (Beckman

Coulter), 10 pg of primer and water up to 20 µl. Thermocycling conditions were 30 cycles of denaturation at 96°C for 20 seconds, annealing at 50°C for 20 seconds and elongation at 60°C for 4 minutes.

Dye-terminated sequences were precipitated in 95% (v/v) cold ethanol/dH_2O (–20°C) in the presence of sodium acetate. Precipitation was performed in 0.5 ml tubes for each sample. After spinning down at 4 °C at 16363 x g for 15 minutes, DNA pellets were rinsed twice with 200 µl of 70% (v/v) ethanol/dH2O (–20°C). The pellet was then vacuum dried in the DNA120 Speed VAC® (Bio-Rad) for 20 minutes and re-suspended in 40 µl of Sample Loading Solution (supplied in the sequencing kit). The sample was then available for analysis in the CEQ8000 sequencer. The CEQ8000 Long Fast Read method was used for all separations. DNA sequences were analysed using the CEQuence Investigator software (Beckman-Coulter).

2.2.5.4 Direct sequencing

Direct sequencing the HA gene (for confirmation of diagnostic test in chapter 3 and gene analysis in chapter 5) and for a portion of the NA gene that covers the H274Y mutation (chapter 4) was performed at OUCRU using the CEQ8000 platform (Beckman Coulter). PCR primers NA1-609F/NA1-811R (table 2.3) were used in these sequencing reactions.

2.2.5.5 Post sequencing analysis

2.2.5.5.a Sequence assembly

Single-stranded DNA sequences generated by different sequencing primers were assembled using the Lasergene software version 6.0 (DNASTAR, Madison, WI, USA) or Vector NTI ContigExpress (Vector NTI 7.1, Invitrogen). This software allows assembly of many small

DNA fragments, supplied as either text files or chromatograms from automated sequencers, into longer contiguous sequences. Assembled sequences were then saved or exported into BLAST (Basic Local Alignment Search Tool, available from http://blast.ncbi.nlm.nih.gov/Blast.cgi), BioEdit software (BioEdit version 7.0.1, Isis, Parmaceutical, Inc, city, USA), or PAUP version 4.0 (Sinauer Associated, Inc, Massachusetts, USA) [138] for sequence comparison, residue analysis and construction of relationship phylogenetic trees.

2.2.5.5.b Multiple sequence alignment and phylogenetic tree analysis

The nucleotide sequences of genes of interest were compared to reference sequences obtained from publicly available sequence databases and/or from reference influenza laboratories (such as Hong Kong University) using BioEdit. The alignment was then edited, if necessary, and was exported as nexus-formatted files into MrModeltest version 2.2 (Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden) [139] for appropriate DNA substitution determination. The generated model was used in construction of Neighbor-joining and maximum-likelihood trees with gaps treated as missing data using PAUP*. Estimates of the phylogenies were calculated by performing 1000 NJ bootstrap replicates.

2.2.6 Pyrosequencing

Pyrosequencing is a real-time DNA sequencing technique based on the kinetics of the polymerase chain reaction. This technique can be used for short sequence (SQA) and single nucleotide polymorphism (SNP) analyses. The technology involves preparation of single stranded DNA template, addition of enzymes and nucleotides, detection of emitted

fluorescence and determination of nucleotide sequences. The chemical process of pyrosequencing involves the release of pyrophosphate (PPi) occurring when a dispensed nucleotide is successfully incorporated into a DNA strand by DNA polymerase during elongation. Through a cascade of enzymatic reactions, PPi is converted into visible light, which is detected with a charge-coupled device camera and quantitatively displayed as a peak in the Pyrogram™ [140]. In addition to SQA and SNP applications, pyrosequencing also allows quantitative analyses of subpopulations present in the sample [140] as the peak height in the Pyrogram is proportional to the ratio of successfully incorporated nucleotides at each position. In this thesis, pyrosequencing was used to assess the presence of wildtype or mutant alleles in influenza virus gene fragments that were amplified by PCR and cloned into a plasmid, that was used to transform *Escherichia coli* bacteria (chapter 4). In addition, pyrosequencing was used to quantitatively analyse for the presence of wild type and mutant H5N1 alleles directly in clinical specimens (chapters 4 and 5).

2.2.6.1 Pyrosequencing primer design

Primers for pyrosequencing were designed using PSQ assay design software (version 1.0.6, Biotage, Uppsala, Sweden). Each primer set consists of 3 primers: a forward and reverse PCR primer (one of which is biotinylated) to amplify the DNA sequence that contains the region of interest, and a sequencing primer that hybridizes close to the position of interest on the (biotinylated) forward or reverse DNA strand. Similar to the design of PCR primers described in section 2.2.3, the reference sequence used in the PSQ software was a consensus nucleotide sequence of the NA and HA gene of avian influenza A H5N1 virus. PCR primers are shown in table 2.3 (primer set number 2, 3, and 4) and sequencing primers are shown in table 2.9.

Table 2.9 Sequencing primers for pyrosequencing analysis.

Primer set*	Pyrosequencing primer	Sequence (5'→3')	Target gene	Application
2	Seq-274F	CGAATTGGATGCTCCTA	NA	Quantitative pyrosequencing analysis of oseltamivir-conferring mutation (H274Y). Qualitative analysis of wild-type (274H) or mutant (274Y) bacterial clones.
3	Seq-HA134F	TCATTAGGGGTGAGCT	HA	Quantitative pyrosequencing analysis of wild-type and mutant variants at position 134 (A134V) in the HA gene.
4	Seq-HA151F	GAAATGTGGTATGGCTT	HA	Quantitative pyrosequencing analysis of wild-type and mutant variants at position 151 (I151F/L) in the HA gene
	Seq-HA186F	TCCTAATGATGCGGC	HA	Quantitative pyrosequencing analysis of wild-type and mutant variants at position 186 (N186D) in the HA gene.

Note: Asterisk (*) indicates numbering is based on table 2.2.

2.2.6.2 PCR and pyrosequencing analysis

The reverse transcription and PCR were performed as described above. PCR product for pyrosequencing was processed according to the manufacturer's instructions (Biotage, Uppsala, Sweden). Briefly, 25µl of each biotinylated PCR product was mixed with 22 µl binding buffer (Biotage) and 3 µl streptavidin-coated sepharose beads (Amersham Biosciences, Uppsala, Sweden). To allow efficient immobilization of the biotinylated DNA product, the mixture was incubated at room temperature in a vortex mixer for 5 minutes. The mixture was then applied to a filtered vacuum to remove unused PCR materials including non-biotinylated PCR primers and dNTPs. Subsequently, the beads were rinsed with 70% v/v ethanol and treated with denaturation solution (Biotage) to remove the non-biotinylated PCR strand. Finally, beads were released directly into a 96 well plate containing 40 µl of annealing buffer (Biotage) and 30 pmoles of sequencing primer. To allow the sequencing primer to anneal to the target DNA,

the plate was heated up to 80 °C for 2 minutes and slowly cooled down to room temperature. The plate was then inserted into the PyroMark^{TM}ID machine (Biotage). Pyrosequencing was performed using Pyro Gold Reagents kits (Biotage). The SNP method was used for all analyses in this thesis. All resulting pyrograms were quantitatively or qualitatively (table 2.9) analyzed using the PyroMark^{TM}ID software (version 1.0.5).

To confirm the quantitative pyrosequencing results of H274Y in clinical specimens (chapter 4), PCR products from direct amplification of the NA gene using the NA1-606F/NA1-811R primer set were cloned into pCR2.1 plasmids as described in the bellow section (section 2.2.7). All white colonies from a single transformation were selected for PCR and subsequent analysis in pyrosequencing. For this application, one of the M13 primers were modified with biotin (table 2.10) and used in combination with the other M13 primer to allow streptavidin capture for pyrosequencing analysis.

Table 2.10 Original M13 and biotin modified M13 primers.

Primer	Sequence (5'-3')	Note
M13F	GTAAAACGACGGCCAG	**Included in the TOPO TA Cloning kit.**
bM13F	Biotin-GTAAAACGACGGCCAG	**Biotin modified at the 5'.**
M13R	CAGGAAACAGCTATGAC	**Included in the TOPO TA Cloning kit.**
bM13R	**Biotin-CAGGAAACAGCTATGAC**	**Biotin modified at the 5'.**

2.2.7 DNA cloning

In this thesis, molecular cloning was performed to generate control standard curves for the quantitative FluA-specific rRT-PCR assay (chapter 4) and the generic one-step rRT-PCR for clade 1 and 2 H5N1 virus detection (chapter 6), and to confirm the results of quantitative H274Y pyrosequencing analysis (chapter 4) using TOPO TA cloning kits (Invitrogen, Carlsbad, CA, USA). TOPO TA cloning kit includes linearized plasmid vector pCR2.1-TOPO, *E.coli* strain TOP 10, plasmid specific primers (forward and reverse M13 primers), pUC18 control plasmids, salt solution, and SOC medium. The TOPO TA cloning kit allows PCR product with an A (deoxyadenosine) overhang at the 3' end (generated by nontemplate-dependent terminal transferase activity of Taq polymerase) to be inserted into a 3'T-overhang in the lacZ operon of a linearized pCR2.1 plasmid, also containing an ampicillin resistance cassette [141]. In the cloning, 1 microliter of purified DNA products was used to be inserted into the pCR2.1-TOPO plasmid. The reaction mixture was then used for chemical transformation of recipient TOP 10 *E.coli* cells. The cells were recovered in SOC medium for 1 hour at 37 °C and were cultured on LB plates containing 50μg/ml ampicillin (to select transformed bacteria) and 40μg/ml X-gal (bromo-chloro-indolyl-galactopyranoside) in dimethylformamide (Invitrogen, Carlsbad, CA, USA). After 16 hour incubation at 37°C, colonies transformed with plasmids containing an insert are easily discriminated from colonies transformed with self-ligated plasmids through their colour: insertion of a fragment causes disruption of lacZ and the bacteria lose the ability to break down X-gal. Because X-gal produces a blue colour when broken down, colonies transformed with self-ligated plasmids turn blue whereas colonies transformed with insert containing plasmids stay white. White colonies were selected for further analysis.

2.2.8 Virus isolation

In this thesis, H5N1 viruses were originally isolated in MDCK (Madin Darby Canine Kidney) cells. Other avian influenza virus subtypes were isolated in embryonated chicken eggs. The isolation was performed in a bio-safety level III laboratory. Before adding of sample to cells or eggs, samples were treated with a cocktail of 7 antibacterial and antifungal agents (table 2.11) in phosphate buffered saline (PBS) pH 7.2 to eliminate growth of all bacteria and fungi present in respiratory, fecal and other non-sterile samples. After addition of these antimicrobial agents, the mixture was vigorously shaken using a vortex mixer, left at room temperature for 30 minutes, and filtered using a 0.45 micro-pore filter (Nalgene Syringe filter, city, USA). The flow through was immediately used for virus isolation.

Table 2.11 Antibiotics and their final concentrations in treated specimens.

No	Antibiotic	Final concentration
1	Penicillin G	2000 U/ml.
2	Streptomycin	200 µg/ml.
3	Polymyxin B	2000 U/ml.
4	Gentamicin	250 µg/ml.
5	Ofloxacin	60 µg/ml.
6	Sulfamethoxazole	200 µg/ml.
7	Nystatin	500 U/ml.

2.2.8.1 Virus isolation in MDCK cells

MDCK cells purchased from the European Collection of Cell Cultures (EACC, Wiltshire, UK) were grown in growth medium (Minimun Essential Medium [MEM]-Eagle with Earle's salts (GIBCO, Invitrogen) supplemented with mycoplasma free fetal bovine serum (Sigma-

Aldrich), HEPES-buffer (GIBCO), MEM Non-Essential Amino Acids (Sigma), L-glutamin (GIBCO) and Penicillin-Streptomycin (GIBCO) in T-25 ml plastic flasks. After 2-3 days of incubation at 37 °C, when the cells reached approximately 80% confluency, growth medium was discarded and the cells were washed twice with MEM-Eagle with Earle's salts 1X (GIBCO). Each T-25 ml plastic flask was then inoculated with 200 µl of sample at 37 °C for 30-60 minutes. Then, all medium was discarded and 5 ml of virus infection medium (MEM-Eagle with Earle's salts (GIBCO) supplemented with MEM Vitamin solution (GIBCO), Folic acid (Sigma), Biotin (Sigma), Bovine Serum Albumin (Sigma) containing 2µg/ml of TPCK-trypsin (New England Biolabs, Ipswich, UK), and containing amphotericin-B (Sigma), L-glutamin, Penicillin-Streptomycin) was added. Cytopathogenic effect (CPE) was visually assessed daily. Cultures were harvested 3 to 7 days after inoculation (CPE≥ 75%).

2.2.8.2 Virus isolation in embryonated chicken eggs

Influenza A viruses can be cultured in the allantoic and amniotic cavities of embryonated chicken eggs [142]. In this thesis, H5N1 and non-H5N1 avian influenza viruses were isolated in the allantois of embryonated chicken eggs (10-11 day old). These eggs were obtained locally. After eggshells were sterilized with iodine, each egg was injected with 200 µl of sample into the allantois and influenza viruses were harvested after two days of incubation at 35°C.

Embryonated chicken eggs were also used in the 'egg-adaptation' experiment to identify possible HA mutations involved in human or avian receptor recognition. In this experiment, MDCK isolated H5N1 viruses were passaged 5 times in eggs (chapter 5).

2.2.8.3 Identification of influenza virus

Avian influenza A H5N1 virus growth in MDCK cells and embryonated chicken eggs was confirmed by the H5-specific (the H5b) and N1 RT-PCR assays (as described in section 2.2.4 and table 2.4).

2.2.8.4 Titration of influenza virus

For determination of analytical sensitivity of the assay described in chapter 6, a representative clade 1 (strain A/Vietnam/CL115/2005(H5N1)) was titrated in MDCK cells. In brief, MDCK cells grown in 6 well-TC plates (Corning Incorporated, Corning, NY14831, USA) were inoculated with serial 10-fold dilutions. After incubation at 37°C for 1 hour, each well was overlayed with 2,5 ml of complete carboxymethyl cellulose liquid overlay medium [carboxymethyl cellulose 3% (GIBCO) in MEM-Eagle with Earle's salts (GIBCO) supplemented with MEM-vitamine (GIBCO), folic acid (Sigma), biotin (Sigma), bovine serum albumin (Sigma), Penicillin-Streptomycin (GIBCO), HEPES (GIBCO), $NaHCO_3$ (Sigma), and TPCK-trypsin (New England Biolabs, Ipswich, UK)] and incubated at 37°C with 5% CO_2 for 2-3 days. Medium was then discarded and the plate was gently washed two times with PBS (Sigma). Subsequently, 1ml cold methanol (Sigma) was added to each well and cell were fixed at -20°C for 30 minutes. After methanol was discarded, the plate was rinsed with tap water. Then 1ml naphtol blue black (Sigma) was added to each well at room temperature for 30 minutes. The plate was then rinsed gently with tap water and was dried at room temperature. The number of plaques was then counted under a magnifier (Bio-Rad). Virus stock at a concentration of 10^4 PFU/µl was then diluted ten-fold in MEM (up to 10^{-2} PFU/µl). From each resulting dilution, viral RNA was extracted and subjected to one-step rRT-PCR as described in section 2.2.4.1.

2.2.8.5 Other influenza virus strains

For sensitivity and specificity analyses of the molecular assay described in chapter 6, in addition to clade 1 human H5N1 viruses isolated from 13/16 enrolled patients, 4 clade 2.3.4 H5N1 viruses isolated from patients admitted to the National Institute of Infectious and Tropical Diseases, Hanoi, Vietnam in 2007, 1 human clade 2.1 H5N1 isolate (A/Indonesia/5/2005(H5N1); kindly provided by Dr Takehiko Saito, The National Institute of Infectious Diseases, Tokyo, Japan, 9 human influenza A viruses of subtype H1N1 (n = 4) and H3N2 (n = 5), isolated from patients with seasonal influenza from Dong Thap Province, Vietnam, in 2006, and 7 avian influenza viruses of subtypes H3 (n = 1), H4 (n = 3), H5 (n = 2), and H6 (n = 1), isolated from poultry in Long An and Vinh Long and Dong Thap Provinces, Vietnam, in 2006 were used. All viruses were cultured in MDCK cells (ECACC, Wiltshire, UK) and were subtyped using subtype-specific RT-PCRs described previously [62, 143].

Chapter 3

HPAI AVIAN INFLUENZA A (H5N1) VIRUS IN HUMANS IN SOUTHERN VIETNAM, 2004-2005

3.1 Introduction

The first recorded instance of human infection with HPAI H5N1 virus occurred in Hong Kong in 1997 during a large-scale poultry outbreak that resulted in 18 infections, 6 of whom were fatal [2]. After that outbreak, further human cases were not identified until February 2003 when avian influenza A H5N1 viruses were confirmed in two Hong Kong residents: a father and his child who had returned shortly after a visit to mainland China. The father succumbed to the infection [6]. Later that year, with increasing and widespread poultry outbreaks, human cases were also reported from Vietnam, Thailand, Cambodia, Indonesia, and Laos. At the time of this writing (February 2011), 519 human cases have been reported from 15 countries in Asia, Eurasia, the Middle East and Africa, with 306 deaths (~60%). Although poultry H5N1 outbreaks have also been reported in Europe, no human infections have been reported from this continent. In Vietnam, since December 2003, 119 confirmed human H5N1 cases have been reported, 59 of them were fatal [5].

Human infection with avian influenza H5N1 virus presents as influenza-like illness with symptoms of fever, shortness of breath and cough. Complications include rapid progression to acute respiratory distress syndrome (ARDS) and multi-organ dysfunction [62, 80]. Factors associated with severity and outcome include age, delayed initiation of treatment, lower respiratory tract damage, leukopenia, thrombocytopenia, hypoalbuminemia, and increased AST and urea nitrogen levels [62, 75, 79, 144]. In this chapter the clinical features of 16 H5N1 infected patients who were admitted to the Hospital for Tropical Diseases (HTD), Paediatric Hospital One (PH1) and Two (PH2) in Ho Chi Minh City, and Dong Thap Provincial Hospital (DTPH), Cao Lanh District, Dong Thap Province, all in southern Vietnam, between January 2004 and January 2005 were retrospectively studied. Until 26 January 2005, the number of patients enrolled in this study accounted for 43% (16/37 cases) of confirmed cases within

Vietnam and 30% (16/54 cases) of total reported cases in the world. [http://www.who.int/csr/disease/avian_influenza/country/cases_table_2005_01_26/en/index.html] [145]. In addition, this chapter also studied virological factors in these patients associated with outcome.

3.2 Results

3.2.1 Identification of H5N1-infected cases

During January 2004 and February 2005, 16 H5N1-infected patients, divided as groups I and II patients (table 3.2), were diagnosed by means of RT-PCR. Diagnosis of influenza A H5N1 infection was performed in the virology laboratory of OUCRU. Group I patients were admitted to studying hospitals in January and February 2004 and were among the first confirmed patients with H5N1 infection in Vietnam. The clinical features of all of these patients (7/7 patients) have been reported previously (appendix 1)[62, 77]. Group II patients were admitted during December 2004 to February 2005 except one patient, Patient 16, who was admitted in February 2004 and was retrospectively confirmed to have H5N1 influenza in stored specimens (appendix 1) [76].

Diagnostic clinical specimens (nose and throat swabs) from group I patients were tested with two H5 specific primer sets (H5a: H5-1/H5-2 and H5b: H5-515/H5-1220) and an N1 specific primer set (N1-1/N1-2). The nucleotide sequences and PCR conditions for these primers are described in chapter 2 (section 2.2.4.1b and table 2.4). The use of these primers for detection of influenza A H5N1 viruses was recommended by WHO at the time of the initial outbreak [62] but had not been rigorously validated for detection in clinical specimens of H5N1 viruses circulating at that time.

After the identification of the above initial 7 patients under outbreak circumstances, there was a demand for more sensitive and specific assays. Therefore, I developed a TaqMan rRT-PCR assays for detection of influenza A H5N1 directly in clinical specimens. The sequences of primers and probes as well as PCR conditions for these assays are described in chapter 2 (section 2.2.4.1b and table 2.6). The FluA-specific assay has been validated and shown to be sensitive (see following sections) and specific for influenza A viruses without cross-reaction with influenza B viruses [146]. The sequences of primers and probes for the H5-specific and N1-specific assays were designed based on consensus sequences of few H5N1 strains available in GenBank at that time (e.g., A/Thailand/3(SP-83)/2004[H5N1], A/Vietnam/1203/2004[H5N1]) using Primer Express software as described in chapter 2. The sensitivity of the H5 and N1 rRT-PCR assays were evaluated by comparing to the FluA-specific assay and the conventional H5b assay. The latter has been shown to be more sensitive than the H5a assay in detection of H5 RNA [62]. Table 3.1 shows that both the H5 and N1 rRT-PCR assays are as sensitive as the FluA-specific assay and 10-time more sensitive than the H5b assay. These assays were tested with RNAs of H3N2 (A/Panama/2007/99), H1N1 (A/NewCaledonia/20/99), and B (B/Singapore/222/79) (kindly provided by Dr Takehiko Saito, National Institute of Infectious Diseases, Tokyo, Japan) and showed that there was no cross-reaction.

Table 3.1: Comparison of the sensitivity of FluA-, H5- and N1-specific rRT-PCRs versus the conventional H5b assay.

Virus RNA	Test	Dilution (10-fold)		
		10^{-5}	10^{-6}	10^{-7}
H5N1 (A/HK/213/97)	FluA-specific rRT-PCR	+	+	-
	H5- specific rRT-PCR	+	+	-
	N1-specific rRT-PCR	+	+	-
	H5b conventional RT-PCR	+	-	-

Note: A plus indicates positive PCR, and a minus indicates negative PCR.

Diagnostic clinical specimens from group II patients were initially tested with the FluA-specific TaqMan rRT-PCR and were subsequently subtyped for influenza H5N1 using the H5 and N1 subtype-specific TaqMan rRT-PCR assays. Further confirmation for the presence of H5N1 viruses in clinical specimens of these 16 patients was performed either by direct HA gene sequencing or virus isolation as described in chapter 2 (sections 2.2.5 and 2.2.8). The final diagnostic results are shown in table 3.2.

Table 3.2 Final diagnostic results

Group	Patient	Conventional RT-PCR (primer sequences are shown in table 2.4 in chapter 2)			Real-time RT-PCR (primer and probe sequences are shown in table 2.6 in chapter 2)			Further confirmation of diagnostic results	
		H5a primer set	H5b primer set	N1 specific	FluA specific	H5 specific	N1 specific	Direct HA gene sequencing	Virus isolation
I	5	+	+	+	*NA*	*NA*	*NA*	+	+
	6	-	+	+	*NA*	*NA*	*NA*	*NA*	+
	7	-	+	+	*NA*	*NA*	*NA*	*NA*	+
	9	+	+	+	*NA*	*NA*	*NA*	+	+
	10	+	+	+	*NA*	*NA*	*NA*	*NA*	-
	11	+	+	+	*NA*	*NA*	*NA*	*NA*	+
	12	+	+	+	*NA*	*NA*	*NA*	+	+
II	1	*NA*	*NA*	*NA*	+	+	+	*NA*	+
	2	*NA*	*NA*	*NA*	+	+	+	*NA*	+
	3	*NA*	*NA*	*NA*	+	+	+	*NA*	+
	4	*NA*	*NA*	*NA*	+	+	+	*NA*	+
	8	*NA*	*NA*	*NA*	+	+	+	*NA*	+
	13	*NA*	*NA*	*NA*	+	+	+	*NA*	+
	14	*NA*	*NA*	*NA*	+	+	+	*NA*	-
	15	*NA*	*NA*	*NA*	+	+	+	*NA*	-
	16	*NA*	*NA*	*NA*	+	-	-	*NA*	+

Note: The table represents diagnostic results of throat swab samples. *NA* denotes not available. A plus (+) indicates a positive result and a minus (-) a negative result.

3.2.2 Demographic information

The sixteen H5N1-infected patients ranged in age from 4 to 35 years with a median of 18 years. Patients 15 and 16 were initially admitted to Dong Thap Provincial Hospital (DTPH) when they both were on the second day of illness and were then transferred to PH1. These patients came from 10 provinces of sounthern Vietnam (table 3.3 and appendix 2). Eight of the sixteen patients were pre-school or attending school children and the rest were young adults (table 3.3).

Table 3.3 Baseline information of patients with confirmed H5N1 infection

Patient	Residential location in Vietnam	Age	Sex	Hospital of admission	Occupation
1	Dong Thap	13	Female	DTPH/PH1	Student
2	Dong Thap	35	Female	HTD	Housewife
3	Tay Ninh	16	Female	HTD	Worker
4	Tien Giang	18	Female	HTD	Seller
5	Ho Chi Minh City	8	Female	PH2	Student
6	Lam Dong	23	Male	HTD	Framer
7	Binh Dinh	24	Male	HTD	Student
8	Vinh Long	26	Female	HTD	Housewife
9	Ho Chi Minh City	13	Male	PH2	Student
10	Soc Trang	16	Female	HTD	Student
11	Lam Dong	18	Male	HTD	Farmer
12	Lam Dong	20	Male	HTD	Farmer
13	Tra Vinh	10	Male	HTD	Student
14	Tra Vinh	35	Female	HTD	Farmer
15	An Giang	25	Female	HTD	Housewife
16	Dong Thap	4	Male	DTPH/PH1	Pre-school boy

3.2.3 Epidemiologic information

The sixteen patients in this study were identified in a period of 5 months in 2 years: January, February and December 2004 (9 patients), and January and February 2005 (7 patients). Of the 16 patients, a history of exposure to poultry or H5N1-confirmed patients was obtained from 14 patients through questionnaires completed by patients or relatives. As shown in table 3.4, Patients 1, and 2 had no direct contact with poultry and the source of exposure in Patient 16 was less clear. For the 11 of 14 patients with a history of exposure, the median time between exposure and onset of illness was 3 days (range: 2 to 4) and the median time between the onset of illness and hospitalization was 6 days (range: 2 to 8).

Patient 2 was the mother of Patient 1. The mother was admitted to HTD 6 days after onset of illness and died on the following day. On the day of the mother's hospitalization, the daughter (Patient 1) also developed symptoms of influenza. Two days later, she was admitted to DTPH and transferred to PH1. Detailed data on contact between mother and daughter were not available. However, shortly before the onset of illness (2 days) in both patients, chickens had died around their home. H5N1 influenza viruses were successfully isolated from throat swab specimens of both mother and daughter, but sequence analyses showed differences in HA between both isolates (see figure 5.1 in chapter 5).

Patient 16 was a four-year old boy with symptoms fitting the case definition of viral encephalitis who was enrolled in a study of viral encephalitis study at PH1 (see following sections). Influenza H5N1 infection in this patient was confirmed by RT-PCR and virus isolation in stored specimens about two months after hospitalization. Similar illness was reported in his sister. The boy became sick on February 10 2004 and was admitted to DTPH on February 12, and transferred to PH1 on February 15. He died on February 17. His sister became sick on 27 January 2004 and was admitted to DTPH on February 1, and died on

February 2 2004. Since there was no clinical suspicion of influenza, no diagnostic specimens were collected. Before their hospitalization, both patients were cared for by their parents who had no other children. The family lived in a one-room house. The sister swam regularly in a canal near their home, and that canal was the source of washing and drinking water for the family. During the previous weeks many dead chickens, discarded by their owners during ongoing poultry outbreaks, had reportedly been floating in the same canal. In addition, backyard poultry had been present in and outside the family's house until February 2004, at which time all were culled as part of routine measures to contain the nationwide outbreak of H5N1 influenza in poultry [76, 147].

Table 3.4 Epidemiologic data of the 16 patients

Patient No.	Admission date	Days since onset of illness	Days between exposure and onset of illness	History of exposure
1	22 Jan 2005	2	2	Many chicken and ducks died around her home. H5N1 influenza in her mother (Patient 2) was confirmed two days before her hospitalization.
2	20 Jan 2005	6	2	Many chicken and ducks died around her home.
3	26 Dec 2004	7	3	Direct handling of sick chickens.
4	5 Jan 2005	6	2	Direct handling of sick chickens.
5	16 Jan 2004	8	3	Direct handling of a sick duck.
6	4 Feb 2004	7	3	Direct handling of sick ducks and chickens.
7	6 Feb 2004	6	Unknown	No history of exposure.
8	18 Jan 2005	4	3	Direct handling of sick chickens.
9	21 Jan 2004	6	2	Frequently attended cockfights, held rooster and chickens. Walked through a live-poultry market 50 meters from house on his way to school.
10	26 Jan 2004	5	3	Direct handling of dead or dying chickens.
11	30 Jan 2004	6	4	Direct handling of 50 chickens including dead chickens.
12	2 Feb 2004	5	3	Direct handling of chickens.
13	4 Jan 2005	4	Unknown	No history of exposure
14	11 Jan 2005	6	3	Direct handling of sick chickens.
15	17 Jan 2005	7	3	Direct handling of sick chicken.
16	12 Feb 2004	2	Unknown	His sister died of undiagnosed disease few days before. Many chickens and ducks in his house and in a canal nearby from which water was used for washing and drinking.

3.2.4 Clinical symptoms at presentation

The clinical features of 16 H5N1-confirmed patients are summarized in table 3.5. At presentation, all patients had fever (median 39.1; range, 37.5 to 40.3). Statistical analysis revealed that the body temperature of patients who died was not different from those who survived (Mann-Witney U test, P=0.18). Fifteen patients presented with symptoms of respiratory infection including cough and rapid respiratory rate (median, 40 breaths per minute; range, 24 to 60) and there was not different in respiratory rate between patients who died and those who survived (Mann-Whitney U test, P=0.85). Patient 16 did not have any symptoms of respiratory infection at presentation, instead he had symptoms of gastrointestinal infection including vomiting and severe diarrhea. No patient had symptoms of conjunctivitis.

Table 3.5 Clinical characteristics of the 16 patients on admission

Patient	Cough	Dyspnea	Sputum	Diarrhea	Myalgia	Temper-ature (°C)	Blood pressure	Respiratory rate (breaths/minute)	Crackles	Wheeze	Time (day) to initiation of treatment (since onset of illness)	Out come
1	+	+	+	+	+	38.5	104/64	40	+	-	2	Died
2	+	+	-	+	-	39.6	110/70	40	+	+	6	Died
3	+	+	+	+	+	40	110/60	40	+	-	7	Died
4	+	+	+*	+	-	40	100/60	60	+	-	6	Died
5	+	+	+	+	-	39.5	110/60	50	+	-	12	Recovered
6	+	+	+*	+	+	38.7	120/80	28	+	-	7	Recovered
7	+	+	+	+	-	39.7	110/80	46	+	+	6	Recovered
8	+	+	+	+	-	39.7	110/60	60	+	-	4	Recovered
9	+	+	+	+	-	38.6	90/60	60	+	-	6	Died
11	+	+	+*	+	+	37.5	100/60	48	-	-	5	Died
12	+	+	+	+	-	38	110/80	26	+	-	6	Died
13	+	+	+*	+	-	39	120/60	40	+	-	5	Died
13	+	+	-	+	-	40	100/60	24	-	-	5	Died
14	+	+	+	+	+	38.5	60/40	48	+	-	6	Died
15	+	+	+	-	-	40.3	106/60	36	+	-	7	Recovered
16	-	-	-	+	-	38	80/30[a]	-	-	-	Not treated	Died

Note: * denotes the sputum was blood stained. [a] blood pressure was measured on the fifth day of illness

3.2.5 Basic laboratory values at presentation

Hematology and biochemical tests for the patients were performed on admission; results are shown in table 3.6. Almost all patients presented with leucopenia, most marked in the lymphocyte subset: the median total leukocyte count on admission was 2,495 per cubic millimeter (range: 567 to 7,300, normal range: 4,000 to 11,000). However, no statistical difference in the total leukocyte count between fatal and surviving patients (Mann-Whitney U test, P=0.74). The median lymphocyte count in 14 patients was 525 per cubic millimeter (range: 129 to 1,020, normal range: 1500 to 4000) and was not different between patients who died and those who survived (Mann-Whitney U test, P=1). In six of these patients, the value of CD4 and CD8 cell counts was available and the median ratio of CD4-positive cells to CD8-positive cells was 0.68 (range: 0.59 to 1.08, normal range 1.6 to 2.0). The median of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) of nine patients was 72.5 Unit per liter (range: 39 to 1,020, normal range <37) and 105.5 Unit per liter (range: 20 to 1058, normal range <40), respectively (table 3.6).

Table 3.6 Laboratory value at presentation*

Patient/ Variable	Leukocyte count (per mm3)	Lymphocyte count (per mm3)	Neutrophil count (per mm3)	Platelet count (per mm3)	CD4: CD8 ratio	ALT level (U/liter)	AST level (U/liter)	Serum creatinine (μmol/liter)	Serum glucose (mmol/liter)	Oxygen saturation during receipt of 40% oxygen (%)	Out come
1	4800	*NA*	*NA*	183000	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	Died
2	567	129	400	137000	*NA*	467	534	105	*NA*	77	Died
3	2040	587	1047	105000	*NA*	*NA*	*NA*	106	*NA*	51	Died
4	4820	882	3800	98500	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	Died
5	1200	300	700	117000	0.71	354	320	34	*NA*	95	Survived
6	2100	700	1300	62000	1.08	89	110	121	4.9	90	Survived
7	2630	550	1980	78400	1.05	42	58	83	*NA*	83	Survived
8	2430	427	1885	85300	*NA*	39	21	89	*NA*	*NA*	Survived
9	2700	900	1300	81000	*NA*	254	1058	14	*NA*	85	Died
10	3000	500	2500	70000	0.62	47	20	71	19	67	Died
11	1700	500	1100	69000	0.75	*NA*	*NA*	89	13.5	81	Died
12	1900	800	1100	62000	0.59	*NA*	*NA*	43	11.7	80	Died
13	2330	386	1760	82400	*NA*	*NA*	*NA*	81	*NA*	89	Died
14	2560	490	1900	55300	*NA*	56	70	85	*NA*	79	Died
15	4070	1020	2730	200000	*NA*	45	101	68	*NA*	*NA*	Survived
16	7300	*NA*	6060	314000	*NA*	1020	400	*NA*	*NA*	*NA*	Died
Median	2495	525	1670	83850	0.68	72.5	105.5	83	*NA*	81	

* Normal ranges are as follows: leukocyte count, 4000 to 11000 per cubic milliliter; neutrophil count, 2200 to 8250 per cubic milliliter; lymphocyte count, 1500 to 4000 per cubic milliliter; CD4:CD8 ratio, 1.6 to 2.0; platelet count, 150000 to 400000 per cubic milliliter; ALT, below 37U per liter; AST, below 40U per liter; serum creatinine concentration, 82 to 106 μmol per litter; and serum glucose concentration, 3.9 to 6.4 mmol per liter. *NA* denotes not available.

3.2.6 Virological findings

3.2.6.1 Validation of quantitative FluA rRT-PCR assay

The FluA rRT-PCR assay (primer-probe sequences and PCR condition are shown in table 2.6 and section 2.2.4.3 in chapter 2). However, prior to use, the assay was validated for sensitivity and quantitative precision.

3.2.6.1.a Analytical sensitivity and linearity

The analytical sensitivity of the FluA rRT-PCR was determined using serial 10-fold dilutions of quantified ssDNA plasmids (10^6 copies to 1 copy). The preparation of control plasmids is described in chapter 2 (section 2.2.4.3). The assay was shown to be extremely sensitive, consistently detecting 10 copies of ssDNA plasmid per reaction (100%; 10/10 replicates). The assay also could detect 1 copy of ssDNA plasmid per reaction (figure 3.1A). However, the detection rate at this level was 60% (6/10 replicate tests). The linearity of reactions using quantified plasmids had a correlation coefficienct of $R^2 = 0.992$ (figure 3.1B).

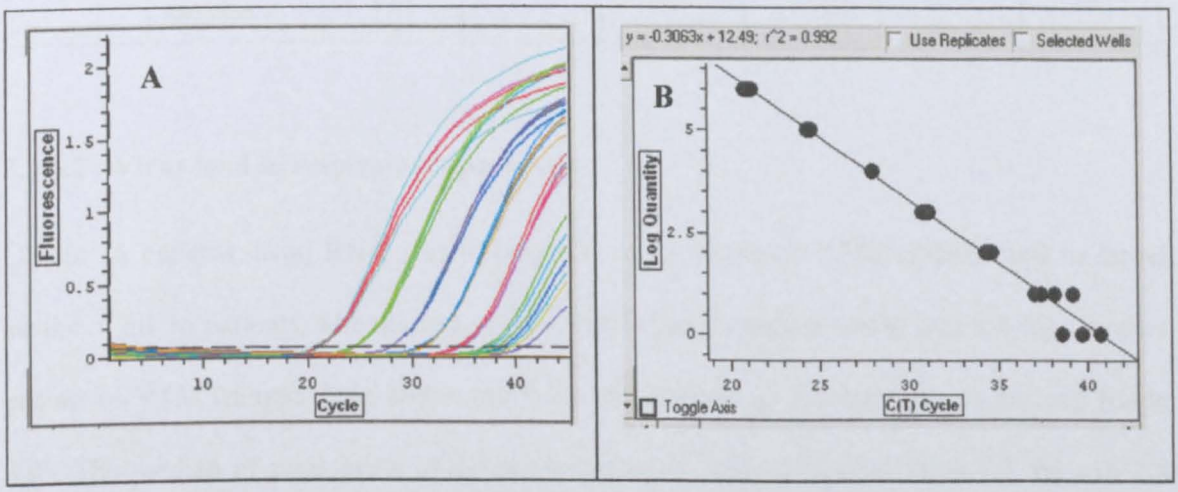

Figure 3.1 Analysis of the analytical sensitivity (A) and linearity (B) of the FluA-specific assay. The test was carried out in quintuplicate using ssDNA plasmid at concentration 10^6 copies to 1 copy (from left to right) per reaction.

3.2.6.1.b Range of quantitation and precision

To determine the reproducibility of the assay, 10-fold dilutions of quantified plasmid (10^6 - 10 copies/reaction) were examined in 5 consecutive days (inter-assay variation) with quintuplicates of each dilution (intra-assay variation). The results showed that the coefficient of variation (CV) of both inter- and intra- assay analyses was less than 1% and 3%, respectively (table 3.7).

Table 3.7 Inter-assay variation of the quantitative FluA assay

Copies per reaction		Threshold cycle (Ct)					
		10^6	10^5	10^4	10^3	10^2	10
Inter-assay	Mean (± SD)	21.62 (0.54)	24.95 (0.24)	28.32 (0.23)	31.67 (0.31)	35.32 (0.26)	38.60 (0.97)
	Coefficient of variation	2.5	0.96	0.81	0.98	0.74	2.5
Intra-assay	Mean (± SD)	20.97 (0.17)	24.43 (0.1)	27.97 (0.04)	30.99 (0.15)	34.5 (0.18)	37.81 (0.18)
	Coefficient of variation	0.81	0.41	0.14	0.48	0.52	0.48

3.2.6.2 Virus load in respiratory specimens

Of the 16 patients, viral RNA was detected in nasal swabs of 12/16 patients and in throat swabs of all 16 patients. The median of viral RNA loads in nasal swabs was 5.4 $\log_{10}$ copies per ml of VTM (ranged from below the level of detection to 7.4 $\log_{10}$ copies per ml) (table 3.9). The median of viral loads in throat swabs was 6.2 $\log_{10}$ (ranged from 4.3 $\log_{10}$ to 7.8 $\log_{10}$). Throat viral load was higher in patients who died compared to those who survived (7.3 log10 vs 6.0 log10) but statistical significance has not been established (Mann-Whitney U test, P=0.221) (figure 3.2 and table 3.8). When plotting the viral loads in throat against the time

after onset of illness, no clear decline of viral loads in throat was observed (Pearman rho correlation R = -308; P = 0.246) and viral RNA was still detectable at high levels up to 8 days after the onset of illness (figure 3.3)

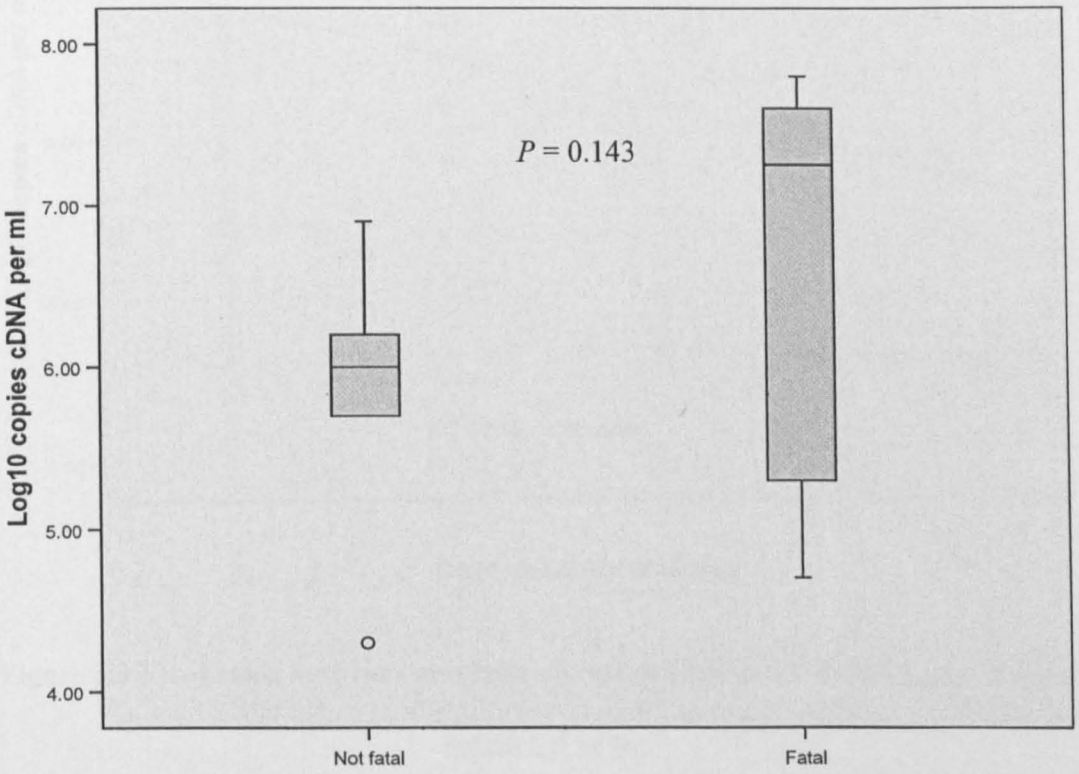

Figure 3.2 Viral loads in throat of patients with fatal and non-fatal outcome. Median, quartiles, range and P value are shown.

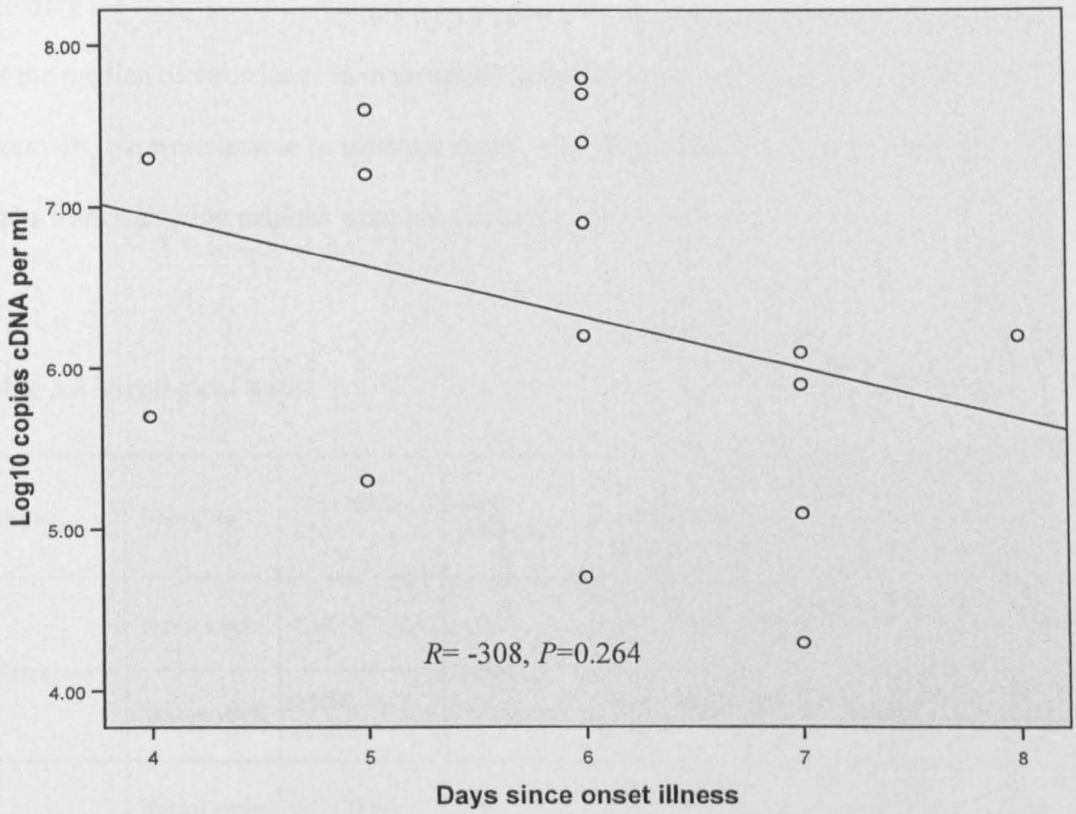

Figure 3.3 Viral loads in throat in relation to the time of onset illness. Circle indicates individual value.

3.2.6.3 Virus detection in non-respiratory specimens

Viral RNA was detectable in blood of 9/11 patients who died but not in the blood of those who survived (0/5). The difference in detection rates in blood between fatal and surviving cases was statistically significant (Fisher Exact test, P=0.005). The median of viral loads in blood was 4.5 $\log_{10}$ per ml of serum. H5N1 virus was successfully isolated from blood of one patient. In the same patient (Patient 16) viral RNA was detectable and virus was isolated from CSF and a rectal swab (table 3.8).

Including the latter patient, viral RNA was detectable in rectal swabs of 5/7 patients who died and the median of virus loads in these swabs was 4.8 log_{10} copies per ml of VTM. Beside from patient 16, we were unable to isolate viruses from remaining rectal swabs (table 3.8). Rectals swabs from surviving patients were not available.

Table 3.8 Virological data

Organ	Specimen	Detectable RNA	Virus isolation	Viral load			
				Median	Fatal	Not fatal	*P* value
Respiratory	Nasal swab	12/16 (76%)	13/16	5.5 (und.-7.6)	5.8 (und.-7.6)	4.7 (und.-5.6)	0.214
	Throat swab	16/16 (100%)		6.2 (4.5-7.8)	7.3 (4.7-7.8)	6.0 (4.3-7.0)	0.147
Non-respiratory	Rectal swab	5/7 (71%)	1/7	4.8 (3.6-5.8_	*NA*	*NA*	*NA*
	Blood	9/16 (56%)	1/6 (17%)	4.5 (3.2-5.7)	4.5 (3.2-5.7)	und.	*NA*
	CSF	1/1	1/1	*NA*	*NA*	*NA*	*NA*

Note: und indicates under detection limit

3.2.7 Treatment and outcome

All 16 patients were treated empirically with broad spectrum antibiotics (ceftriaxone, gentamicin, amikacin) on admission. Fifteen patients were treated with the neuraminidase inhibitor oseltamivir at a dosage of 2mg/kg/day or 75mg twice daily. The median time since onset of illness to antiviral therapy was 6 days (range, 2 to 12 days). However, no statistical difference in illnessday at treatment between fatal and surviving patients was observed (Mann-Whitney U test, P=0.17). Antiviral treatment was initiated immediately up on admission in 14 out of 15 patients (table 3.9). Treatment for patient 5 was delayed for 4 days since admission

because this was the first patient with H5N1 disease identified in southern Vietnam and it took this period of time from clinical suspicion to a laboratory diagnosis of this new suddenly emerging pathogen. Treatment was continued for up to five days in 10 patients, five of whom survived. The median time of hospital stay for the five surviving patients was 25 days (range, 8 to 34 days).

Patients 5, 10, and 11 received 1 to 2 mg of methylprednisolone per kilogram four times a day for one, three and four days, respectively.

Patients 1, 5, and 7 required continuous positive airway pressure with supplemental oxygen. Patients 2, 3, 9, 10, 11, 12, 13, and 14 required mechanical ventilation within a short time (48 hours) after hospitalization.

Despite antiviral treatment and the implementation of intensive supportive health care, 11 patients died in this series, resulting in a case fatality rate of 69%. The median time to death from onset of illness was 10.5 days (range, 5 to 21 days).

Patient 16 was the only patient who was not treated with neuraminidase inhibitor oseltamivir because he had no typical symptoms of H5N1 influenza. This patient was identified during analysis of samples from patients enrolled in study on viral encephalitis in PH1 [76]. On presentation in DTPH, the patient had fever, headache and severe diarrhea that resulted in clinical diagnosis of gastrointestinal bacterial infection.

The patient was treated with intravenous (IV) fluids, paracetamole, ceftriaxone, and gentamicin. As his clinical condition worsened after 3 days of treatment (increasing of diarrhea and drowsiness), he was transferred to PH1. On admission, physical examination showed tachypnea (36 breaths per minute), bilateral crackles and wheezes, and a Glasgow Coma Score (GCS) of 12. The patient was treated with IV fluids, and antibiotic therapy was changed to ceftazidime and amikacin. Approximately 12 hours after admission, he had

generalized convulsions and became comatose (GCS 7). A clinical diagnosis of viral encephalitis was made and treatment was extended with phenobarbital and mannitol. The patient died two days after.

Table 3. 9 Treatment, duration of hospital stay and outcome

Patient	Days since onset of illness	Oseltamivir treatment start since onset of illness	Duration of treatment (days)	Duration of hospital stay (days)	Outcome
1*	2	2	5	7	Died
2	6	6	1	1	Died
3	7	7	5	12	Died
4	6	6	5	15	Died
5	8	12	5	27	Recovered
6	7	7	5	25	Recovered
7	6	6	5	34	Recovered
8	4	4	5	10	Recovered
9	6	6	3	11	Died
10	5	5	5	8	Died
11	6	6	3	3	Died
12	5	5	1	1	Died
13	4	5	1	1	Died
14	6	6	5	6	Died
15	7	7	5	8	Recovered
16#	2	Not treated	0	3	Died

Note: * Diagnostic specimens were obtained on day 5 of illness. # Diagnostic specimens were obtained on day 7 of illness.

3.3 Discussion

Since the re-emergence of influenza virus A H5N1 among birds in 2003, the virus has reached endemic levels in Vietnam, continues to cause poultry outbreaks, and is associated with sporadic human infections [4, 59]. At the time of this writing (February 2011), 119 infections have been reported from Vietnam, 59 of which were fatal [5]. 2005 was the year with the highest number of confirmed cases (61 cases) followed by 2004 (29 cases) [5]. The total number of cases in Vietnam in these two years was 90 accounting for 76% of the total reported cases from Vietnam [5]. In this thesis, the clinical data of human H5N1 disease are presented from 16 cases. Most human H5N1 infections were detected in provinces of northern Vietnam [http://wwwmohgovvn/homebyt/vn/upload/info/attach/1132044424781_KemtheoBaocao9073 doc, accessed on March 19, 2009], where we have limited access to samples and data. Despite that, the clinical features in most of these cases appear to be characteristic of most cases of human disease caused by recently emerged H5N1 virus.

For the current series, the prominent clinical features on admission were those of a severe influenza syndrome with fever, cough, diarrhea, and shortness of breath. The incubation period from exposure to poultry and the onset of illness was two to four days. The most striking laboratory findings were thrombocytopenia, increased levels serum transaminases, and especially marked lymphopenia with a pronounced inversion of the CD4:CD8 ratio in the six patients in whom it could be measured. The patients were all children or young adults (4 – 35 year-old). These clinical features at presentation (of most patients) were similar to those in the concurrent outbreaks of human H5N1 disease in Thailand, Cambodia and other countries from which data are available [75, 79, 80, 123, 148].

Patient 16 was the only patient who had a clinical course that significantly differed from the rest of the patients in the current series. On presentation, while other patients had a clinical syndrome of severe influenza-like illness, this patient had clinical symptoms that led to an initial clinical diagnosis of infectious diarrhoea. A similar patient with clinical symptoms of fever and severe diarrhea alone on presentation has also been identified in Thailand in 2004 [93]. However, the most striking clinical feature of this patient was the progression of illness from severe diarrhea to convulsions and coma within a few days after hospitalization. Although clinically apparent pneumonia developed during a late stage of illness (the last day of his life), respiratory disease was not considered as the most relevant clinical problem of this patient. Instead, acute viral encephalitis was considered as the most likely diagnosis. Acute encephalitis is a rare complication of influenza infection and has particularly been reported in Japanese patients. Successful isolation of virus from CSF specimens is extremely rare [149]. However, in this patient, H5N1 virus was isolated from blood and CSF samples suggesting the virus may have caused acute encephalitis. This notion was supported by evidence for the presence of virus particles in neuron cells of an H5N1 infected patient [94]. Studies in ferrets and mice have shown that H5N1 virus has a capability to cause systemic infection and encephalitis [150-152]. However, it remains unclear how H5N1 virus reaches the central nervous system: haematogenously through crossing of the blood–brain barrier or continuously by spread from peripheral nerve endings [150, 151, 153].

The mortality in our case series was similar to the mortality rate of laboratory confirmed cases reported to WHO [5] but significantly higher than that of a case-study in 29 patients identified in the same period of time in northern Vietnam [144]. However, the reasons for this difference are unclear.

Despite the relatively late presentation in the course of illness, viruses could be isolated from respiratory specimens of most H5N1 patients. Compared to nasal specimens, viral RNA levels in throat swabs were much higher. Highest viral loads in throat occurred in patients who died suggesting that the level of viral replication influences outcome. Viral RNA was detectable in blood of most patients who died but not in those who survived suggesting that, in addition to high virus loads in the respiratory tract, hematogenous spread is also associated with poor outcome. Beside the implications for pathogenesis and transmission, these observations indicate that throat swabs should be used as diagnostic specimens for H5N1 infection. In the mean time this has been widely accepted, in part based on our observations [124].

Although feacal material potentially contains PCR inhibitors, viral RNA could be detected in rectal specimens of the majority of patients (5/7). This confirms earlier reports of viral RNA detection in gastrointestinal specimens [76, 96]. However, H5N1 virus was only isolated from rectal swab of one patient [76]. This may be due to limitations of culture sensitivity or loss of replication-competent virus during long-term storage of the swabs. In addition, the limited success in isolation from rectal swabs could also be due to the presence of non-specific inhibitors in feacal materials that inhibit viral replication during cultivation [154].

The detection of viral RNA in serum of more than half of the subjects, including 4 of 5 patients with H5N1 RNA-positive rectal swabs, suggests that gastrointestinal infection may be a result of viral dissemination from the respiratory tract. However, data from this study also suggest that initial gastrointestinal infection could have occurred at least in one patient who presented with diarrhoea and in whom H5N1 virus was isolated from the rectum. Observational studies have suggested that gastrointestinal infection could occur following oral ingestion of infected food or virus particles [80] and this has been confirmed in studies using animal models [153].

The timing of hospitalization of these 16 patients coincided with concurrent outbreak waves of avian H5N1 influenza virus among poultry in Vietnam [64]. Most of the patients in the current series had close contact with poultry during the week before the onset of illness. The contact in ten patients of the current case series involved holding, killing, or culling sick or dead chickens or ducks for consumption within the patient's home environment or small homesteads nearby, where a relatively small number of chickens were kept. A case-control study of 28 patients who had H5N1 influenza infection during 2004 in Vietnam showed that preparing sick or dead poultry for consumption is the major risk factor of H5N1 infection [155]. As in other studies reported so far [79], none of the patients in this case series were involved in mass culling of poultry or worked on large poultry farms suggesting that people who have daily closed contact with poultry may have some form of pre-existing immunity to the virus. However, this could not be established in a sero-survey among cullers and farmers [156].

All the patients of the current series were children and young adults. Previous demographical analyses of 169 cases have revealed that incidence and fatality are highest in people at these ages. In contrast, people at the age of 40 or older seem less likely to be infected by H5N1 viruses [157]. This age distribution contrasts with seasonal influenza that mostly affects elderly persons [158]. This skewed nature of age distribution of H5N1 infection may be explained by differences in exposure or cross-protection of pre-existing immunity acquired from natural infection or vaccination with heterotypic influenza vaccine in older persons [159, 160].

Two possible family clusters were identified in this study. The first cluster consisted of a mother and her child. When considering the timing of their illnesses, mother-to-child transmission could have occurred. However, genetic analysis of the HA gene sequences of

isolated viruses revealed that they are not indentical (>1% divergence; 15 nucleotide difference in the HA1) and clustered differently in a phylogenetic tree (figure 5.1 in chapter 5) suggesting that mother-to-child transmission was very unlikely. This is because HA gene sequence identity is the only convincing evidence for person-to-person transmission [86, 87]. Nevertheless, in this family cluster, the possibility of mother-to-child transmission can not be fully excluded because genetic changes may have occurred during infection and/or culture [chapter 5]. The second cluster consisted of a brother-sister pair, however clinical samples from the sister (sister of Patient 16) were not available therefore phylogenetic relationship analyses could not be performed. Nevertheless, the interval between their illnesses (9 days) makes the possibility of sister-to-brother transmission unlikely. The source of their infection remained unclear, but could have occurred from ingestion of water from the canal near their home that might have had high virus concentrations due to healthy infected ducks or dead chickens. No illness was reported in family members of the other fourteen patients, even though they seemed to have had very similar exposure to poultry. Similar observations have been reported from a surveillance study in Cambodia [82]. These findings suggest that a range of other factors (e.g., viral virulence and host genetics) may be involved in efficient bird-to-human (and human-to-human) transmission.

Oseltamivir was administered to fifteen of the patients, ten of whom died. This high fatality rate may be partially due to the delay of hospitalization resulting in late initiation of antiviral therapy which is believed to be a major factor affecting outcome [79]. Although one patient survived even when treatment was delayed until day 12, and another patient died despite initiation of treatment when maximum clinical benefits could be expected (within two days after onset of illness), the effect of the use of and early initiation of oseltamivir in H5N1 infection has been suggested, but in uncontrolled observations [79].

Amantadine and rimantadine (adamantanes) were not given to any of these patients because molecular and in vitro susceptibility analyses revealed a high rate of resistant viruses [29, 161]. Indeed, genetic analysis of the M2 gene of H5N1 virus strains isolated from 13 patients in the current series showed that they all had mutations conferring amantadine and rimantadine resistance in the M2 gene [chapter 5].

In vitro studies have shown that cells infected with H5N1 produce a broad range of proinflammatory cytokines and chemokines, including IFNα, IL6, IL8, IL10, IP10, RANTES, and MCP1 [111, 162], and do this to a greater extent than when infected by seasonal human influenza viruses. Previously, we have found that, in accordance with in vitro findings, patients with H5N1 infection have high plasma levels of those cytokines and chemokines, which correlate with viral loads in throat and with poor outcome. These observations suggest that, besides high levels of viral replication, the ensuing inflammatory response contributes to the pathogenesis of H5N1 disease [36].

While there is a theoretical benefit of immunomodulatory treatment, the challenge may be to balance the beneficial effects of immunomodulation and the potential hazardous effects of interfering with the immune response when there is ongoing viral replication.

Corticosteroids were given in three of our patients, one of whom died. The number of patients treated with corticosteroids in this current series was too small to allow the assessment of any effect. However, experience from a retrospective case study of 29 patients in northern Vietnam showed that methylprednisolone has no effect on outcome [144]. Recently, retrospective analysis from clinical data from 67 patients in Vietnam revealed that, in contrast to expectations that steroids would be helpful for modulating the intense inflammatory responses to the infection and improve outcome [36], the use of corticosteroids was a risk factor for fatal outcome [75]. Nevertheless, data in these studies could be biased by non-

random selection of more severe cases for treatment with steroids. For this reason, we believe that controlled clinical studies to assess the role of corticosteroids or other immunomodulatory strategies remain important and needed.

Although the results of diagnostic tests using conventional RT-PCR with the H5b and N1 primer pair were reliable, after the first wave of human outbreaks [73, 163] [http://nursingcribcom/wp-content/uploads/image/flu%20virusPNG, accessed on July 27, 2010], diagnostic testing was switched to rRT-PCR because of concerns about sensitivity, time to results and reliability of the assay. Although the infection in 2 of 9 patients diagnosed by rRT-PCR was not confirmed by virus isolation or direct sequencing of the HA gene, the test results are not doubted because, in addition to the fact that these patients had typical radiographic and clinical features of severe H5N1 disease: 1) real-time diagnostic assays using TaqMan probes are general excellent in sensitivity and specificity and do not require post-amplification analysis limiting the chances of contamination [135, 164], 2) three different genes (M, HA and NA) were amplified, 3) the risk of contamination has been minimized by physical separation of lab rooms for preparation of reagents, extraction of nucleic acids, and amplification and analysis, respectively, combined with a strict unidirectional work flow [77].

In conclusion, data from this study show that typical symptoms at presentation of this small case series were fever, cough, and shortness of breath. Nevertheless, H5N1 infected patients may have atypical symptoms at presentation and unpredicted manifestation during hospital stay. Such atypical symptoms of H5N1 infection could be a limiting factor for establishment of early suspicion, diagnostics and treatment as well as timely outbreak control.

Chapter 4

OSELTAMIVIR TREATMENT: VIRAL LOAD AND THE EMERGENCE OF RESISTANCE

4.1 Introduction

NA inhibitors are the most important class of drugs for the treatment of influenza. These drugs exert their effect through inhibition of the viral NA. Currently available NA inhibitors are oseltamivir (Tamiflu; Roche) and zanamivir (Relenza; GlaxoSmithKline). They bind to the active site of the NA enzyme, and thereby interfere with hydrolysis of host cell receptors bound to the HA of newly formed viruses budding out of infected cells and thus prevent the release of progeny viruses and a new cycle of infection (figure 4.1) [125, 165]. Zanamivir has a poor oral bioavailability, and is therefore administered directly via inhalation, which limits its usefulness in systemic illness, in elderly and very young patients (<7 years old), in comatose patients and in people with asthma or chronic obstructive pulmonary disease [http://wwwfdagov/cder/news/relenza/defaulthtm, accessed on 28 August 2009]. In contrast, oseltamivir is administrated orally which favors the use of this drug in treatment and prophylaxis [166].

Standard dosage of oseltamivir for treatment of influenza in adults and adolescents (≥13 years) is 75 mg twice daily for 5 days, and weight-adjusted twice daily dosages (~2mg per kg body weight) for five days in children ≥ 1 year old [http://wwwrocheusacom/products/tamiflu/pipdf, accessed on January 9, 2009]. Treatment with oseltamivir reduces the duration and severity of acute uncomplicated influenza in otherwise healthy children and adults [167, 168], but clinical benefit in uncomplicated disease is optimal when treatment is started within 48 hours of onset of illness [169].

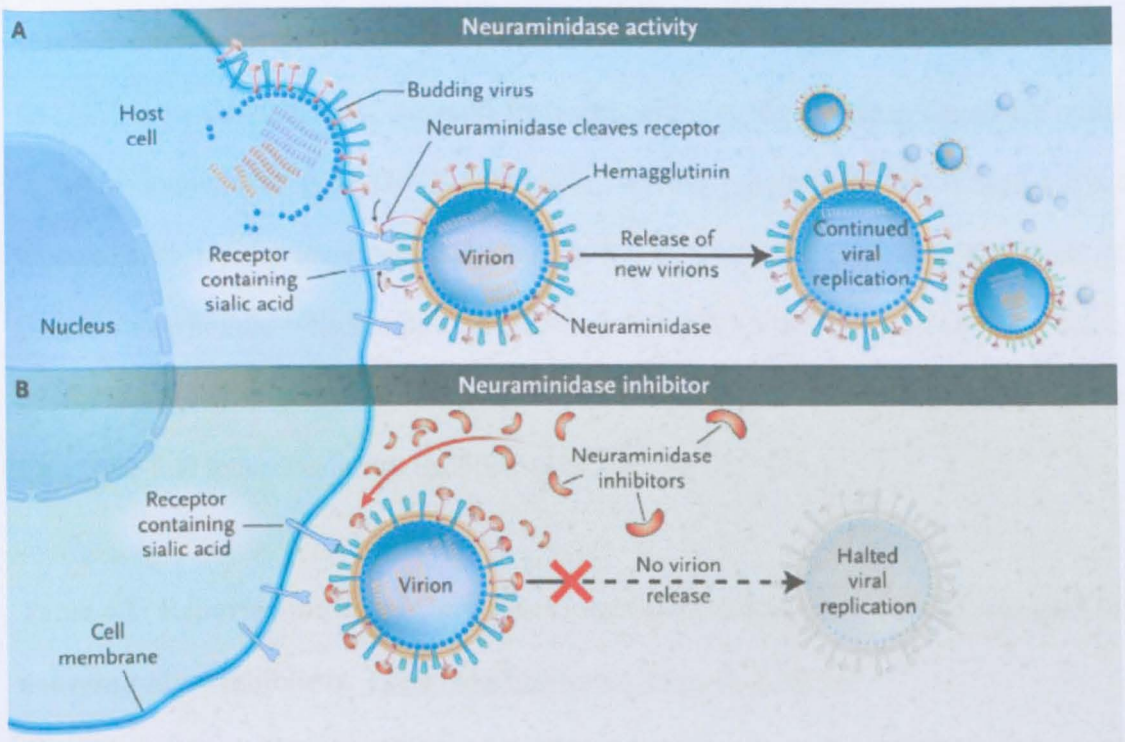

Figure 4.1 Mechanism of action of neuraminidase inhibitors.

Neuraminidase catalyses the cleavage of host cell receptors important for release of progeny influenza virions (panel A). The release of progeny virions is blocked by neuraminidase inhibitors (Panel B) [figure reproduced from Moscona A [125]].

Unlike adamantane drugs, resistance to neuraminidase inhibitors has occurred infrequently in clinical trials. Estimated resistance rates vary from 4% to 8% in outpatient children [168, 170] and 0.4% to 1% in adults [171] after one course of treatment. However, recent treatment studies in Japanese children showed oseltamivir-resistance conferring mutations in the NA gene in 16.7% (7/43) of H1N1 and 18% (9/50) of H3N2 virus strains isolated from specimens obtained after therapy [172, 173]. These high resistance rates possibly reflect primary infections associated with high replication rates combined with possible underdosing at that time due to different (non-weight-based) dosing schedules in Japan at that time. In late 2007, surveillance for antiviral susceptibility of influenza viruses in European countries revealed that

approximately 14% of circulating H1N1 were oseltamivir resistant possessing a His274Tyr (H274Y) mutation (table 4.1), the most frequently encountered resistance associated mutation, in the neuraminidase gene [174]. Since then, resistant seasonal H1N1 viruses have been detected with rapidly increasing frequency in Europe and other regions in the world [175], [http://wwwwhoint/csr/disease/influenza/h1n1_table/en/indexhtml, accessed on March 25 2010]. In vitro studies have shown that H1N1 viruses with the H274Y mutation exhibit more than 1000-fold reduction in susceptibility to oseltamivir [175].

Table 4.1: Reported mutations in the neuraminidase conferring reduced susceptibility to neuraminidase inhibitors. Table modified from Crusat et al., [176].

Influenza subtype	Amino acid substitution	Reduced susceptibility to
N1	H274Y	Oseltamivir Peramivir
N1	N294S	Oseltamivir
N1	G248+I226V	Oseltamivir Zanamivir
N1	Y155H	Oseltamivir Zanamivir
N2	R292K	Zanamivir Oseltamivir Peramivir
N2	E119V	Oseltamivir
N2	E119G	Zanamivir
N2	E119A	Zanamivir
N2	E119N	Zanamivir
N2	E41G	Oseltamivir
N2	Q226H	Oseltamivir

Oseltamivir is the recommended treatment of avian H5N1 influenza virus infection in humans [http://wwwwhoint/csr/disease/avian_influenza/guidelines/ClinicalManagement07pdf, accessed on January 9, 2009] and is believed to improve outcome [79]. However, as with seasonal and pandemic H1N1 viruses [177], H274Y oseltamivir resistant variants may emerge during treatment.

While monitoring H5N1 viral load during oseltamivir treatment, I identified two patients in whom oseltamivir-resistant variants with an H274Y mutation (N2 numbering) in the NA gene emerged [77]. H5N1 viruses with such a mutation have been shown to have an approximate 1000 fold increase of IC50 values for oseltamivir [128].

This chapter describes the identification of mutant viruses in these patients, treatment and disease progress in one patient, and the kinetics of mutant virus replication in the other. I also discuss possible causes of resistance emergence and possible treatment options to improve outcome.

4.2 Results

4.2.1 Patients and clinical samples

Patients included in our virus load monitoring study were patients with 1) confirmed H5N1 infection, 2) who received at least one standard dose (75 mg) of oseltamivir, and 3) from whom at least one nose or throat sample in 2 ml of VTM was obtained during treatment. Following these criteria, 8 out of 16 H5N1-infected patients (see chapter 3) were selected for this study. These 8 patients are Patients 1 to 8 shown in chapter 3. Baseline information and available clinical samples from these patients are shown in table 4.2.

Table 4.2 Patients and clinical specimens.

Patient	Day of illness	Throat swab (days since admission)$	Outcome
1	2	3 and 8	Died on day 8th day if illness
2	6	1 and 2	Died on day 7 of illness
3	7	1, 2, and 6	Died on day 19th of illness
4	6	1, 3, 4, 6, and 9	Died on day 21th of illness
5	12	1, 2, and 3	Survived
6	7	1, 2, 3, 5, and 7	Survived
7	6	1, 2, 3, 4, and 6	Survived
8	4	1, 3, 5, 10, and 11	Survived

Note: $ day of admission was denoted as day 1. See table 3.9 in chapter 3 for more information.

4.2.2 Treatment and outcome

All patients were treated with the recommended dosages of oseltamivir [177], and the drug was given orally or through nasogastric tubes in case patients were intubated. Patient 1 was a 13 year old girl who presented to Dong Thap provincial hospital (DTPH), Dong Thap province, on January 22, 2005 with a one-day history of fever and cough. The day before, her mother - Patient 2 - died of influenza A (H5N1) infection, one day after start of oseltamivir therapy. Therefore, H5N1 infection was immediately suspected at presentation of patient 1 and she was given an initial 75-mg dose of oseltamivir and transferred to PH1 in Ho Chi Minh

City, where she was given another dose of oseltamivir 6 hours later. Treatment with standard dose of oseltamivir twice daily was continued.

Patients 2-8 presented to our hospitals relatively late in the course of their illness. Patient 2 was the mother of Patient 1. She was admitted to our hospital on day 6 of illness. She was treated with a standard dose of oseltamivir and died one day after admission. Patients 3 and 4 presented to our hospital on day 6 and 7 of illness, respectively. They completed a full course of 5 days of oseltamivir, but both died (on day 19 and 21 of illness, respectively). Patients 5-8 presented at our hospital on day 8, 7, 6 and 4 of illness, respectively. Although oseltamivir therapy was initiated relatively late, they all recovered (see chapter 3 for further details).

4.2.3 Viral RNA load during treatment

Viral load in throat swab specimens was measured using quantitative FluA rRT-PCR as described in chapter 2 (section 2.2.4.3) and validations of this assay for quantitative analyses are shown in chapter 3 (section 3.2.6.1). Viral load data shown in this chapter were derived from a single run. As shown in table 4.3, the influenza A (H5N1) viral RNA load in the admission throat swab specimen of Patient 4 was the highest among all current samples at admission. The mean viral RNA load on admission was 6.9 $\log_{10}$, ranging from 5.7 to 7.7 $\log_{10}$.

Viral RNAs in throat swab samples of Patients 5, 6, and 7 were undetectable with quantitative rRT-PCR analyses after two days of treatment. After 5 days of treatment, viral RNA was still detectable in samples of Patients 3 and 4. Strikingly, RNA of H5N1 virus was still detectable in samples obtained on the day 2nd and 3rd after completion of oseltamivir treatment from Patients 1 and 4, respectively (table 4.3).

Table 4.3 Influenza A (H5N1) RNA loads in throat specimens and results of virus culture and direct sequencing

Patient	Pharyngeal influenza A (H5N1) virus load (copies/ml)										
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	**Day 11**
1	*NA*	*NA*	*NA*	*NA*	5.4*$	*NA*	*NA*	6.7*$	*NA*	*NA*	*NA*
2	5.9*$	7.6$	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
3	6.0*	6.2	*NA*	*NA*	4.5	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
4	7.7$	NA	5.1$	5.9	*NA*	5.2	*NA*	*NA*	5.9*$	*NA*	*NA*
5	5.7*	5.3	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
6	6.7*	5.7	0	*NA*	0	*NA*	0	*NA*	*NA*	*NA*	*NA*
7	5.7*	4.6	0	0	*NA*	0	*NA*	*NA*	*NA*	*NA*	*NA*
8	**6.1***	*NA*	**4.7**	*NA*	**3.6**	*NA*	*NA*	*NA*	*NA*	**0**	**0**

Note: Viral load is presented as $\log_{10}$ copies per ml of VTM. Quantitative rRT-PCR analyses were performed in batch-wise fashion. NA indicates that a sample was not available. 0 indicates negative RT-PCR result. Brown indicates oseltamivir treatment. * indicates successful isolation of H5N1 viruses from these specimens. $ indicates direct sequencing was performed.

Viral RNA loads shown in table 4.3 were used to plot against the timing of sample collection and oseltamivir treatment, and are displayed in figure 4.2.

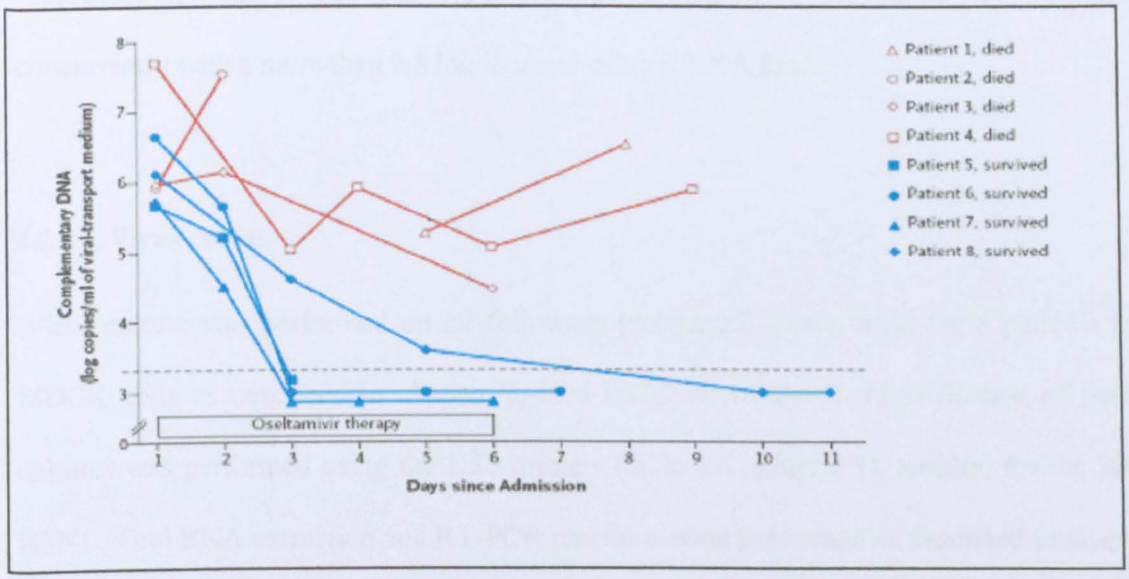

Figure 4.2 Avian influenza A (H5N1) in throat swabs of 8 patients. Blue lines represent patients who survived from influenza A (H5N1) infection. Red lines represent patients who died. The dashed-horizontal line denotes the limit of detection of the assay. Figure was modified from de Jong MD, Thanh TT et al., [77].

Figure 4.3 shows viral loads in throat swabs rapidly declined to undetectable levels after two days with oseltamivir therapy in 3 surviving patients (Patients 5, 6, and 7). In the fourth surviving patient (Patient 8), viral load remained detectable at low level at day 4 of oseltamivir treatment.

Viral RNA loads remained detectable at high levels on the last day of oseltamivir treatment in three of the four patients who died (Patients 1, 3 and 4) (figure 4.2). After completion of treatment, increasing viral RNA loads were detected using quantitative realtime RT-PCR in respiratory samples from patients 1 and 4. Patient 2 died during the second day of admission concurrently with a more than 1.5 log increase of viral RNA loads.

4.2.4 Virus culture

Virus culture was performed on all follow-up pharyngeal swabs from the 8 patients using MDCK cells as described in chapter 2, in a BSL3 environment. Identification of positive cultures was performed using the H5b primers (table 2.4, chapter 2), specific for the HA of H5N1. Viral RNA extraction and RT-PCR reactions were performed as described in chapter 2 (section 2.2). An example of H5 specific RT-PCR results is shown in figure 4.3.

From 28 clinical throat swab samples available from the eight patients (table 4.2), nine influenza A H5N1 virus isolates were cultured. Six isolates were cultured from admission throat swab samples of Patients 2, 3, 5, 6, 7, and 8. Three isolates were cultured from throat swab samples collected during and 2 days after completion of oseltamivir treatment of Patient 1, and from the sample collected three days after the completion of oseltamivir treatment of Patient 4.

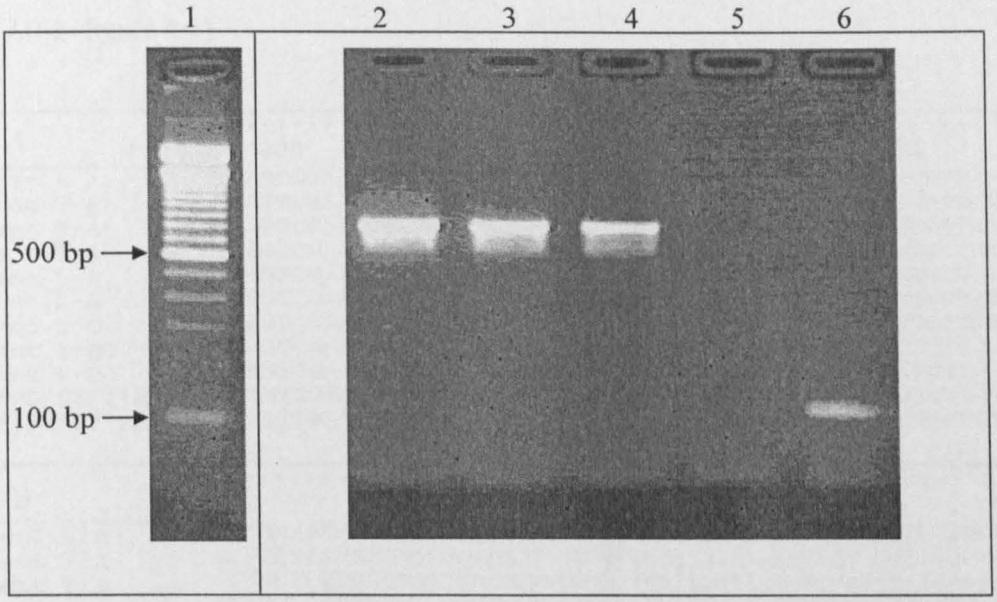

Figure 4.3 Identification of cultured H5N1 viruses using H5 specific RT-PCR. Lane 1 is a 100bp ladder. Lanes 2, 3, and 4 are amplicons indicative of the presence of H5N1 virus, with an expected size of 708 bp, from culture supernatant of follow-up specimens of Patients 1 and 4. Lanes 5 and 6 are mock extraction control and negative reaction control, respectively. The PCR band (~100bp) present in the negative control lane likely represents primer-dimer formation .

4.2.5 NA gene sequences of isolated viruses

Sequencing of the NA gene of the 9 H5N1 virus isolates was performed at the Department of Microbiology, Faculty of Medicine, University of Hong Kong, Hong Kong SAR, China, and at the Virology Laboratory of the OUCRU as described in chapter 2.

DNA sequencing reactions were performed using capillary sequencing in an ABI Prism 3700XL sequencer as described in chapter 2. The sequencing results generated by 6 individual sequencing primers were assembled and edited using Lasergene software version 6.0. The

sequences were then aligned and compared to reference sequences using BioEdit software version 7.0.1 (figure 4.4).

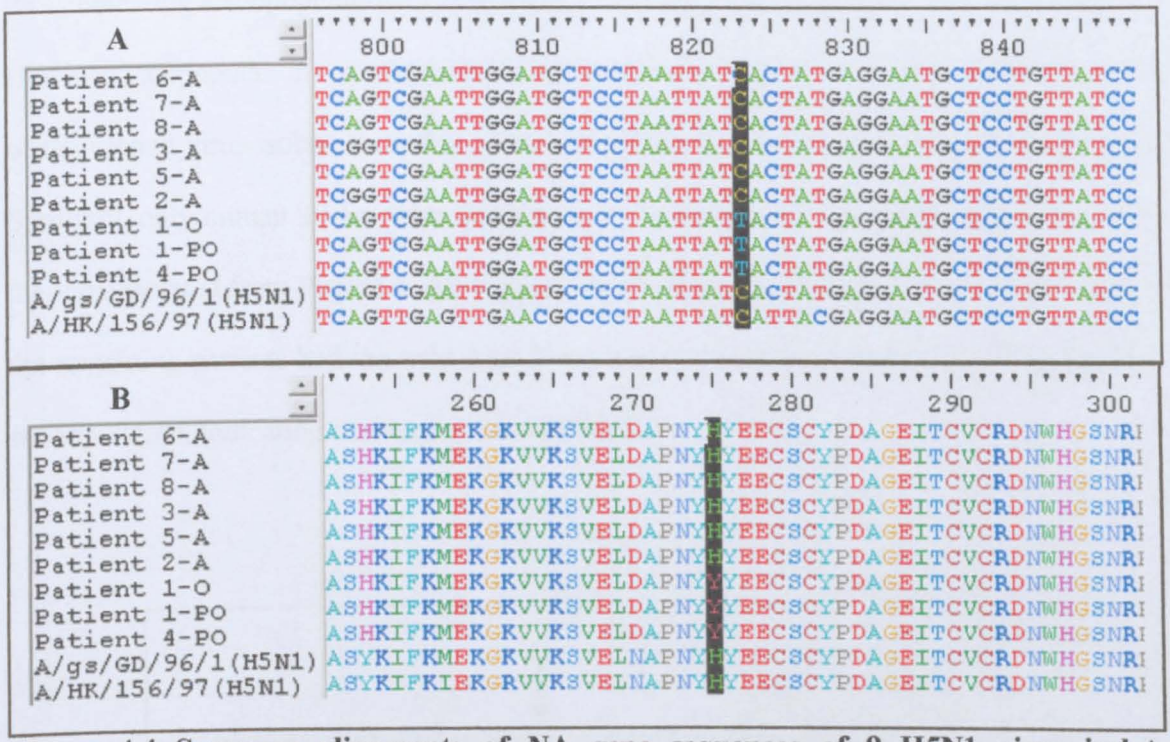

Figure 4.4 Sequence alignments of NA gene sequences of 9 H5N1 virus isolates. A/GS/GD/1/96(H5N1) and A/HK/156/97(H5N1) are reference NA sequences. The use of reference sequences allows positioning of the residue of interest. Suffixes denote viruses were isolated from samples collected at admission (A), during (O) and post (PO) oseltamivir therapy. Residues in black highlight the nucleotide (panel A) and amino acid (panel B) of interest.

Sequencing results show that the three viruses isolated from Patients 1 and 4 have a C to T substitution at position 823, resulting in an amino acid change from histidine (H) to tyrosine (Y) at position 274 of NA, analogous to the earlier reported mutation conferring oseltamivir resistance (figure 4.4).

I further analyzed the sequencing chromatogram and found that the first virus isolate (isolated from the sample collected on day 4 of treatment) from Patient 1 had a double peak at position 283, indicating a subpopulation of wild-type 274H variants among 274Y mutants (figure 4.5). However, subpopulations of wild-type variants were not observed in the raw sequencing traces of the second virus isolate from this patient (isolated from the sample collected 3 days later). Similarly, only mutant alleles were detected when analyzing the sequencing chromatogram of the virus isolated from Patient 4. All 6 viruses isolated from admission throat swab samples of the remaining patients had the wild-type 274H residue, without the presence of mutant 274Y subpopulation, indicating oseltamivir susceptibility.

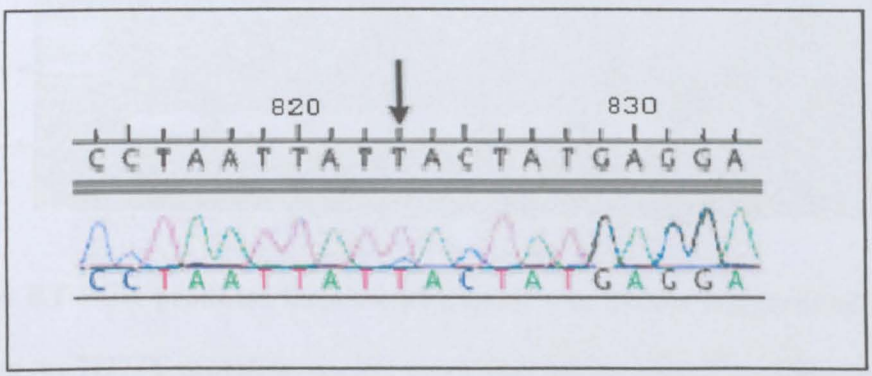

Figure 4.5 Sequencing traces of the NA gene of H5N1 virus isolated from Patient 1 on the fourth day of treatment. The arrow indicates the predominant T peak, with a smaller C peak at nucleotide position 823. Histidine (H) and tyrosine (Y) are encoded by AAC and TAC, respectively. A C→T mutation at this position results in the substitution of tyrosine (Y) for histidine (H) at amino acid 274.

4.2.6 Direct sequencing

To exclude the possibility of resistant variants emerging or disappearing during virus isolation, I performed direct sequencing in clinical samples collected at admission, during and after oseltamivir treatment from Patients 1, 2 and 4. Primers for RT-PCR amplification of a portion of the NA gene that covers the nucleotide of interest (chapter 2, table 2.3) were designed using Primer Express software version 2.0 and PCR reactions were performed as described in chapter 2 (section 2.2.4.1b). The results of RT-PCR amplification are shown in figure 4.6.

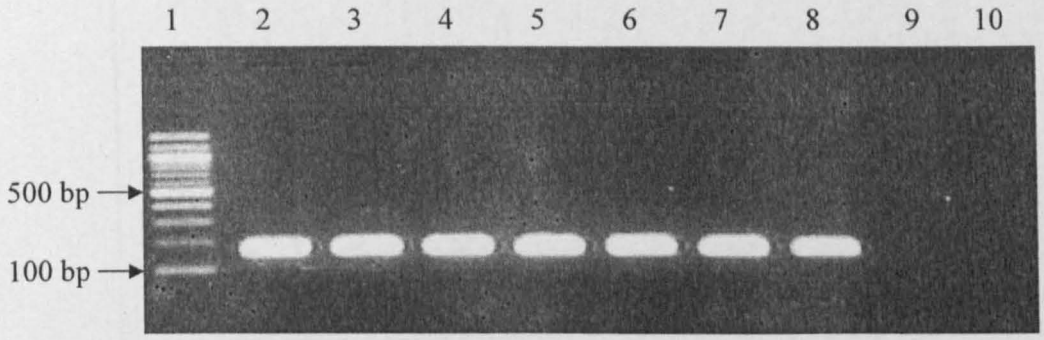

Figure 4.6 RT-PCR products from amplification of a 203bp fragment of the NA gene that covers the H274Y mutation. Lane 1 is a 100bp ladder. Lanes 2 to 8 are amplification of 7 clinical samples obtained from Patients 1 (2 samples), 2 (2 samples) and 4 (3 samples). Lanes 9 and 10 are mock extraction control and negative reaction control.

Sequencing reactions were performed using capillary sequencing in the CEQ800 sequencer as described in chapter 2. Chromatograms were checked for the presence of subpopulations.

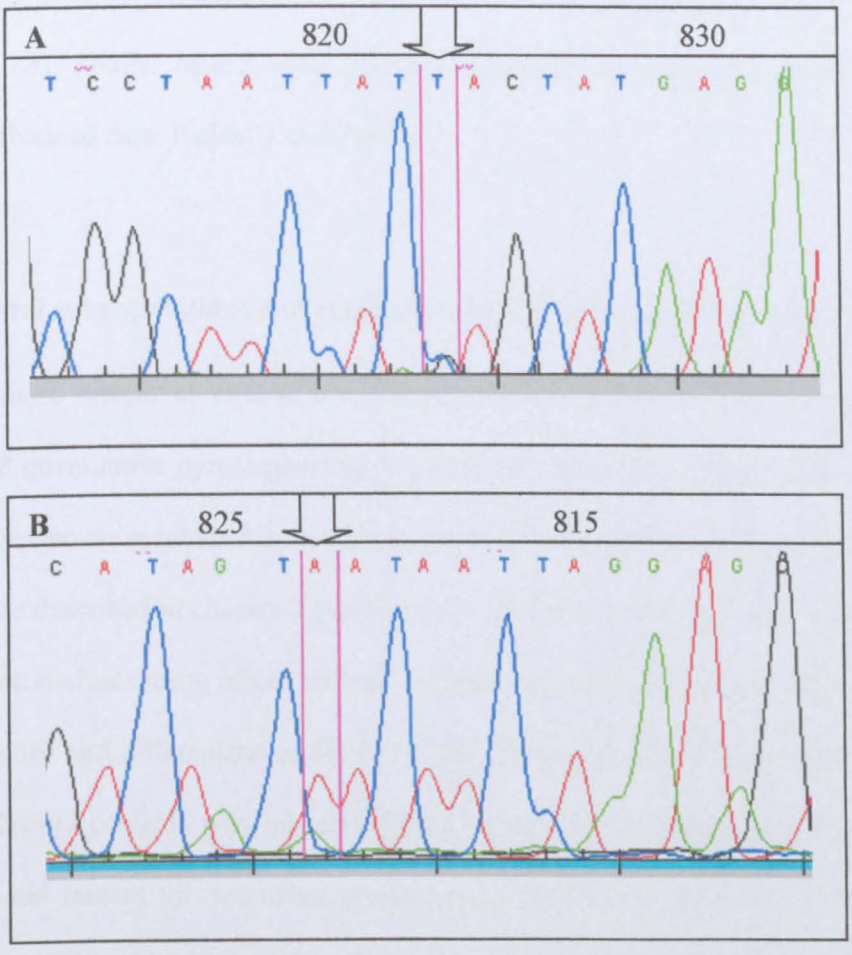

Figure 4.7 Sequencing traces of the NA gene of H5N1 virus in clinical specimen obtained on the fourth day of oseltamivir therapy from Patient 1. Arrows indicate a mixture of wild-type and mutant variants in the forward (Panel A) and reverse (Panel B) sequence). Histidine (H) and Tyrosine (Y) are encoded by GAG and ATG (from left to right) in the reverse sequence, respectively.

Sequencing analyses of diagnostic samples of Patients 2 and 4, and a sample taken after 1 day of oseltamivir treatment of Patient 2, and a sample taken after 3 days of oseltamivir treatment of Patient 4 revealed wild-type 274H viruses only. Analysis of the chromatogram of the forward strand from the first sample of Patient 1 revealed a double peak of equal height corresponding to a T and a C at nucleotide position 823 (figure 4.7A). In the reverse sequence

showed the peak corresponding to a C at position 823 (wild-type) was lower than the T peak (figure 4.7B). Similar results were obtained when analyzing the raw sequences of the last samples obtained from Patient 1 and Patient 4.

4.2.7 Viral subpopulations and replication kinetics of mutant viruses in vivo

To gain more insight in viral subpopulations and replication kinetics of mutant viruses, I performed quantitative pyrosequencing analysis for clinical specimens. PCR and sequencing primers are shown in tables 2.3 and 2.9, respectively, and pyrosequencing assays using these primers are described in chapter 2 (section 2.2.6.2). Prior to use, the assay had been validated in triplicate analyses using mixed mutant - wildtype plasmids at different concentrations (10 to 10^6 copies/μl) and different ratios (0, 5, 10, 20, 40, 60, 80, 90, 95 and 100%). Wildtype and mutant plasmid controls were generated by cloning of PCR products from amplification of wildtype and mutant viruses using primers NA1-609F/811R (table 2.3 in chapter 2) were cloned into qCR2.1 plasmids as described in chapter 2 (section 2.2.7). Based on the validation result shown in figure 4.8, quantitative pyrosequencing data with mutant variants less than 10% were considered to be unreliable. Quantitative data generated by pyrosequencing were further verified using cloning of PCR products (of the NA1-609F/811R primer set) followed by SNP pyrosequencing, as described in chapter 2 (section 2.2.6). Examples of pyrosequencing analysis are shown in figure 4.9, and data of viral quasispecies generated by pyrosequencing and cloning followed by sequencing are shown in table 4.4.

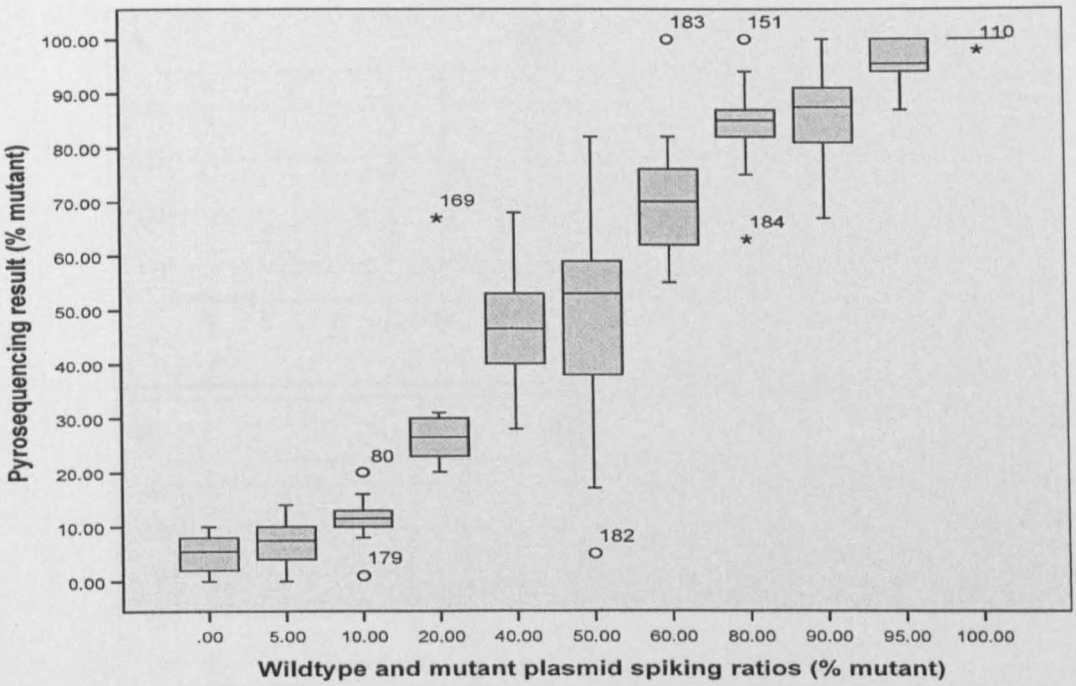

Figure 4.8 Pyrosequencing results of plasmid spiking experiments. Eighteen replicates were performed for each spiking ratio. Median, quartiles and range are shown. Spiking ratios and pyrosequencing are well correlated (Spearson, $P < 0.001$).

Table 4.4 274Y mutant variants in clinical specimens of Patient 1, 2, and 4

Patient	Sample (date of collection since admission)	274Y mutant (%) in pyrosequencing * (STDV)	274Y mutant (%) in cloning followed by sequencing (analyzed clones)
1	8	69.5(±12.6)	71.4 (10/14)
2	1	<10	ND
	2	<10	0 (0/156)
4	1	<10	ND
	4	<10	0 (0/120)
	6	83.4 (±2.3)	80 (4/5)
	9	89.6 (±4.5)	ND

Note: (*) The displayed value is the mean of 3 independent analyses. ND indicates verification was not performed.

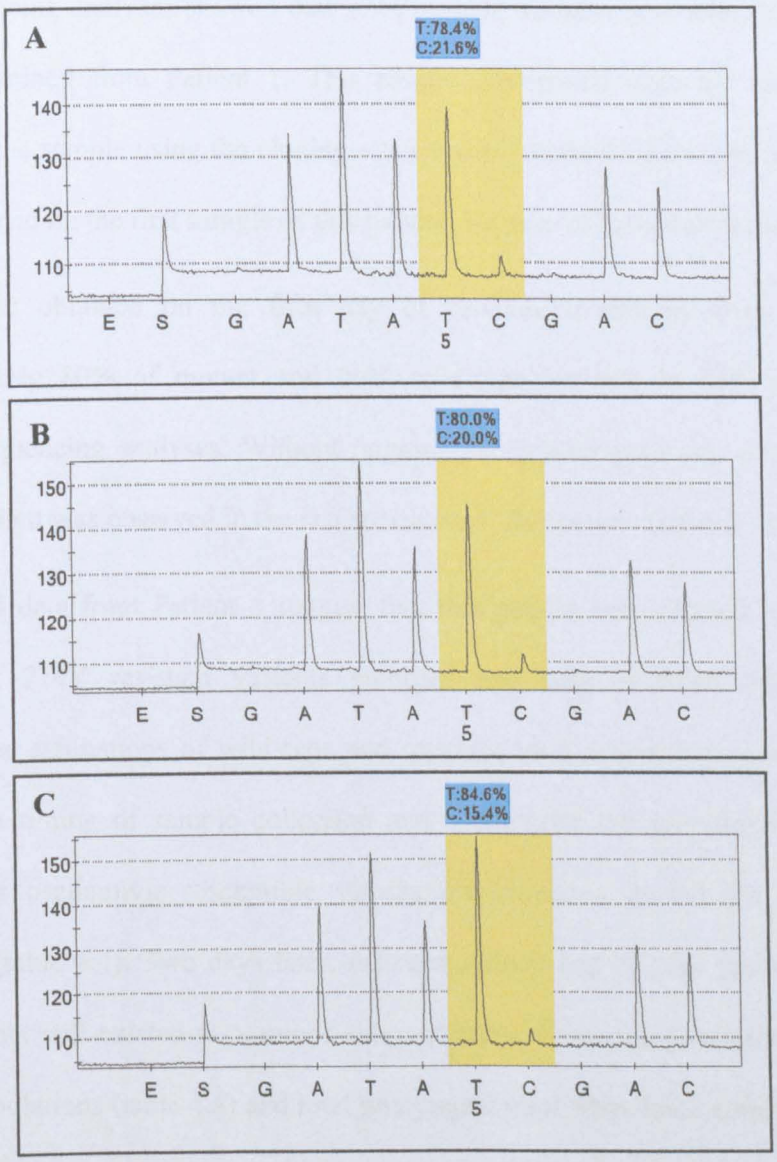

Figure 4.9 Representative pyrograms estimating mutant virus in clinical specimens. Panel A, B, and C are the last sample of Patient 1, five-day oseltamivir treated and the last sample of Patient 4, respectively. Yellow indicates the C→T mutation. Blue highlights estimated results. Horizontal axis indicates the order of nucleotide dispensation.

Pyrosequencing analyses showed that 274Y mutant variants predominate (69.5%) in the last sample obtained from Patient 1. This result corresponds with the result obtained when analyzing the sample using the cloning - sequencing method (table 4.4). These analyses were not performed on the first sample of this patient, because of insufficient sample volume.

The sample obtained on the fifth day of oseltamivir therapy from Patient 4 showed approximately 80% of mutant and 20% wild-type variants in both pyrosequencing and cloning-sequencing analyses. Without ongoing oseltamivir pressure, a similar proportion of mutant variant was observed in the last specimen of this patient (table 4.4).

Virological data from Patient 4 suggest that this patient was infected by a 274H wild-type virus, and 274Y resistant variants emerged and were selected out during treatment. Quantitative estimations of wild-type and resistant viral proportions were made and plotted against the timing of sample collection and oseltamivir therapy (figure 4.10). This figure shows that oseltamivir susceptible viruses predominated during the first three days of treatment (table 4.4). Two days later, resistant viruses had become predominant while wild-type variants still existed as a minor subpopulation. From the proportions of wild-type and mutant populations (table 4.4) and total pharyngeal viral RNA loads (table 4.3), estimations of absolute wild-type and mutant RNA copies were made by multiplying these values and plotted against admission days and treatment (figure 4.11). This figure shows that wild type virus continues to decline throughout the course of treatment, albeit not to undetectable levels, while resistant virus rapidly emerges and increases to high titers between days 3 and 5 of treatment. After stopping treatment wild-type virus starts to rise again while resistant viruses continue to increase, both indicating ongoing viral replication. Overall, these observations suggest suboptimal suppression of wild-type virus during oseltamivir treatment in this patient that allowed virus containing the resistance-conferring mutation to emerge and selectively outgrow

wild-type viruses. The emergence of the resistance mutation likely happened between days 3 and 5 of treatment.

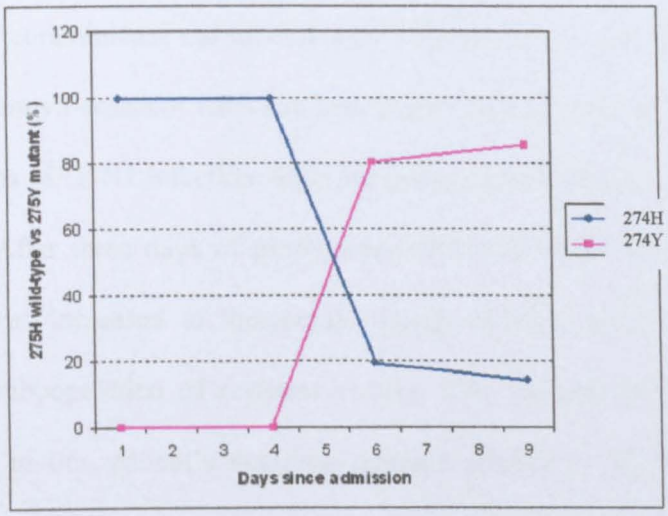

Figure 4.10 Proportion of 274H wild-type and 274Y mutant variants in Patient 4.

Note: treatment was given from day 1 to day 6.

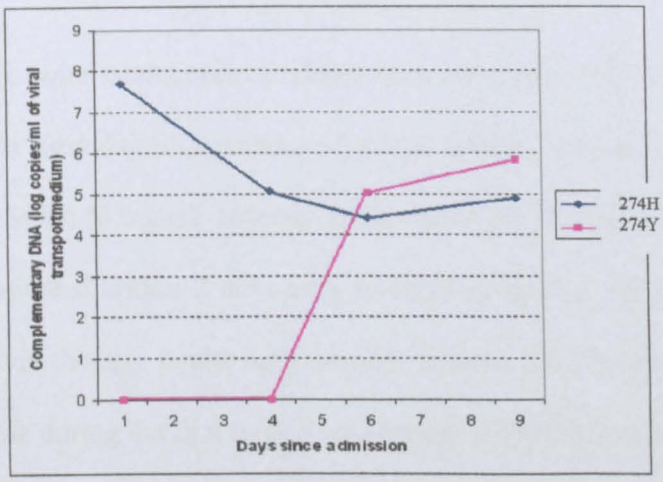

Figure 4.11 Viral RNA loads of 274H wild-type and 274Y mutant variants in Patient 4.

Data for generating this figure were obtained from multiplying the virus loads shown in table 4.3 with estimated virus subpopulation shown in table 4.4. Note: treatment was given from day 1 to day 6.

4.3 Discussion

In this study, I describe the emergence of oseltamivir resistant variants containing a H274Y change in the viral neuraminidase during oseltamivir therapy in two patients. Before these two cases, H274Y oseltamivir-resistant virus had been reported in another Vietnamese patient who developed symptoms of H5N1 infection while receiving oseltamivir at a prophylactic dose of 75mg once daily. After three days of prophylaxis, H5N1 infection was diagnosed and the oseltamivir dose was increased to therapeutic dosage. Further analyses of the specimen revealed a minor subpopulation of resistant viruses. This patient survived but the role of antiviral treatment in this patient's outcome remains unclear [128]. In contrast, the two patients described in the current study developed antiviral resistance during treatment with therapeutic doses and both succumbed to the infection after 2 and 9 days after completion of therapy.

Patient 1 was treated with oseltamivir at doses that were relatively high for her weight, particularly during the first day of treatment when the interval time to the second dose was shorter than recommended (6 hours). Notably, unlike most H5N1-infected patients, treatment in this patient was initiated within 2 days after onset of symptoms when maximum clinical benefits of oseltamivir therapy could be expected. Indeed, the clinical conditions of this patient remained stable during the first three days of treatment with localized lung damage and without the need for supplemental oxygen (appendix 3). However, on the fourth day of treatment, her condition suddenly worsened and she progressively became dependent on supplemental oxygen (appendix 3). A chest radiograph obtained on this day showed widespread progression of pneumonia in the right lung and at that time there was laboratory evidence of hepatic involvement (appendix 3). At the time of her death, viral load in her throat

had increased. These findings suggest that the emergence of drug resistance significantly contributed to therapy failure, followed by progression of disease and death of this patient.

In the second patient (Patient 4), as in most of H5N1-infected patients, oseltamivir treatment was initiated relatively late in the course of illness when pneumonia had already progressed. Nevertheless, virological analyses revealed the patient responded well to treatment initially during the first two days of therapy, and viral load in her throat was, although still detectable, declining sharply. While only wild-type virus was detectable on the third day of treatment, H274Y resistant variants predominated two days later when the viral load had increased again. The emergence of resistant virus and fatal course after initial virological response to treatment also suggests a causative role of resistance in the outcome of this patient.

The progression of disease and increase in viral load after stopping treatment in both patients suggest that the H274Y change does not confer major reductions in viral fitness. Indeed, reverse genetic studies have shown that oseltamivir-resistant 274Y mutant H5N1 viruses replicate as efficiently as wild-type 274H viruses in cell cultures and retain their high virulence in mice [178] in contrast to resistant 274Y seasonal influenza variants [179].

The emergence of resistant influenza A (H5N1) variants during oseltamivir treatment was unexpected, but may have been predictable in view of observations in human influenza H1N1 and H3N2 viruses. Although the resistance rate in adults is around 0.4 to 1% [171], resistance rates of up to 18% have been reported in Japanese children who received oseltamivir treatment [172]. The difference in resistant rates between adults and children may be due to the lack of pre-existing immunity that allows relatively uninhibited viral replication which provides a greater opportunity for resistant virus to emerge. In addition, suboptimal drug levels may have been an additional reason for high resistance rates in Japanese children since at that time a non-weight-based dosing regimen potentially led to underdosing of young children. Because

no pre-existing immunity against H5N1 influenza virus exist in the human population, all human cases can be regarded as primary infections. Our previous studies have indeed shown that H5N1-infected patients experience prolonged viral shedding and have higher viral loads in the throat as compared to those with human influenza H1N1 or H3N2 [36]. Furthermore, studies in animals show more efficient replication of the recent H5N1 influenza viruses when compared to H5N1 virus strains which caused the Hong Kong outbreak in 1997, and higher doses and more prolonged treatment with oseltamivir for protection [180]. The standard dose of oseltamivir is based on clinical studies in uncomplicated seasonal influenza and it remains unclear at present whether higher doses are needed for treatment of H5N1 influenza and other forms of severe influenza. A multinational randomized controlled clinical study evaluating the efficacy of standard dose versus double dose oseltamivir in hospitalized severe influenza, including H5N1, is currently ongoing in Vietnam, Thailand and Indonesia [181]. In conclusion, human H5N1 infections are characterized by high rates of viral replication while some doubts remain concerning the adequacy of drug levels: this combination provides fertile grounds for resistance development and our observations of resistance development in 2 of 7 patients followed throughout the course of treatment should thus perhaps not be surprising.

Indeed, the presence of subpopulations of wild-type variants in specimens obtained during and after oseltamivir treatment in the two patients in whom drug resistant viruses emerged, together with the evidence of their active replication, suggests that treatment with a standard dose of oseltamivir incompletely suppressed viral replication. Besides allowing the infection to proceed, such incomplete suppression provides opportunities for the selection of drug resistant variants, particularly when resistance only requires a single amino acid change in the second highest variable protein.

The presence of oseltamivir-resistant 274Y mutant viruses in clinical specimens was initially confirmed by Sanger sequencing. However, because Sanger sequencing does not allow quantitative analysis, an assay based on pyrosequencing was used to further estimate the presence of mutant variants in clinical specimens. Validation data using plasmids revealed that this pyrosequencing assay could reliably detect mutant variants when present at 10% or higher. This detection limit is consistent with previously published assays for H274Y detection [252] and can be explained by the high background noise in pyrosequencing that requires sufficient amount of sequencing template to produce light signal exceeding this background noise, thereby allowing reliable detection [140]. Virus mixtures can also be analyzed using the recently developed 454 pyrosequencing, which produces more accurate quantitative data [182]. However, 454 pyrosequencing was not available at the time.

Structural studies have classified neuraminidase of influenza A viruses into two groups: 1 and 2 [183]. The neuraminidase of N1, N4, N5, and N8 influenza viruses belongs to group 1, and the neuraminidases of other subtypes belong to group 2 [183]. Group 1 neuraminidases contain a bulky and conserved tyrosine at position 252 that is close to the binding site. Tyr252 interacts with His274 to create a binding pocket for oseltamivir. A substitution of tyrosine for histidine at position 274 prevents this conformation leading to failure of oseltamivir to bind and thus oseltamivir resistance [184]. In contrast, the histidine at position 274 in the neuraminidase of group 2 viruses is not involved in creation of the binding pocket, therefore a mutation at this position would not cause drug resistance [184]. Zanamivir binds to the active site of neuraminidase without the need for the creation of binding pocket and is therefore not influenced by the presence of tyrosine at position 274 providing an option for treating patients with H274Y mutant variants [125].

Because pharmacokinetic analyses were not performed, we do not know whether the emergence of drug resistance is a consequence of suboptimal druglevels (eg. due to altered pharmacokinetics in severely ill patients or suboptimal nasogastric drug administration during intubation) resulting in inadequate suppression of viral replication or not. As most of patients infected with H5N1 virus, during hospitalization one of the two patients in whom resistant virus emerged had diarrhea which may lead to suboptimal biological availability of drug. Nonetheless, adequate absorption of the drug has been shown in two H5N1-infected patients even when administering the drug through a nasogastric tube and even in the presence of diarrhea [185].

Seven out of the eight patients in the current series were treated with oseltamivir relatively late in the course of illness when, in uncomplicated human influenza virus infections, the efficacy of treatment is suboptimal. However, clinical benefit of antiviral treatment is still expected when viral replication is ongoing. In this study, such benefit may be suggested by the rapid viral clearance in the four patients who recovered. However, based on this limited number of cases we cannot draw any conclusion on the efficacy of oseltamivir treatment for H5N1 influenza, but studies with larger numbers do suggest this effect [79]. Nevertheless, to avoid resistant mutant develop during treatment and thereby improve outcome, strategies such as using higher doses and prolonged regimens of oseltamivir or combination therapy should be considered. The potential antiviral efficacy and clinical efficacy of treatment with higher dose and longer treatment time are currently under evaluation in a larger randomized controlled trial in patients with severe influenza [181]. However, preliminary results showed that higher dose and longer treatment have similar antiviral and clinical efficacy compared to standard dose regiment (Prof. Menno de Jong, personal communication). Protocols to study combination treatment with zanamivir, particularly the intravenous form, are under

development [181]. Combination treatment with oseltamivir and amantadine has shown improved survival rates in mice infected with amantadine susceptible H5N1 viruses [186], but whether such combination benefits humans deserves further study. Clinical studies to assess combination treatment with other existing antiviral drugs, particularly ribavirin which has been shown to have synergistic effect in combination with oseltamivir and amantadine in vitro [187], should also be considered. Such combination regimens are currently under investigation in immunocompromised patients with influenza (Prof. Menno de Jong, personal communication).

Chapter 5

GENETIC ANALYSIS OF INFLUENZA A (H5N1) VIRUSES ISOLATED FROM HUMANS IN SOUTHERN VIETNAM IN 2004 – 05

5.1 Introduction

Avian influenza A (H5N1) viruses caused poultry outbreak and human infections for the first time in 1997 in Hong Kong. Genetic analyses revealed that the virus responsible for the outbreak possessed the HA gene most likely derived from A/Goose/Guangdong/1/96(H5N1) and other internal genes derived from A/Quail/HongKong/G1/97(H9N2)) and A/Teal/HongKong/W312/97(H6N1) [1, 48, 51]. Although the H5N1 virus responsible for the Hong Kong outbreak has been eliminated as a result of strict quarantine and infection control measures, its putative precursors remained in nature and have reassorted with other avian viruses, generating multiple new genotypes [45, 46, 51].

In 2003, genotype Z emerged as the predominant genotype and spread to many countries in South East Asia including Vietnam, Thailand, Cambodia, Laos, Indonesia, and Malaysia [9]. Phylogenetic analyses for the HA gene revealed viruses belonging to genotype Z found in birds in Vietnam, Thailand, Cambodia, and Malaysia formed a sublineage of closely related viruses termed clade 1. Those that caused epidemics among birds in Indonesia belonged to another sublineage termed clade 2.1. From 2005 onward, genotype Z H5N1 viruses have spread to Middle East, Africa and Europe. The viruses found in these regions belong to a single sublineage: clade 2.2 [45, 46]. Clade 2.3 viruses are more divergent with at least 4 sub-clades (2.3.1 to 2.3.4) have been identified but mostly from China [46].

Sequence analyses for host-specific residues have shown that H5N1 viruses isolated from humans have genetic signatures of avian rather than human influenza viruses [29, 188]. However, some clade 1 H5N1 virus strains have mutations in the HA gene associated with enhanced human-type receptor affinity [189]. Recently, it has been shown that the selection of these mutations occurs naturally in humans [190].

This chapter aims to understand the molecular characteristics of H5N1 viruses isolated from 13 patients from southern Vietnam in 2004-05, to analyse drug resistance and virulence markers, and to investigate HA residues that are potentially involved in human receptor recognition.

5.2 Results

5.2.1 Virus isolates and sequencing

As shown in table 5.1, 17 virus strains were isolated from 13/16 enrolled patients. Sequencing for these strains was performed on the ABI 3170XL sequencer as described in chapter 2. Sequences of all eight gene segments were obtained from 10 strains. Gene sequences of most strains were deposited to Gen Bank (see accession numbers in appendix 11) except those of the A/VNM/CL107-13/2005(H5N1), A/VNM/CL2009-28/2005(H5N1), A/VNM/PEV16C/2004(H5N1), A/VNM/PEV16B/2004(H5N1) and A/VNM/PEV16R/2004(H5N1) (appendix 12).

Table 5.1 Virus isolates and gene sequences

No	Virus isolate	Sample of isolation (day since initiation of oseltamivir therapy)	Patient	Gene sequence available (bp in length)								Genotype
				PB2	PB1	PA	HA	NP	NA	M	NS	
1	A/VNM/CL2009/05*	Throat swab (3)	1	2236	1947	2166	1527	1459	1280	826	840	Z
2	A/VNM/CL2009-28/05	Throat swab (6)		1970	-	-	1620	1459	1366	826	840	ND
3	A/VNM/CL119/05*	Throat swab (0)	2	2236	1947	2166	1527	1459	1280	826	840	Z
4	A/VNM/CL100/05*	Throat swab (0)	3	2236	1947	2166	1527	1459	1280	826	840	Z
5	A/VNM/CL107-13/05*	Throat swab (8)	4	2270	-	-	1659	1459	1366	826	840	
6	A/VNM/CL01/04*	Throat swab (0)	5	2280	1947	2166	1648	1459	1280	826	840	Z
7	A/VNM/CL26/04*	Throat swab (0)	6	2236	1947	2166	1694	1459	1280	826	840	Z
8	A/VNM/CL36/04*	Throat swab (0)	7	1270	1947	2166	1527	1459	1280	826	840	Z
9	A/VNM/CL115/2005*	Throat swab (0)	8	2236	1947	2166	1527	1459	1280	826	840	Z
10	A/VNM/CL02/04*	Throat swab (0)	9	854	-	-	1578	-	1280	826	840	ND
11	A/VNM/CL17/04*	Throat swab (0)	11	-	1947	2166	1526	1459	1280	826	840	ND
12	A/VNM/CL20/04*	Throat swab (0)	12	2236	-	2166	1694	-	1280	826	840	ND
13	A/VNM/CL105/2005*	Throat swab (0)	13	2236	1947	2166	1527	1459	1280	826	840	Z
14	A/VNM/PEV16T/04*	Throat swab (0)	16	2236	1947	2166	1527	1459	1280	826	840	Z
15	A/VNM/PEV16C/04	CSF (0)		2236	1947	2166	1527	-	1280	826	840	ND
16	A/VNM/PEV16S/04	Serum (0)		2236	1947	2166	1527	-	1280	826	840	ND
17	A/VNM/PEV16R/04	Rectum swab (0)		2236	1947	2166	1527	1459	1280	826	840	Z

Note: A dash (-) indicates that gene sequence was not available. Asterisk indicates selected viruses for genotype and genetic clade determination. ND = not determined.

5.2.2 Phylogenetic analysis

To determine the viral genotype and genetic clade, I performed phylogenetic analysis for the available sequence length of all eight segments. Only one virus isolate from each individual patient was selected for analysis (13 virus isolates from 13 patients) (table 5.1). Reference sequences for virus genotypes and clades were obtained from Dr Gavin J.D Smith, State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong, Hong Kong SAR, China. Prior to construction of maximum-likelihood trees in PAUP* version 4.0 [138], nucleotide sequences were aligned using BioEdit version 7.0.1 (Isis, Parmaceutical, Inc, USA), and appropriate DNA substitution models were determined using MrModeltest version 2.2 [139] as described in chapter 2. Trees were rooted to distantly related sequences of other avian influenza viruses e.g., A/Tern/HA/61(H5N3), A/Parrot/Ulster/73(H7N3), and A/Equine/Prague/1/56(H7N7) [52]. Results of phylogenetic analysis indicated that all virus isolates in this study had HA and NA genes derived from the Goose/Guangdong/1/96-like (Gs/GD/1/96-like) lineage (figures. 5.1 and 5.2). Analysis of the remaining 6 gene segments shows that 9 of the 13 isolates belonged to genotype Z (figure 5.3 and appendices 6-10). The remaining 4 isolates could not be determined to their genotype, because sufficient gene sequence data were not available (table 5.1).

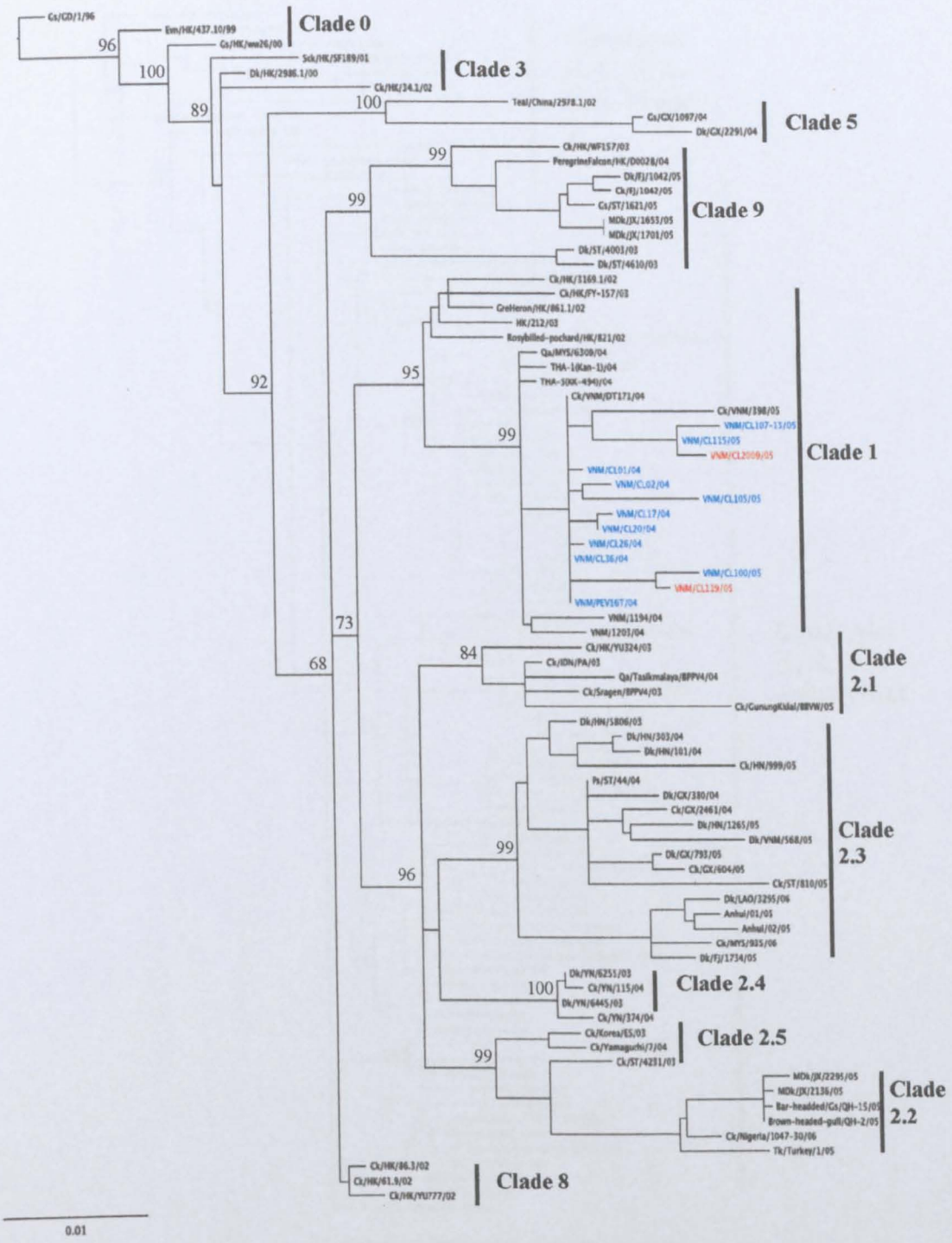

Figure 5.1 ML phylogenetic relationship tree of the HA gene of the 13 virus strains (in red and blue). Red indicates viruses isolated from two patients of the same family. Number above the branches indicates neighbour joining bootstrap value. Tree was rooted to A/Tern/HA/61(H5N3).

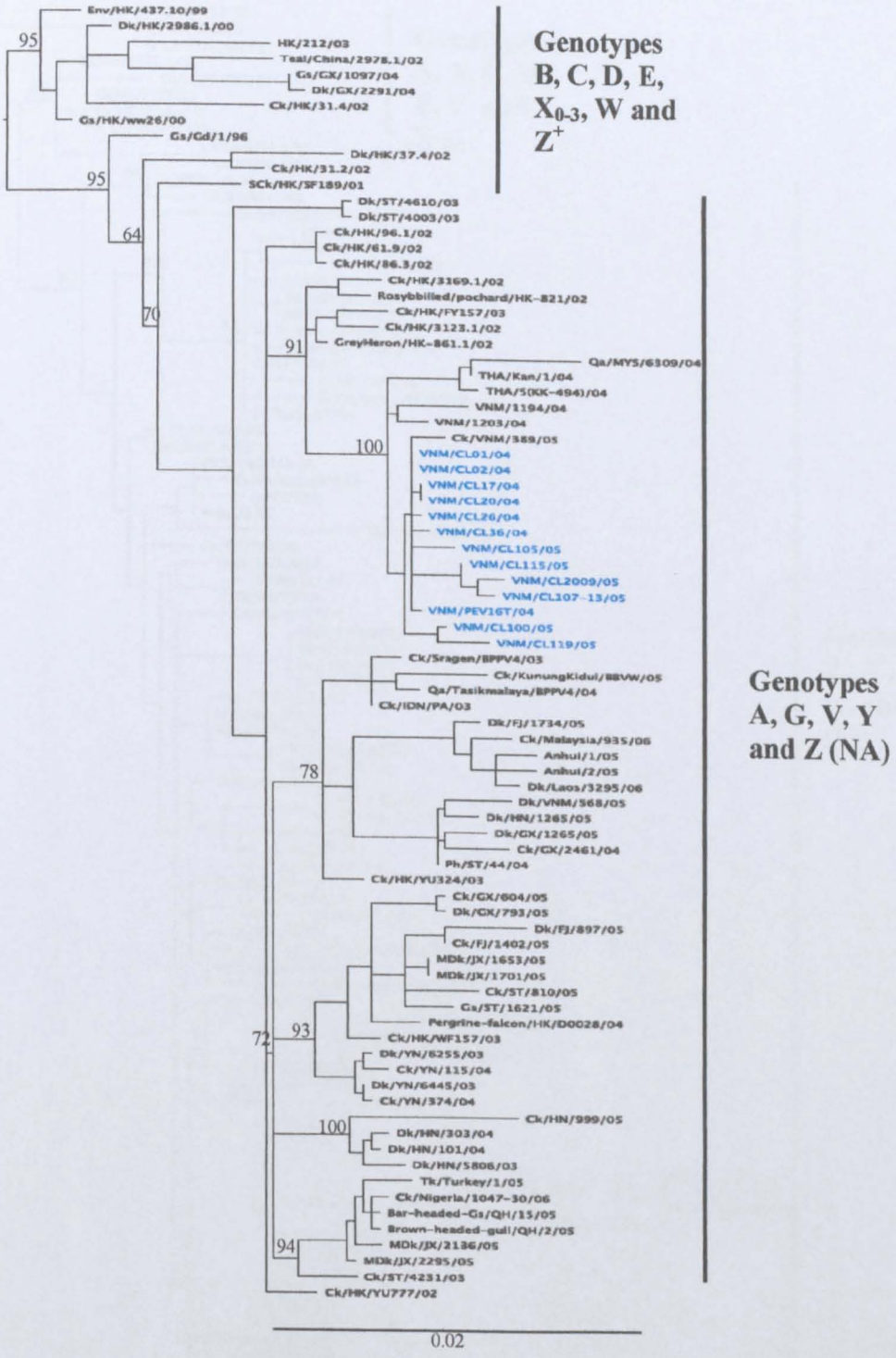

Figure 5.2 ML phylogenetic relationship tree of the NA gene of the 13 virus strains (in blue). Number above the branches indicates neighbour joining bootstrap value. Tree was rooted to A/Parrot/Ulster/73(H7N3).

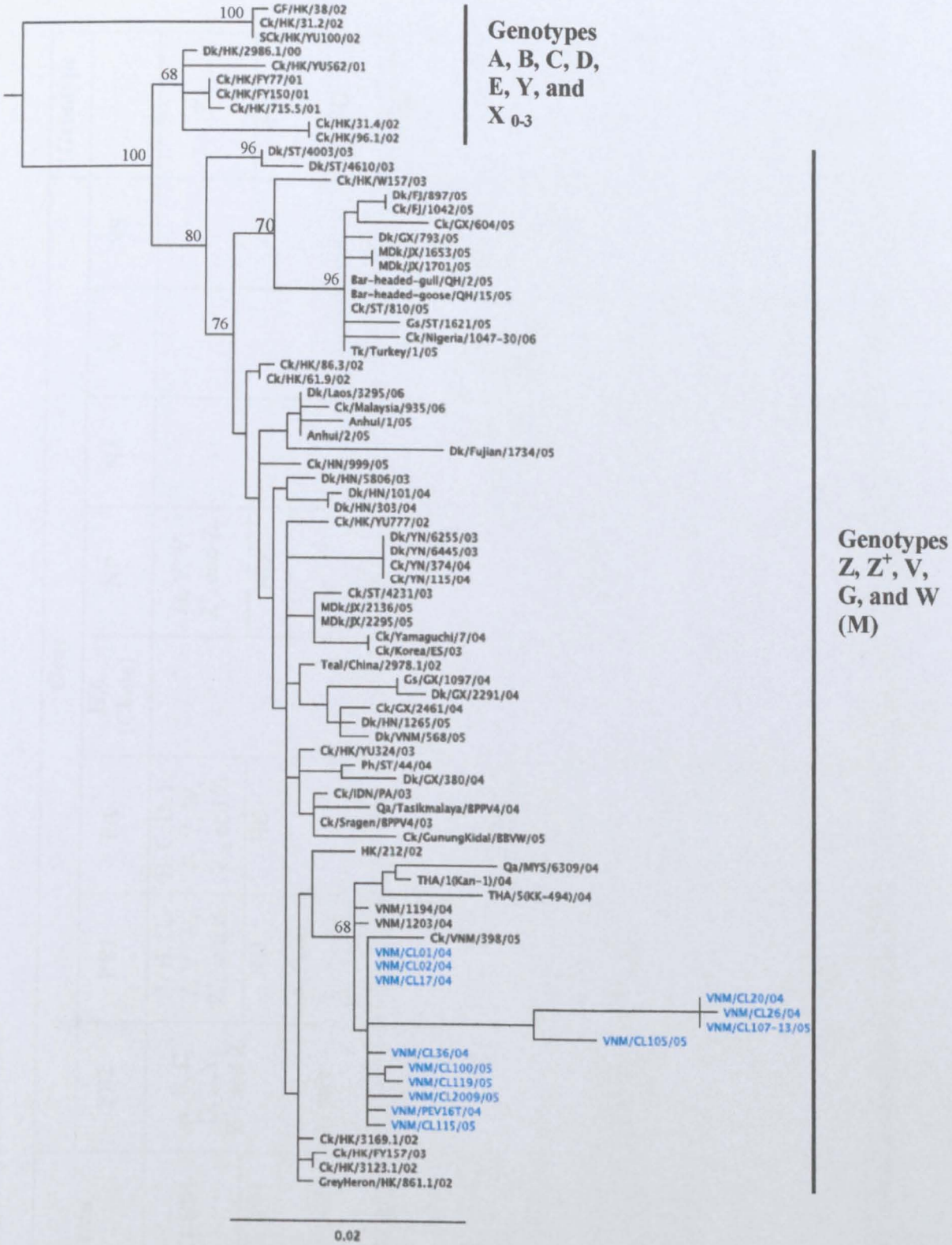

Figure 5.3 ML phylogenetic relationship tree of the M gene of the 13 virus strains (in blue). Number above the branches indicates neighbour joining bootstrap value. Tree was rooted to A/Equine/Prague/1/56(H7N7).

Table 5.2 Virus genotype and clade

Virus strain	Gene								Genotype
	PB2	PB1	PA	HA (Clade)	NP	NA	M	NS	
A/VNM/CL1/04	A, B, C, D, E,Y, Z^+, and **Z**	A, B, G, Y, V, W, Z^+, and **Z**	B, C, D, E, G, Y, W, Z^+, and **Z**	1	G, V, Y, Z^+, and **Z**	A, G, V, Y, and **Z**	G, V, W, Z^+, and **Z**	A, B, C, D, E, V, Y, W, G, Z^+, and **Z**	**Z**
A/VNM/CL2/04		ND	ND		ND				ND
A/VNM/CL17/04	ND	A, B, G, Y, V, W, Z^+, and **Z**	B, C, D, E, G, Y, W, Z^+, and **Z**		G, V, Y, Z^+, and **Z**				ND
A/VNM/CL20/04	A, B, C, D, E,Y, Z^+, and **Z**	ND							ND
A/VNM/CL26/04		A, B, G, Y, V, W, Z^+, and **Z**							**Z**
A/VNM/CL36/04									
A/VNM/CL100/05									
A/VNM/CL105/05									
A/VNM/CL115/05									
A/VNM/CL119/05									
A/VNM/CL2009/05*									
A/VNM/PEV16T/04*									
A/VNM/CL107-13/05		ND	ND						ND

Note: Asterisk (*) indicates representative strain from Patients 15 and 16. ND = not determined.

5.2.3 Molecular characterization

5.2.3.1 HA and the cleavage site

In order to present a more complete picture of the HA gene properties, the deduced amino acid sequences of the HA gene of all index virus isolates were aligned and compared. As shown in table 5.3, all virus isolates had multiple basic amino acids (QRERRRKKR/G) at the cleavage site, which is a molecular trait of highly pathogenic avian influenza virus that permits viral replication in organs beyond the respiratory and gastrointestinal tracts of birds [17, 18]. While the A/VNM/CL105/05 virus had a K (Lys) at position -8 of the cleavage site, the rest of viruses had an R (Arg) at this position. The viruses had at least seven potential glycosylation sites (Asn-xxx-Ser/Thr/Cys) in HA1 which may be associated with virulence [191] (at positions 10-12, 11-13, 22-24, 154-156, 165-167, 193-195, and 286-288).

The receptor binding site residues at amino acid 222 and 224 of the HA1 were Gln and Gly, respectively, indicating 'avian-type' A α2,3 Gal receptor specificity. None of the viruses had the Asn182Lys or Gln192Arg mutations, which are associated with increased 'human-type' SA-α2,6 Gal receptor specificity [189].

The amino acid sequences of HA1 were compared to the prototype vaccine strain [http://wwwwhoint/csr/disease/avian_influenza/guidelines/2strains2006/en/, accessed on April 13, 2010], A/VN/1194/04 (H5N1) strain, isolated from a Vietnamese patient in 2004 [162]. The A/VN/1194/04 (H5N1) has a single amino acid (Met175Leu) that is different from the HA1 sequence of current avian isolates and the isolates presented here (table 5.3).

Glycan-array analyses have shown that the VN/1194/04 is unable to bind to 'human-type' SA-α2,6 Gal receptors [189]. Therefore, comparing HA1 sequences of our human isolates to that of this strain may reveal mutations potentially associated with increased binding to SA-α2,6

Gal. As shown in table 5.3, all viruses had at least one mutation, in addition to Met175Leu that differed from A/VNM/1194/04. The virus A/VNM/CL105/05 had an Ala134Val (A134V) mutation while other three viruses A/VNM/CL17/04, A/VNM/CL20/04, and A/VNM/CL01/04 each had a single mutation at positions 96(Asn→Asp) and 186(Glu→Asp) that was not found in any of the HA sequences of H5N1 influenza virus currently available in Genbank (as of April 2009).

Interestingly, the A/VNM/CL2009-28/05 had an Ile151Phe (I151F) mutation. This mutation was not found in any others, including A/VNM/CL2009/05, isolated from the same patient 3 days earlier from a throat swab, suggesting possible adaptive changes to replication in humans.

Viruses were unable to isolate from the admission throat swab sample of Patient 10, but because viral RNA load in this sample was sufficiently high (4.92E+07 RNA copies/ml clinical specimen, see chapter 4) that allowed me to perform direct HA gene sequencing. The obtained sequence of HA1 was compared to that of the isolate (A/VNM/CL107-13/05) cultured from this patient 7 days later and this revealed 100% identity. Similarly, no amino acid differences were found among the HA gene sequences of 4 virus isolates cultured from different organs (respiratory tract, gastrointestinal tract, blood and central nervous system) of Patient 16 (tables 5.1 and 5.3).

Table 5.3 HA1 amino acid sequences of MDCK cultured viruses

Virus strain	Comparison of mature HA1 sequence																
	Connecting peptide sequences	14	53	83	84	94	96	133	134	140	151	175	186	188	189	203	219
A/VNM/1194/04	QRERRRKKR/G	E	R	A	N	D	N	S	A	K	I	L	E	T	K	I	V
A/VNM/CL01/04	QRERRRKKR/G	E	R	A	N	D	N	S	A	K	I	M	D	T	K	I	V
A/VNM /CL02/04	QRERRRKKR/G	E	R	A	N	D	N	S	A	E	I	M	E	I	K	V	V
A/VNM /CL17/04	QRERRRKKR/G	E	R	A	N	D	D	S	A	K	I	M	E	T	K	I	V
A/VNM /CL20/04	QRERRRKKR/G	E	R	A	N	D	D	S	A	K	I	M	E	T	K	I	V
A/VNM /CL26/04	QRERRRKKR/G	E	R	A	N	D	N	S	A	K	I	M	E	T	K	I	V
A/VNM /CL36/04	QRERRRKKR/G	E	R	A	N	D	N	S	A	K	I	M	E	T	K	I	V
A/VNM /CL100/05	QRERRRKKR/G	E	K	A	D	N	N	S	A	R	I	M	E	T	R	I	I
A/VNM /CL105/05	QKERRRKKR/G	E	R	V	N	V	N	A	V	K	I	I	E	I	K	I	V
A/VNM/CL107-13/05	QRERRRKKR/G	E	R	A	N	N	N	A	A	K	I	M	E	A	K	I	V
A/VNM /CL115/05	QRERRRKKR/G	E	R	A	N	N	N	S	A	K	I	M	E	A	K	I	V
A/VNM /CL119/05	QRERRRKKR/G	E	K	A	N	N	N	S	A	R	I	M	E	T	R	I	V
A/VNM /CL2009/05	QRERRRKKR/G	E	R	A	N	N	N	S	A	K	I	M	E	A	K	I	V
A/VNM/CL2009-28/05	QRERRRKKR/G	E	R	A	N	N	N	S	A	K	F	M	E	A	K	I	V
A/VNM /PEV16T/04	QRERRRKKR/G	K	R	A	N	D	N	S	A	K	I	M	E	T	K	I	V
A/VNM /PEV16C/04	QRERRRKKR/G	K	R	A	N	D	N	S	A	K	I	M	E	T	K	I	V
A/VNM /PEV16S/04	QRERRRKKR/G	K	R	A	N	D	N	S	A	K	I	M	E	T	K	I	V
A/VNM /PEV16R/04	QRERRRKKR/G	K	R	A	N	D	N	S	A	K	I	M	E	T	K	I	V
Consensus sequence of HPAI H5N1 viruses	QRERRRKKR/G	E	R	A	N	D	N	S	A	K	I	M	E	T	K	I	V

Note: A/VNM/1194/04 is a reference virus strain. Bold and underlined capital letters indicate amino acid difference as compared to A/VNM/1194/04 and the concurrent avian consensus sequence.

5.2.3.2 NA and oseltamivir resistance mutation

All human H5N1 viruses in this study had a 20 amino acid deletion (aa 49-68) in the stalk of the NA molecule. This deletion is believed to restore the balance between HA affinity and NA activity allowing H5N1 virus to replicate and transmit efficiently in land-based birds [29] and is one of the molecular characteristics of genotype Z viruses [45]. As a result of this deletion, our viruses lacked one potential glycosylation site at amino acids 50-52 in the NA sequence. The remaining three potential glycosylation sites were at positions 88-90, 146-148 and 237-237.

I also analyzed the single amino acid substitution from His to Tyr at position 274, that is associated with resistance to oseltamivir, in the catalytic site of the NA enzyme [128]. As described in the previous chapter (chapter 4), three of the viruses isolated from two patients during and after oseltamivir treatment had Tyr at position 274 indicating oseltamivir resistance (see chapter 4).

5.2.3.3 Mutation associated with adamantane resistance

M2 protein is the target of the M2 inhibitors; the adamantane drugs [192]. However, influenza A viruses can become resistant to this class of drugs by acquiring a single mutation at any of the following four sites in the transmembrane region of M2: 26 (Leu26Ile/Phe), 27 (Val27Ala/Gly/Ile/Thr), 30 (Ala30Ser/Thr/Val) and 31 (Ser31Asn/Arg) [161, 193]. The H5N1 viruses that caused human infections in 2003-2004 in Vietnam and Thailand were adamantane resistant [29, 194]. Previous studies showed that all H5N1 strains isolated from Vietnam, Thailand and Cambodia during 2003-05 had dual Leu26Ile - Ser31Asn mutations in the M2

protein [161]. In accordance with these findings, I found that all the viruses of the current study possessed similar dual M2 mutations associated with adamantane resistance.

5.2.3.4 Virulence determinants and host-specific markers

In addition to the multiple basic amino acids in cleavage sequence of HA, other factors may contribute to the increased virulence of H5N1 viruses [195]. Studies in pigs have shown that a single amino acid change from Asp to Glu at position 92 in the NS1 protein is associated with virulence [113]. However, Glu92 in the NS1 was not found in our viruses. All viruses did have a PDZ binding motif Glu-Ser-Glu-Val (ESEV) (table 5.4) in the carboxy terminus of the NS1 which may correlate with virulence in mammals and disease severity in humans [116].

Lys627 (instead of Glu) and Asn701 (instead of Asp) in PB2 protein are important for efficient replication and transmission of H5N1 viruses in mammals [32, 103]. The presence of these residues in virus strains of this study has also been examined and shown in table 5.4. Of the twelve strains with available PB2 gene sequences, eight strains had Lys627, three strains had Asn701, and one strain neither had Lys627 nor Asn701. Interestingly, all the strains with Lys627 had Asp701, while three of the four strains with Glu627 had Asn701, suggesting that Lys627 and Asn701 may play a role in human adaptation. Of the eight strains with PB2 Lys627, five strains were isolated from patients with fatal outcome. However, a statistically significant correlation between the presence of PB2 Lys627 and disease outcome, could not be established (Fisher Exact Test, P=1).

Table 5.4 Virulence determinants and host specific markers

Virus	Virulence determinant (Low→High)				Host specific marker (Avian→Human)			
	NS1		PB2		PA		NP	M2
	D92E	C-terminal	D701N	E627K	V100A	Q/T/S400L	D375G/E	I28V
A/VNM/CL01/04	D	ESEV	D	**K**	V	S	D	V
A/VNM /CL02/04	D	ESEV	**N**	E	V	S	D	V
A/VNM/CL17/04	D	ESEV	-	-	V	S	D	V
A/VNM /CL20/04	D	ESEV	D	**K**	V	S	D	V
A/VNM /CL26/04	D	ESEV	D	**K**	V	S	D	V
A/VNM /CL36/04	D	ESEV	D	**K**	V	S	D	V
A/VNM /CL100/05	D	ESEV	D	E	V	S	D	V
A/VNM /CL105/05	D	ESEV	D	**K**	V	S	**E**	V
A/VNM/CL107-13/05	D	ESEV	D	**K**	V	S	D	V
A/VNM /CL115/05	D	ESEV	**N**	E	V	**F**	D	V
A/VNM /CL119/05	D	ESEV	**N**	E	**L**	S	D	V
A/VNM/CL2009/05	D	ESEV	D	**K**	V	S	D	V
A/VNM/PEV16T/04	D	ESEV	D	**K**	V	S	D	V

Note: Underlined indicates the representative strain of single patient. A dash (-) indicates sequence data were not available.

Recent studies have revealed that Lys627 is one of the 13 important markers for avian viruses to be biologically active in humans [188]. The remaining markers in PB2 (Ser199, Met475, Asn567, and Arg702), PA (Leu28, Asn55, and Ser552), NP (Asp16, Pro283, Tyr313, Lys357), and M1 (Ala121) proteins reported by Finkelstein et al. [188] were not found in our isolates. I have also examined the presence of other human specific markers reported by Shaw et al [196] and found that all the viruses had the human specific marker Val28 in the M2 protein. This marker was also found in all clade 1 viruses isolated from Thai patients [29]. VNM/CL105 was the only virus possessing the human specific marker Glu375 in the NP protein (table 5.4). Interestingly, two virus strains: VNM/CL119/05 and VNM/CL115/05 each had a single amino acid change (Val100Leu and Ser400Phe, respectively) in the PA protein. The human specific markers reported by Shaw et al., in 2002 at these two sites were Ala100 and Leu400 [196].

5.2.4 Mutations in HA gene (HA1) potentially involved in human-type receptor recognition

As shown in table 5.3, some H5N1 virus strains have amino acid changes in the HA1 relative to the consensus sequence of avian influenza A H5N1 virus and the VNM/1194/04 strain. I hypothesized that these mutations may be involved in adaptation to the human host or recognizing human-type receptors.

To identify if these mutations may be involved in human adaptation 7 virus strains isolated in MDCK cells (table 5.5) were passaged in the allantoic cavities of embryonated chicken eggs, where cells express only avian-type receptors (SA-α2,3-Gal receptor) [197]. After five passages, 'egg-adapted' viruses were harvested, and HA1 (aa 18-342) was sequenced on the CEQ8000 platform as described in chapter 2. The deduced amino acid HA1 sequence of 'egg-adapted' virus was compared to that of the parent MDCK isolates. As shown in table 5.5, after

five passages in eggs, the Asp (D) residue at position 186 of VNM/CL01/04 had mutated back to Glu (E), which is commonly found in strains of avian origin.

The MDCK VNM/CL105/05 parent strain had five amino acids in HA1 that differed from the VNM/1194/04 strain. After five passages in eggs, the Val (V) at position 134 had mutated back to Ala (A) (table 5.5) while the other four residues were retained. Interestingly, the Phe (F) residue at position 151 in HA1 of the VNM/CL2009-28/05 had not mutated back to reversed to Ile (I), but changed to Leu (L) (table 5.5).

To confirm the presence of E186D, A134V, and F151L. I performed quantitative pyrosequencing analyses directly on original clinical specimens as well as on primary and egg-adapted virus isolates. The design of PCR primers and sequencing primers for pyrosequencing as well as pyrosequencing procedures are described in chapter 2. As shown in table 5.6, no subpopulation of the 'human adapted' 186D variant (0%) was detected in the clinical specimen from which the VNM/CL01/04 was isolated. However, in the MDCK parent virus, this variant was predominant (94.6%) while the 'avian' 186E variant was detected as a minor subpopulation (5.6%), suggesting emergence and selection of 'human' 186D during culture in MDCK cells and not during replication in vivo in the patient.

Direct sequencing of the throat swab specimen from which the VNM/105/05 virus was isolated revealed 26% 'avian' 134A and 74% 'human' 134V. In the MDCK parent strain 46% was A and 54% V, while in the egg-adapted strain 96.5% was A and 3.5% V.

The 'human' 151F variant was predominant (94.9%) in the throat swab specimen of VNM/CL2009-28, while the I and L variants were detected as minor subpopulations (2.5% and 2.6% respectively) (table 5.5). The 151F variant was also predominant in the MDCK parent strain, whereas subpopulations of I and L were not detected. However, in the egg-adapted strain, the L variant was predominant (98%) (table 5.6).

Table 5.5 HA1 amino acid reserve and change after five generations in egg

Virus strain		Amino acid changes in HA1														
		53	83	94	96	133	134	140	151	175	186	188	189	198	265	323
A/VNM/CL1/01	MDCK parent	-	-	-	-	-	-	-	-	M	D	-	-	-	-	-
	Egg-adapted	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-
A/VNM/CL2/04	MDCK parent	-	-	-	-	-	-	E	-	M	-	I	-	V	-	-
	Egg-adapted	-	-	-	-	-	-	E	-	M	-	I	-	V	-	-
A/VNM/CL20/04	MDCK parent	-	-	-	D	-	-	-	-	M	-	-	-	-	-	-
	Egg-adapted	-	-	-	D	-	-	-	-	M	-	-	-	-	-	-
A/VNM/CL105/05	MDCK parent	-	V	V	-	-	V	-	-	I	-	I	-	-	-	K
	Egg-adapted	-	V	V	-	-	-	-	-	I	-	I	-	-	-	K
A/VNM/CL115/05	MDCK parent	-	-	N	-	A	-	-	-	M	-	A	-	-	-	-
	Egg-adapted	-	-	N	-	-	-	-	-	M	-	A	-	-	-	-
A/VNM/CL119/05	MDCK parent	K	-	N	-	-	-	R	-	M	-	-	R	-	-	-
	Egg-adapted	K	-	N	-	-	-	R	-	M	-	-	R	-	-	-
A/VNM/CL2009-28/05	MDCK parent	-	-	N	-	A	-	-	F	M	-	A	-	-	I	-
	Egg-adapted	-	-	N	-	A	-	-	L	M	-	A	-	-	I	-
A/VNM/1194/04(H5N1)*		R	A	D	N	S	A	K	I	L	E	T	K	I	M	R

Note: (-) indicates amino acid residue is identical to the VNM/1194/04. * indicates the virus has a single amino acid (position 175) that differ to avian strains. Blue indicates residues that changed during egg adaptation.

Table 5.6 Subpopulation estimation at the three positions of interest in HA1

Virus strain		*HA position (H5 numbering)*	*Amino acid (%)*	
			Wild-type	Mutant
VNM/CL01/04	Throat swab	186	E (100)	D (0)
	MDCK parent		E (5.4)	D(94.6)
	Egg-adapted		E (97.1)	D (2.9)
VNM/CL105/05	Throat swab	134	A (26)	V (74)
	MDCK parent		A (46)	V (54)
	Egg-adapted		A (96.5)	V (3.5)
VNM/CL2009-28/05	Throat swab	151	I (2.5)	F 94.9, L (2.6)
	MDCK parent		I (0)	F (100), L (0)
	Egg-adapted		I (0)	F (2), L (98)

5.3 Discussion

Insight into the molecular aspects of avian influenza A H5N1 virus infections, particularly those influencing the susceptibility to antiviral drugs, the host specificity and transmissibility, and virulence are crucial for disease management, infection prevention, and outbreak control. Studies in this chapter provide more such details on H5N1 viruses isolated from humans in southern Vietnam in 2004-2005.

Data obtained from phylogenetic analysis confirm that most (9/13) of H5N1 virus isolates collected during this study belonged to genotype Z. This genotype emerged in southern China in 2002, became the predominant genotype and subsequently spread to Vietnam in 2003 [45, 46] causing large-scale poultry outbreaks and sporadic human infection [62]. Because of unavailable sequence data, we were unable to genotype the remaining (4/13) strains. However, since other known genotypes (such as G and V) have never been detected in southern Vietnam during that time [45, 46, 59, 198], these isolates most likely also belong to the prevalent genotype Z.

Analysis of known host-specific markers revealed that all the viruses of this study had very few (2-3) human markers. Molecular analyses of the genotype Z viruses isolated from Thai patients in 2004 revealed similar findings [29]. These data suggest that these genotype Z viruses are purely avian viruses.

In this study, a striking finding was that 75% (8/12) of the human viruses had E627K mutation. This mutation was not found in any H5N1 virus strains isolated from poultry in Vietnam during 2004-2005 [198]. This mutation is believed to confer increased viral replication and virulence in mammals [33]. In this study, a direct correlation between this mutation and disease outcome in patients could not be established. The E627K mutation has also been found in H5N1 viruses of the Qinghai-like lineage (clade 2.2) that spread to Middle

East, Africa and Europe [46, 199], but it seems not to correlate with virulence since the fatality rate of infection to these viruses was low [5, 200]. These observations together with sequence comparison of avian versus human strains [188] as well as studies in vitro [105] suggesting that the E627K mutation may be one adaptation of the virus to mammalian host. Data from this study also suggest that the lack of E627K may be compensated by the presence of D701N in PB2 gene. Recently, this compensatory role of D701N in term of transmissibility has been confirmed by studies in animals using reverse engineered H5N1 viruses [34].

All the viruses had dual mutations conferring amantadine resistance in the M2 gene. It has been shown that influenza viruses with these mutations have no replication defect [201]. H5N1 viruses with these mutations may be acquired through the massive use of amatadine in poultry farming in China [118]. In Indonesia, low percentage (6.3%) of virus strains isolated in 2004-2005 had M2 mutations [161]. However, in recent years, without apparent widespread use of amantadine, analyses for M2 mutations of human specimens revealed a rapid increase in frequency from 25% in 2005 to 78% in 2006 and 87% in 2007, and patients with the M2 mutated viruses had a significant higher virus load in throat as compared to those with wild-type viruses [169]. In addition, M2 mutated viruses were mostly found in patients with fatal outcome [202]. These data may suggest a possible, but unknown role for these mutations in replication and virulence.

Phylogenetic analysis of the HA gene revealed that all the viruses belong to clade 1 that predominated in Vietnam, Thailand, and Cambodia during 2003-05 [29, 123, 198]. Previous studies showed that the clade 1 viruses isolated from Vietnam during 2003-05 belonged to two different genetic groups (sub-lineage): virus strains from southern Vietnam belonged to group S, whereas those from northern Vietnam belonged to group N [198]. Recently, group N viruses found in northern Vietnam have been replaced by the recently emerged clade 2.3.4

H5N1 viruses [59], but this trend has not been observed in Southern Vietnam [203]. The most recently isolated H5N1 virus from southern Vietnam (February 2010, paper in preparation) still belonged to clade 1.

Three viruses isolated from two patients had the oseltamivir-resistance conferring H274Y mutation in the NA gene. H5N1 viruses with this mutation have been shown to be approximately 1000-fold less susceptible to oseltamivir, but remain susceptible to zanamivir [128]. Reverse genetic studies have shown that mutant H5N1 viruses can replicate as efficient as wild-type viruses [178]. At present, three patients with selected oseltamivir resistance during treatment have been described - two in this thesis [77] and one in northern Vietnam [128]. This mutation has, thus far, not been found in any other genetic lineage of H5N1 [5].

Sequence comparison of the HA gene of H5N1 virus isolates cultured from different organs of the same patient (Patient 16) revealed 100% identity, suggesting no tissue-specific adaptive mutations were selected for during systemic viral dissemination in this patient. This is in accordance with data from HA gene sequencing analysis reported by Buchy et al., [123] in which no difference in the HA sequences of H5N1 viruses isolated from the respiratory tract, serum, and rectum was found.

Although glycoarray analyses of reverse engineered H5N1 viruses possessing the HA and NA genes of clade 1 viruses and the remaining genes from A/Puerto Rico/8/34 (H1N1) virus have shown that viruses with an Ala134Val substitution are unable to bind to 'human-type' SA-α2,6 Gal receptors [189], our data in this study suggest that Ala134Val, residing in the 130 loop of the receptor binding domain, may affect receptor specificity and play a role in human adaptation. However, this residue may not function alone since the virus strain with this mutation had four other mutations in the HA1. Direct sequencing revealed that this mutation was selected in vivo during replication in a human patient. Previously, Auewarakul et al., have

shown that this mutation is selected during viral replication in a Thai patient with fatal outcome [190]. However, in their studies, Ala134Val was co-selected with the Leu129Val mutation [190]. Reversely engineered viruses with an Ala134Val substitution were unable to recognize SA-α2,6 Gal, but virus strains with both the Ala134Val and Leu129Val mutations can bind to 'human-type' SA-α2,6 Gal receptors [190].

Data from this study suggest that the I151F mutation possibly was selected during viral replication in humans. It has been shown that the amino acid residue at position 151 (155 in H3 numbering) is involved in receptor binding [204]. The HA of avian influenza H5 viruses that preferentially recognizes 'avian-type' SA-α2,3 Gal receptors has I151, whereas that of human influenza H3 preferably recognizes 'human-type' SA-α2,6 Gal receptors and has T151 [204]. Although further studies are needed to address the role of F151 in receptor binding, I postulate that the F151 may be involved in 'human-type' SA-α2,6 Gal binding since this residue was selected in humans, and was outcompeted in the alantoic cavities of embryonated chicken eggs where cells express only 'avian-type' SA-α2,3 Gal receptors [197].

All the H5N1 virus strains of this study were initially isolated in MDCK cells. Previous studies have suggested that H5N1 influenza viruses [123], in contrast to human influenza viruses [205, 206], may quickly adapt to MDCK cells during in vitro culture. Data from my sequence analysis revealed that the Glu186Asp mutation in a single virus strain was possibly selected during culture in MDCK cells. Interestingly, Glu186Asp (Glu190Asp in H3 numbering) is one of the two human-type specific receptor determinants of H1N1 influenza viruses [207]. Although glycan microarray analysis showed that reversely engineered H5N1 viruses with this mutation alone are unable to bind to 'human-type' SA-α2,6 Gal, its role in efficient replication of H5N1 in MCDK cells should be evaluated particularly in light of the current interest of cell culture-based vaccine production [208].

Chapter 6

A REAL-TIME RT-PCR FOR DETECTION OF CLADE 1 AND 2 H5N1 INFLUENZA A VIRUS USING LOCKED NUCLEIC ACID (LNA) TAQMAN PROBES

6.1 Introduction

Highly pathogenic avian influenza A (H5N1) viruses cause sporadic infections in humans, and are associated with severe respiratory disease with a mortality of about 60% [5]. Since the re-emergence of human H5N1 influenza virus infections in January 2003 [71], 505 human cases have been documented in 15 countries in Asia, Africa, and Europe [5]. Genetic studies have revealed that most of the viruses isolated from humans and poultry belong to genotype Z [45, 46]. The worldwide distribution of this genotype has resulted in the establishment of at least two genetically and geographically distinct clades: clade 1 and 2 [209]. Clade 1 H5N1 viruses have been isolated from poultry and humans in Vietnam, Thailand, and Cambodia, and from poultry in Laos and Malaysia [54, 210, 211]. Clade 2 viruses have a larger genetic diversity and are divided into 5 sub-clades (2.1 to 2.5) [209]. Clade 2.1 viruses have been found only in Indonesian poultry and humans [54]. Clade 2.2 viruses have caused poultry outbreaks and human infections in the Middle East, Africa, and Europe [5]. Clade 2.3 viruses are further divided into four sub-clades (2.3.1 to 2.3.4) [209]. Recently, clade 2.3.4 viruses have become predominant in China and have also been reported in Hong Kong, Laos, Malaysia, Thailand, and North-Vietnam [59, 198]. In Vietnam, clades 1 and 2.3.4 have co-circulate among poultry and have both caused human infections [4, 59].

The circulation of more than one virus clade poses a challenge for laboratory diagnostics, since methods for detection of H5N1 infection usually rely on clade specific amplification of the HA gene [212-214]. Although rapid antigen tests, virus isolation, and serological tests can be used to diagnose H5N1 infection across all clades, these methods have limited use for routine diagnostics because of the inability to subtype, the low sensitivity, and the requirement of biosafety level 3 laboratory facilities limit their use for routine diagnostics. The accepted reference method for routine diagnosis of H5N1 infections is real-time RT-PCR (rRT-PCR)

[129]. Compared to conventional RT-PCR, rRT-PCR has a smaller risk of cross-contamination, higher sensitivity and specificity, and shorter per sample laboratory turnaround. Several rRT-PCR assays for H5N1 detection have been described [214-218], but few of them have been specifically designed for the detection of both clades [217, 218]. In addition, clinical evaluation has not been performed for most of these assays [214, 216-218]. Therefore, a well evaluated single assay for detection of both clade 1 and 2 H5N1 viruses in human clinical samples is still needed.

Locked nucleic acid (LNA) is a new nucleotide analog that was first synthesized in 1998 [219]. LNA nucleotides contain common nucleobases (A, T, G, and C) in which the ribose ring is 'blocked' by a methylene bridge connecting the 2'-O atom and the 4'-C atom (figure 6.1) which reduces the conformational flexibility of the ribose and increases the local organization of the phosphate backbone [219, 220]. LNAs are linked by the same phosphate backbone found in DNA or RNA (figure 6.1) that allow them to easily incorporate into the sequences [219, 220]. It has been shown that LNAs improve affinity for complementary DNA and RNA [220]. Incorporation of a single LNA residue increases the melting temperature of the sequence from 0.6 to 9.6 °C as compared to the same sequence without LNA residue [220]. LNA oligonucleotides have cellular delivery and physiological stability and are therefore very useful for biological studies especially those that focus on gene silencing and gene expression [221-225]. LNA oligonucleotides have also been evaluated as RNA-binding drugs for treament of cancer and metabolic diseases [226, 227]. In recent years, LNAs have been extensively used as molecular probes for direct isolation of messenger RNAs, *in situ* detection of micro-RNAs, and detection of single point mutations [136]. LNAs have also been used as probes in real-time PCR enabling a more flexible probe design and improving probe binding and amplification efficiency [136, 228, 229, 230]. This chapter aims to use LNA as a

Taqman probe to develop a real-time RT-PCR assay that allows direct detection of clade 1 and 2 H5N1 viruses in clinical specimens.

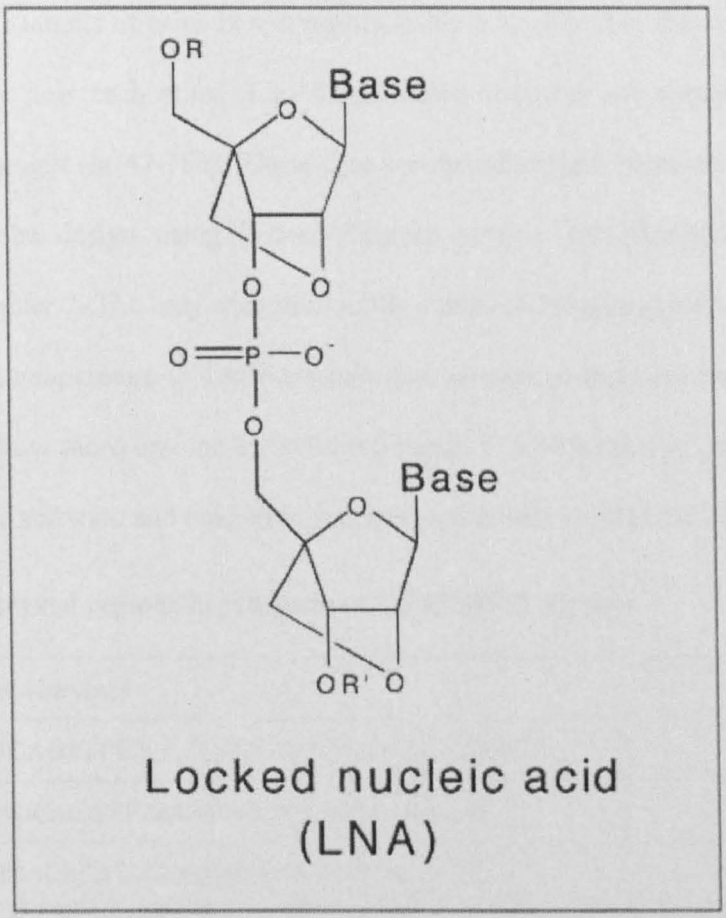

Figure 6.1 Locked nucleic acids and their linkage. Figure reproduced from Braasch et al., [220].

6.2 Results

6.2.1 Real-time RT-PCR with original TaqMan probe

6.2.1.1 Primer and probe design

Nucleotide sequences of full length H5 HA genes of H5N1 virus (N=312) were retrieved from Influenza Virus Resource available at

http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/multiple.cgi [131] and were subsequently aligned using BioEdit version 7.0.1 (Ibis Biosciences). Investigation of sequence alignment revealed 9 highly conserved regions of 25 to 45 nucleotides in length that can serve as targets of primers and probes (table 6.1). The first four conserved regions are located very near each other (1 to 4 nucleotide distance) and expand a region >130 nucleotides in length (nt 47-180). These four conserved regions were chosen as target for primer and probe design using Primer Express version 2.0 (Applied Biosystems) as described in chapter 2. The only exception in the design of primers and probes for this study was the melting temperature of TaqMan probe that was set equal to the melting temperature of primers to allow more options for primer finding. The sequences of primers and probes generated by the software and used in this chapter are shown in table 2.7 in chapter 2.

Table 6.1 Conserved regions in HA gene of HPAI H5N1 viruses

No	Conserved sequence	HA gene position*
1	GTGATCAGATTTGCATTGGTTACCATGCAAACAACTC	47 - 83
2	ACAGAGCAGGTTGACACAATAATGGAAAAGAA	85 - 116
3	ACTGTTACACATGCCCAAGACATACTGGAAA	121 - 151
4	GACACACAACGGGAAGCTCTGCGATCTA	153 - 180
5	GATTGTAGTGTAGCTGGATGGCTCCTCGG	208 - 236
6	CCGGAATGGTCTTACATAGTGGAGAAGGCCA	268 - 298
7	TGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAA	362 - 406
8	AGGGGTGAGCTCAGCATGTCCATAC	435 - 459
9	AATGTGGTATGGCTTATCAAAAAGAACA	484 - 511

Note: * Positioning is based on the 1st nucleotide of the starting codon

6.2.1.2 Real-time RT-PCR

Realtime RT-PCR using the designed primers and probes demonstrated favourable sensitivity, detecting up to 10 copies of the control plasmid per reaction (figure 6.2). However, the endpoint fluorescence was low - approximately 0.1 fluorescent unit at the end of the reaction (cycle 45) when using 120 nM of TaqMan probe. This low fluorescent signal suggests insufficient amount of probe used or inefficient probe binding and is a challenge for intepretation of results. To improve this, instead of increasing probe concentration or modifying the probe with minor groove binders, I modified the probe with LNA residues. Evaluation and use of LNA probe are shown in the following section.

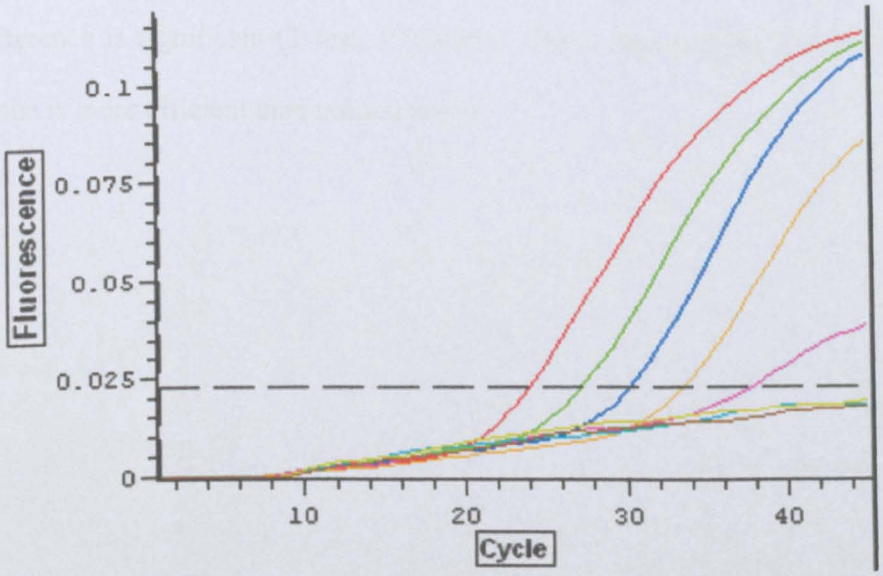

Figure 6.2 Fluorescent graph showing results of realtime RT-PCR using single stranded control plasmids. The assay was carried out on these plasmid concentrations 10^5 – 10^1 (from left to right) in triplicate however only one series of plasmid concentration are shown.

6.2.2 Realtime RT-PCR using LNA probe

6.2.2.1 Modification of normal probe into LNA probe

Five LNA residues were incorporated into the original probe. Residues modified with LNA are all adenin nucleotides (chapter 2, table 2.7) that did not cause *in silico* self-complementary secondary structures (tested by LNA tools available at http://lnatools.com/hybridization) or form primer-dimers. Incorporation of LNA residues resulted in an increase in melting temperature of the probe (average 1°C per modification) (chapter 2, table 2.7).

The binding efficiency of the LNA modified probe was evaluated by comparing its endpoint fluorescence against the original probe. Figure 6.3 illustrates that the LNA modified probe has a higher endpoint fluorescent signal compared to original probe. In reactions using the same concentration of probe and template, the mean Ct value of reactions using LNA probe was 2.93 (the mean of 15 replicates) cycles earlier than reactions using the normal probe and this difference is significant (T test, P=0.0001). These data suggest that the binding of the LNA probe is more efficient than normal probe.

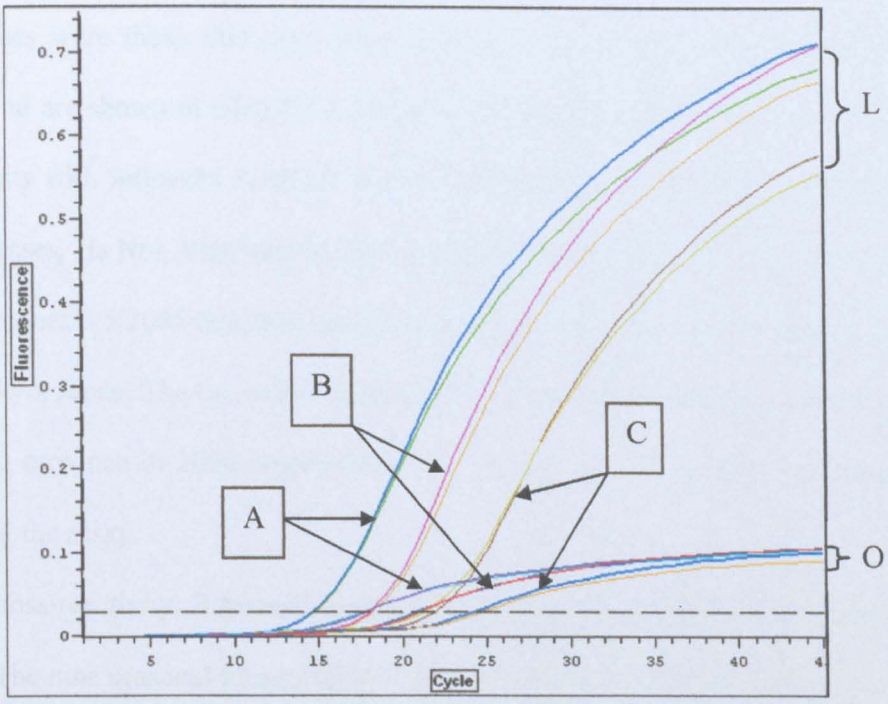

Figure 6.3 Fluorescent signals of FAM emitted by original (O) and LNA (L) TaqMan probes. Each color curve indicates a single rRT-PCR reaction containing 120 nM of either probes. A, B, and C: reactions using 4000, 400, and 40 PFU of the virus A/Vietnam/CL115/2005, respectively.

6.2.2.2 Analytical sensitivity and specificity

The analytical sensitivity of the LNA Taqman rRT-PCR for the detection of the HA gene of H5N1 was determined on three occasions using serial 10-fold dilutions of a representatitive clade 1 virus (A/Vietnam/CL115/2005). The assay consistently detected the HA gene of the virus at a concentration of less than 0.5 PFU per reaction. The analytical sensitivity of the assay was also determined using single-stranded quantified DNA (ssDNA) plasmids and the detection limit of the assay was 10 copies of ssDNA plasmids per reaction.

To test for specific detection of influenza A H5N1, the assay was used with 12 clade 1, 4 clade 2.3 and 1 clade 2.1 human H5N1 viruses, and 2 avian clade 1 H5N1 viruses. The 12 human

clade 1 viruses were those that have been isolated from patients described in this thesis (chapter 3) and are shown in table 5.1 in chapter 5. The four clade 2.3 viruses were isolated from 4 patients with influenza A H5N1 admitted to the National Institute of Infectious and Tropical Diseases, Ha Noi, Viet Nam in 2007 (appendices 12.5.8-12.5.11). The clade 2.1 virus was the A/Indonesia/5/2005 that was kindly provided by The National Institute of Infectious Diseases, Tokyo, Japan. The two avian clade 1 H5N1 viruses were isolated from sick chickens in Vinh Long province in 2006 (appendices 12.5.12-12.5.16). All viruses were successfully detected using the assay.

To test for cross-reactivity, 9 human seasonal viruses and 5 non-H5 avian influenza virues were tested. The nine seasonal viruses (H1N1; n=4 and H3N2; n=5) used in this analysis were isolated from clinical specimens obtained from patients with influenza symptoms presenting to Dong Thap Provincial Hospital in 2006. The five avian viruses (H4N6; n=3, H6N1; n=1, and H8N3; n=1) were isolated from ducks in Dong Thap and Long An provinces in 2006. The subtpye of these avian and human viruses were confirmed by RT-PCR as described by Wright et al., [3] and Lee et al., [143] and sequencing (appendice 12.5.6 and 12.5.7). The result showed that there was no cross-reactivity with these viruses, indicating specificity for influenza A viruses of subtype H5.

6.2.2.3 Evaluation of sensitivity and specificity using clinical specimens

The sensitivity of the assay was clinically evaluated in 58 human specimens with clade 1 (n=10), clade 2.1 (n=25), or clade 2.3 (n=23) H5N1 viruses. The ten clade 1 clinical specimens were diagnostic specimens obtained from 10 out of 16 patients described in chapter 3 (Patients 2-7, 9-13). The twenty five clade 2.1 clinical specimens were diagnostic specimens that were obtained from 25 patients in Indonesia in 2006-07. These specimens were sent to

The National Institute of Health Research and Development in Jarkata, Indonesia for diagnosis of influenza A H5N1 infection. H5N1 viruses in these specimens were confimred using the FDA-approved CDC assay [231]. Since only clade 2.1 viruses have been reported in humans in Indonesia [54], these clinical specimens most likely contained clade 2.1 viruses. The 23 clade 2.3 clinical specimens were diagnostic and follow-up specimens obtained from 4 patients admitted to the National Institute of Infectious and Tropical Diseases, Hanoi, Vietnam in 2007 and from which clade 2.3 viruses were isolated and used in the above section (section 6.2.2.2). My rRT-PCR assay using LNA TaqMan probe detected H5 virus in 56 of these samples indicating a 97% sensitivity. The sensitivity was 100% for clade 1 and clade 2.3, and 92% for clade 2.1 (table 6.2).

Table 6.2 H5N1 clinical samples and rRT-PCR results

Samples / virus clade	NS	TS	TA	Plas	PF	Stool	Total	rRT-PCR positive
Clade 1	2	7	1	0	0	0	10	10
Clade 2.1	7	17	1	0	0	0	25	23
Clade 2.3	5	7	6	2	2	1	23	23
Total	14	31	8	2	2	1	58	56
rRT-PCR positive	13	30	8	2	2	1	56	

Note: NS = Nasal swab; TS = Throat swab; TA = Tracheal aspirate; Plas = Plasma; PF = Pleural fluid.

The specificity of the assay in clinical specimens was assessed by analyzing influenza A H1 or H3 positive samples (n = 19) and influenza negative (n = 29) respiratory samples. These clinical specimens were obtained during 2004-07 from patients with influenza like illness presented to Hospital for Tropical Diseases. The 19 samples with influenza A H1N1 and H3N2 were confirmed using a RT-PCR assays described by Wright et al [3] and the CDC [231]. The 29 influenza A negative samples were those that were negative in FluA specific real-time RT-PCR assay (as described in chapter 2) and/or FluA_CDC assay [231]. The result showed that all of these samples were negative in the LNA_H5 assay indicating 100% specificity.

6.2.2.4 Sensitivity of LNA assay against CDC assay in detecting clade 2.3 viruses

The CDC assay for detection of influenza A H5N1 consists of a generic influenza A specific (FluA_CDC) and two H5 specific assays: asH5a and asH5b. These assays have been aproved by the USA FDA [231] for use in diagnosis of human clinical specimens. Since 2006, CDC assays have been used as universal diagnostic assays for influenza H5N1 infection in laboratories of the South East Asia Infectious Diseases Clinical Research Network [181]. During seting up the LNA assay, I have compared the sensitivity of the LNA assay against the CDC assays in diagnosis of 23 clinical specimens with clade 2.3 H5N1 viruses. These clinical specimens are the clade 2.3 specimens used in the above section (section 6.2.2.3). Table 6.3 shows that the Ct value of the LNA H5 assay is significantly lower than the Ct value of the FluA_CDC, asH5a and asH5b, and the detection rate of the LNA assay is significantly higher compared to both the CDC H5 assays.

Table 6.3 Sensitivity of LNA_H5 assay vs CDC assay in 23 clinical specimens with clade 2.3 H5N1 viruses

Test	FluA_CDC	asH5a	asH5b	LNA_H5
Positive (%)	23 (100)	9* (39)	14 (61)	23 (100)
P value (Fisher exect test)	1	<0.001	0.0015	
Median of Ct value	34.7	38.85	37.25	29.85
P value (Mann-Witney U test)	0.007	0.005	0.0017	

Note: * indicates that these specimens were positive in the asH5b assay

6.3 Discussion

Recent evidence of co-circulation of clade 1 and clade 2 H5N1 viruses in South East Asia has highlighted the need for RT-PCR assays that allow detection of both genetic clades. In this chapter, I have developed a single step rRT-PCR assay using an LNA TaqMan probe for direct detection in clinical samples of H5 genes from both clades of H5N1 viruses. This assay was shown to be sensitive, specific, and rapid (approximately 3.5 hours after RNA extraction). The primers and probe used in this study were designed to target a highly conserved region in the HA gene of H5N1 viruses. Despite that, to ensure amplification of both clade 1 and 2 RNA, one and two degenerated bases were incorporated into the forward and reverse primers,

respectively. Incorporation of degenerated bases into primers (and probes) was frequently observed in recent published assays for influenza A H5N1 detection [231, 232]

The assay using the original probe was shown to be sensitive, detecting 10 ssDNA plasmids per reaction. However, because of its inadequate binding, modification with LNA residues was performed. The residues modified with LNA were chosen according to LNA design guidelines published by You. Y et al., [233] and LNA provider available at http://www.exiqon.com/oligo-tools. When using LNA modified probe, an increase in binding efficiency was observed but without increasing sensitivity. The assay using LNA probe could also detect 10 ssDNA plasmids and was capable to detect less than 0.5 PFU of H5N1 viruses per reaction. Data from this chapter suggest that incorporation of LNA residues may be a good option to improve the binding efficiency of TaqMan probes, particulartly when the probe has a low melting temperature. Indeed, it has been shown that incorporation of LNA residues is a practical way to improve probe-binding efficiency [234].

The LNA assay was shown to be specific for the detection of influenza A of subtype H5. The H5 gene of clades 1, 2.1, and 2.3 was amplified from both virus isolates and human clinical specimens. Cross-reaction with virus isolates from other influenza A subtypes was not observed, and no positive results were obtained when analyzing 48 clinical samples from patients with either seasonal influenza or non-influenza respiratory illness.

Clinical evaluation was performed on 58 stored clinical specimens from 39 patients infected with either clade 1, 2.1 or 2.3 viruses and showed high concordance when compared to initial diagnostic RT-PCR and/or cell culture results. To my knowledge, the number of H5 positive clinical specimens used in this study is larger than in any other previously published assays [215]. Nevertheless, my LNA assay failed to detect virus in a nasal swab and a throat swab.

This may be due to RNA degradation during long-term storage and multiple freeze-thaw cycles.

The LNA assay was shown to be more sensitive than the FDA aproved CDC assay in detecting clade 2.3 viruses in 23 clinical specimens. In these specimens, while my LNA assay could detect the presence of the HA gene of H5N1 virus in all (23/23 specimens), the asH5b could only detect 14/23 and asH5a 9/23. This difference is significant indicating that the LNA assay is more sensitive than the H5 specific CDC assays. The indicator of this sensitivity could be Ct value of reaction that was significantly lower in the LNA assay compared to that of the CDC H5 assays. It should be noted that all specimens positive in the asH5a were also positive in the asH5b suggesting that the use of CDC assay may result in misdiagnosis of a substantial proportion of specimens. For this reason, the LNA assay has been chosen as a supplementary assay (in addition to the FDA CDC assay) for diagnosis of influenza A H5N1 infection in laboratories within the South East Asia Infectious diseases Clinical Research Network [235].

The clade 2.2 H5N1 viruses have shown a widespread global distribution. Therefore including viruses and clinical specimens of this sub-clade in assay validation is important. However, this LNA assay has not been evaluated in clade 2.2 because viruses and specimens were not available to us. Nevertheless, *in silico* analyses indicates that this assay should be capable in detecting viruses of this sub-clade.

In conclusion, in this chapter I have developed a highly sensitive and specific rRT-PCR assay using LNA TaqMan probe for the detection of H5N1 influenza A virus of both clades 1 and 2 directly in clinical specimens. Using this assay, reliable diagnostic results can be obtained in a few hours, thus would be important for timely clinical management and outbreak control.

Chapter 7

FINAL DISCUSSION

7.1 Problems surrounding H5N1 influenza

Since the re-emergence of H5N1 influenza outbreaks in poultry and humans in 2003, the virus has spread to a large part of the world and has continued to cause sporadic human infections with high morbidity and fatality [5]. Although current world-wide attention is focused to the newly emerged influenza A H1N1 virus that has rapidly spread globally [236], avian influenza A H5N1 is still a cause of concern because of its ability to give rise to a pandemic strain, either through human adaptation or reassortment with circulating human viruses and particularly with the pandemic H1N1 virus. The latter has been shown to cause outbreaks in humans, pigs and turkeys providing several hosts for potential co-infection with H5N1 viruses. Current data on H5N1 disease still suggest that if an avian influenza pandemic occurs, the outcome would most likely be very grim. Defensive strategies for this event include the stockpiling of antiviral drugs and mass-production of vaccines.

Antiviral drugs would be the first line of defense for such an influenza pandemic. However, because of limited options, treatment for H5N1 disease relies largely on the NA inhibitor oseltamivir carboxylate. This drug is effective against all influenza A subtypes and is recommended for stockpiling and use during outbreaks and pandemics [http://wwwsearowhoint/LinkFiles/Avian_Flu_Stockpile_Oseltamivirpdf, accessed on May 22, 2009]. However, to be used effectively, besides early identification of cases and timely administration of the drug, a better understanding of the clinical characteristics of H5N1 disease and the viral and human responses to treatment are required to determine the optimal treatment strategy. Furthermore, the use of this drug alone for H5N1 influenza treatment may result in the emergence of resistant variants causing treatment failure [77] and the risk of subsequent spread of resistant viruses [178]. Therefore, while waiting for the availability of new antiviral drugs which are under clinical development [237, 238] or new administration

routes for zanamivir [79, 239] – the drug that oseltamivir resistant virus remains susceptible to-, combination treatment with other existing antiviral drugs is an important alternative option for treatment of severe influenza and is currently under investigation for severe seasonal influenza (Menno de Jong, personal communication).

Vaccines are the most important weapon for pandemic prevention [9]. Human H5N1 influenza vaccines are currently being developed and evaluated in Europe, USA, Japan, Australia, China, and others [236]. In Europe, 4 vaccines have been licensed. Those are the Prepandrix (adjuvanted vaccine) of GlaxoSmithKline, Daronrix (inactivated vaccine) of GlaxoSmithKline, Focetria (subunit vaccine) of Novartis, and Celvapan (inactivated cell-cultured vaccine) of Baxter [236]. However, due to their high genetic diversity, these vaccines seem not to offer sufficient protection for all circulating H5N1 lineages [236]. Therefore, the successful use of H5N1 vaccines always requires an understanding of the genetic and, if possible, the antigenic properties of circulating H5N1 viruses in different regions of the world that would be helpful in selection of appropriate vaccine. Thus, monitoring for the molecular changes of H5N1 viruses from humans and poultry remains of great importance.

7.2 Findings in this thesis

Studies in this thesis (chapter 3) revealed that most patients have typical symptoms of severe respiratory disease, compatible with severe influenza at presentation. Abnormalities observed on chest radiographs included extensive bilateral infiltration, lobar collapse, focal consolidation, and air bronchograms. The prominent laboratory factors were marked lymphopenia along with neuropenia and thrombocytopenia; increased levels serum transaminases. Although rare, patients with H5N1 disease may present with atypical symptoms (e.g., gastrointestinal and CNS infections) that may result in misdiagnosis. The

finding of this case together with those found in Thailand [93] indicate that accurate clinical diagnosis of all H5N1 infections is challenging and thus, during influenza H5N1 outbreaks in poultry, suspected cases should not only be involved patients with symptoms of respiratory infection but also those with unexplained gastrointestinal and CNS infections.

Data from Chapter 4, together with other larger case studies [79], showed that oseltamivir may be benefit to patients with H5N1 infection, but this chapter also describes the emergence of oseltamivir resistance in two patients under standard dose treatment with ensuing failure and fatal outcome. The confirmation of resistant viruses in these patients highlights the need for alternative treatment options to prevent resistance emergence and to improve outcome.

Chapter 5 showed that all H5N1 virus strains in this study belonged to clade 1, genotype Z H5N1 viruses. Some of them had mutations in the HA1 gene that potentially involved in the recognition of 'human-type' receptors and almost all of them had changes in the PB2 gene associated with improved replication in and transmission between mammals. Although the data in this chapter may not be relevant for appropriate vaccine selection, findings from this study are relevant in the sense that they show evidence of rapid adaptation during replication in humans and highlight the need for close monitoring of such changes during human infection as part of pandemic preparedness. In addition, our findings show that changes in the main antigenic protein (HA) occur in different ways in different patients. Moreover, if an H5N1 virus would acquire pandemic properties through human adaptation, it is not unlikely that the sequence of genetic changes leading to successful human to human transmission may also partially affect antigenicity and thus vaccine efficacy. Finally, our observations indicate that sequences from cultured viruses should be interpreted with caution as 'adaptive' changes may also be selected for in tissue culture. Ideally, monitoring for adaptive changes should be done in sequences from direct clinical specimens.

Chapter 6 describes a diagnostic assay which may be useful for timely and accurate diagnosis of human H5N1 infection in regions where different genetic clades cocirculate. Since individual disease management and pandemic preparedness depends on adequate diagnostic capacity, reliable and rapid diagnostics are of greatest importance.

7.3 Future areas of interest

7.3.1 Evolution of H5N1 viruses

Since late 2005, human H5N1 infection has not been detected in southern Vietnam until April 2009 when H5N1 virus was confirmed in a 3 year-old child in Dong Thap province [240]. This long absence of human infections may have been partially a result of nationwide poultry vaccination, poultry rearing and selling regulations, and aggressive public education started in mid-2005 [147]. Despite all that, avian influenza A H5N1 virus was still circulating in domestic birds, possibly in an unvaccinated subpopulation [203], posing a continuous risk of sporadic human infection [240]. The current concern is that avian influenza A H5N1 viruses can recombine with other influenza subtypes circulating in humans, particularly the recently emerged 2009 pandemic influenza virus A (H1N1) that is causing world-wide infections, to form a new strain with combined high pathogenicity of H5N1 and successful human to human transmission of 2009 H1N1. Therefore, if further human H5N1 cases are found, we will continue to closely monitor the characteristics and molecular evolution of the virus by isolation of the virus in MCDK cells and whole genome sequencing of clinical specimens and isolates.

Beside human H5N1 viruses, we believe that the evolution of H5N1 virus in poultry should also be closely monitored to understand the level of evolution and the circulating lineages. For

those reasons, we will conduct a retrospective study to monitor the evolution of H5N1 influenza viruses in poultry in southern Vietnam since 2005 to date. In this study, approximately 100 H5N1 virus strains which were isolated and stored by the local animal health services during 2005-2009 will be sequenced. Sequence data from these strains will be analyzed for their evolution, their antigenic variations, and identification of host-specific markers and mutations possibly involved in recognizing human-type receptors, virulent determinant markers etc. After this study, we will continue our collaboration with the animal health services to keep monitoring the evolution of avian influenza A H5N1 viruses and if possible we will expand our collaboration to study other avian influenza A subtypes (e.g., H3N8, H4N6, H6N1, H9N2 etc.,) that are circulating in poultry and their evolution.

7.3.2 Mutations conferring human-type receptor recognition

Data in this thesis provide evidence for possible occurrence of adaptive mutations in the HA gene of H5N1 influenza virus during infection supporting the notion that continuing transmission of H5N1 virus from poultry to humans may provide a greater opportunity for the virus to adapt to humans resulting in a pandemic strain [190]. At least two mutations (A134V and I151F/L) in the HA gene could be involved in recognizing human-type SA α2,6 receptors. Previously, Auewarakul P et al. has shown that the A134V mutation does influence SA α2,6 receptor binding in the presence of Leu129Val [190]. However, our virus with this mutation did not have L129V, but instead several other mutations (A83V, D94V, A133S, and I188T) therefore it remains interesting whether the A134V mutation also influences SA α2,6 receptor binding in the presence of other mutations. In addition, the role of I151F/L in SA α2,6 receptor binding has yet to be established. I have planned to study the role of these two and other emerging mutations in the recognition of SA α2,6 receptor using glycan arrays in

collaboration with Dr Ten Feizi at the Glycosciences lab, Imperial College, London and Dr Mikhail Matrosovich at the Institute of Virology, Philipps University, Germany.

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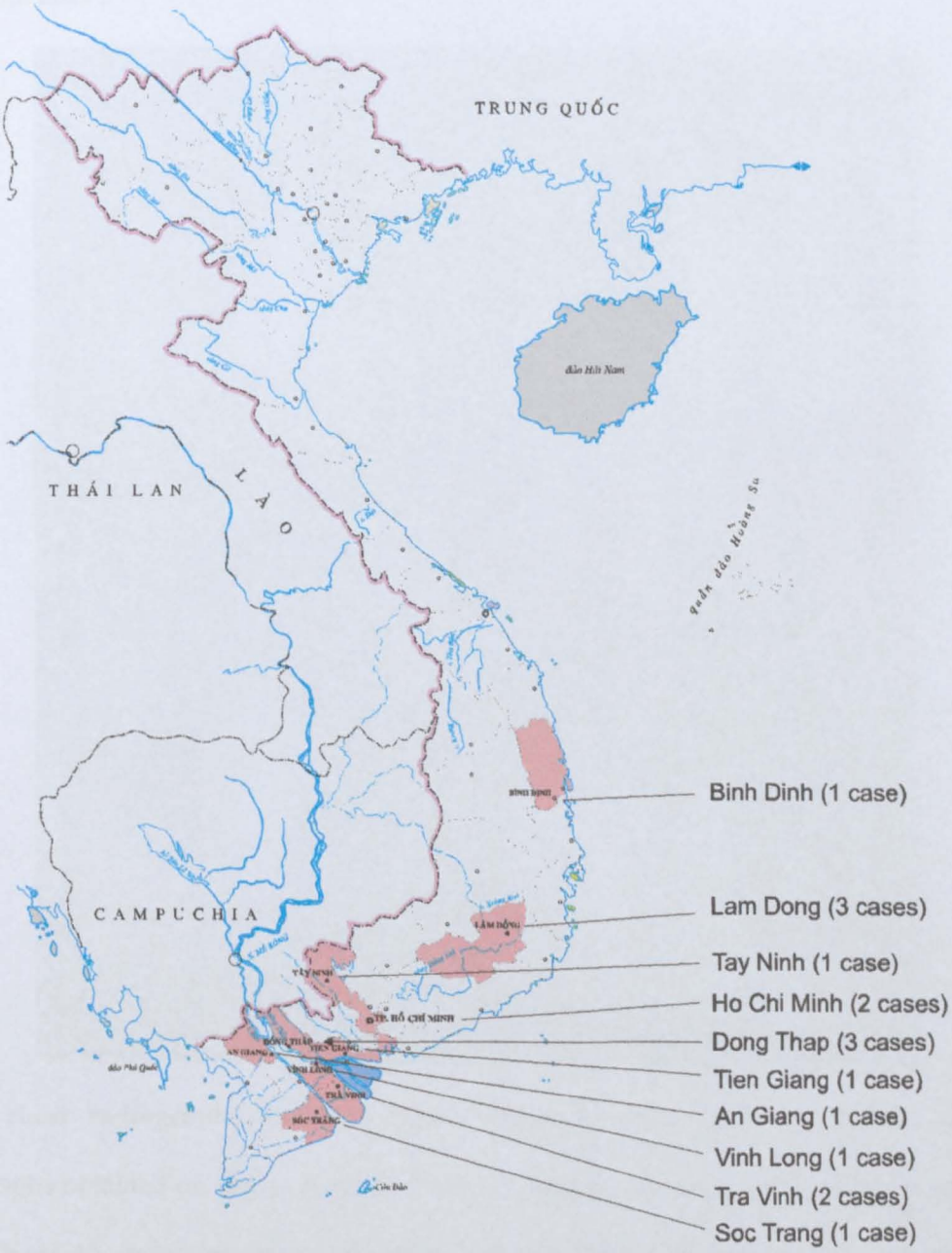
APPENDICES

1. Appendix 1

Published clinical data

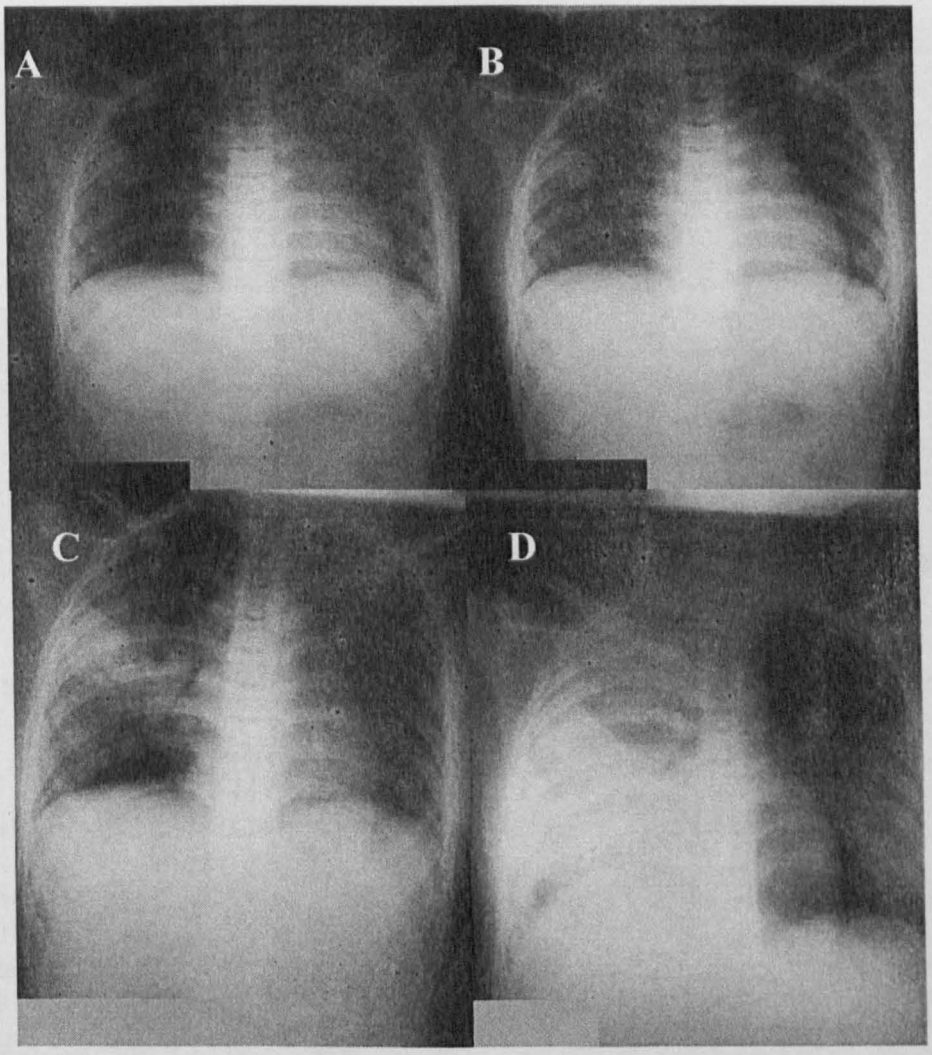
Patient in this thesis	Relevant to published cases	Note
Patient 1	Patient 1 in reference [77]	Clinical data from these 16 cases have been pooled to those from the North in order to investigate prognostic factors associated with fatal outcome in reference [74]. Virological data produced from all patients have been published in reference [36].
Patient 2	Patient 2 in reference [77]	
Patient 3	Patient 3 in reference [77]	
Patient 4	Patient 4 in reference [77]	
Patient 5	Patient 5 in reference [77] and Patient 5 in reference [62].	
Patient 6	Patient 6 in reference [77] and Patient 9 in reference [62]	
Patient 7	Patient 7 in reference [77] and Patient 10 in reference [62]	
Patient 8	Patient 8 in reference [77]	
Patient 9	Patient 6 in reference [62]	
Patient 10	Patient 7 in reference [62]	
Patient 11	Patient 8 in reference [62]	
Patient 12	Not published as case study or case series.	
Patient 13	Not published as case study or case series.	
Patient 14	Not published as case study or case series.	
Patient 15	Not published as case study or case series.	
Patient 16	Patient 2 in reference [76]	

2. Appendix 2



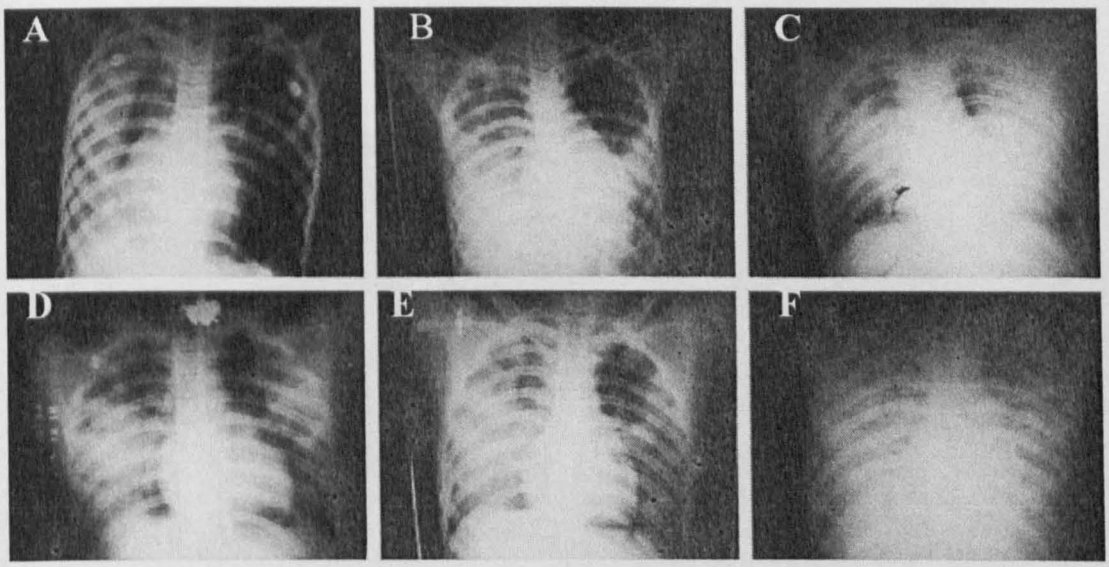
Map of Vietnam showing the geographical location of the 16 enroled cases

3. Appendix 3



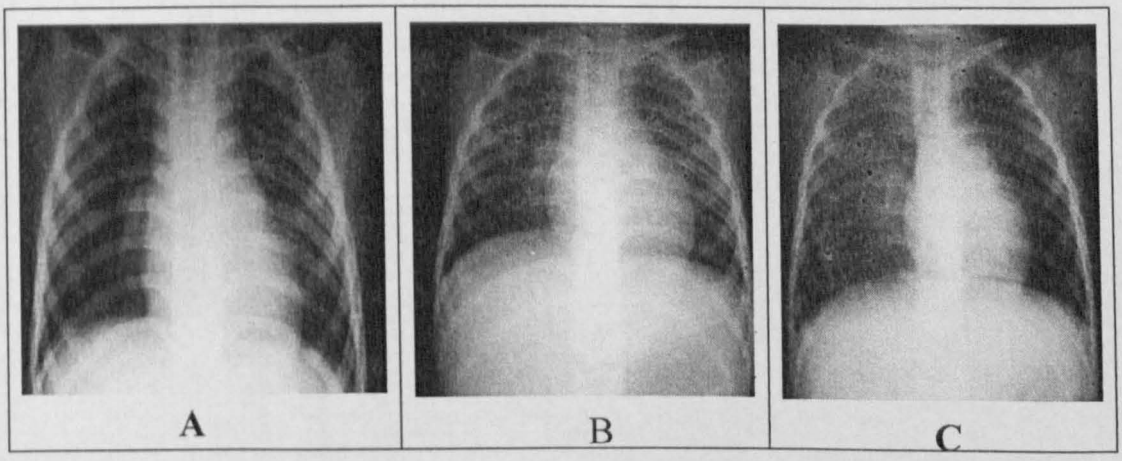
Serial chest radiographs obtained from Patient 1. Panel A, B, C, and D are chest radiographs obtained on day 1, 3, 4 and 7 since treatment started respectively showing small focal (Panel A), minimal progression of the infiltrate (Panel B), focal infiltration (Panel C), and extensive infiltration in the right lung with extension to the left lung. Supplemental oxygen was given on day 4 of treatment and intubation and ventilation were performed on day 6.

4. Appendix 4



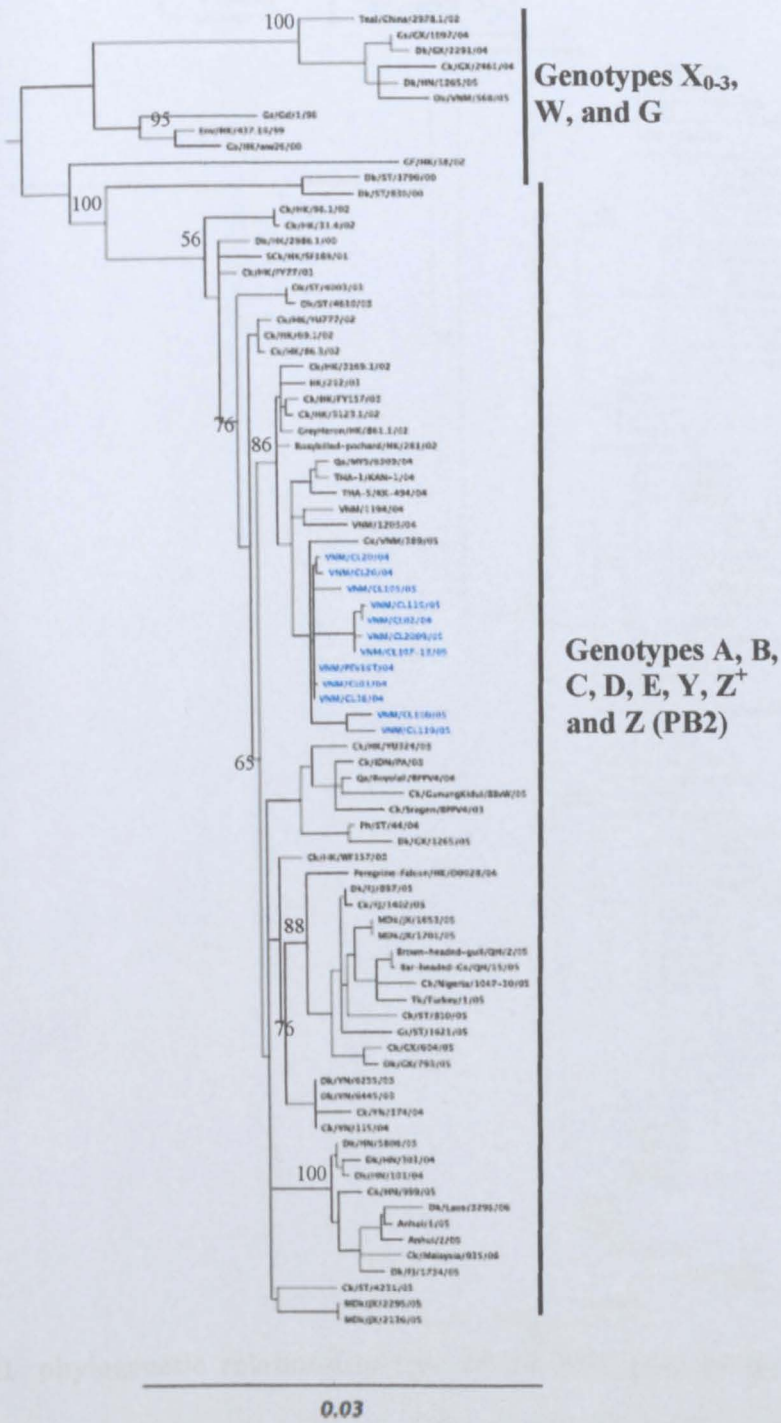
Serial Chest radiographs obtained on admission and during hospital stay from Patient 5, 10, and 12 (Panel A, B, and C) respectively, showing widespread consolidation and interstitial shadowing. The progression of pneumonia in Patient 10 is shown in the chest radiograph in panels D, E, and F, that were obtained on days 5^{th}, 7^{th}, and 10^{th} of illness, respectively.

5. Appendix 5



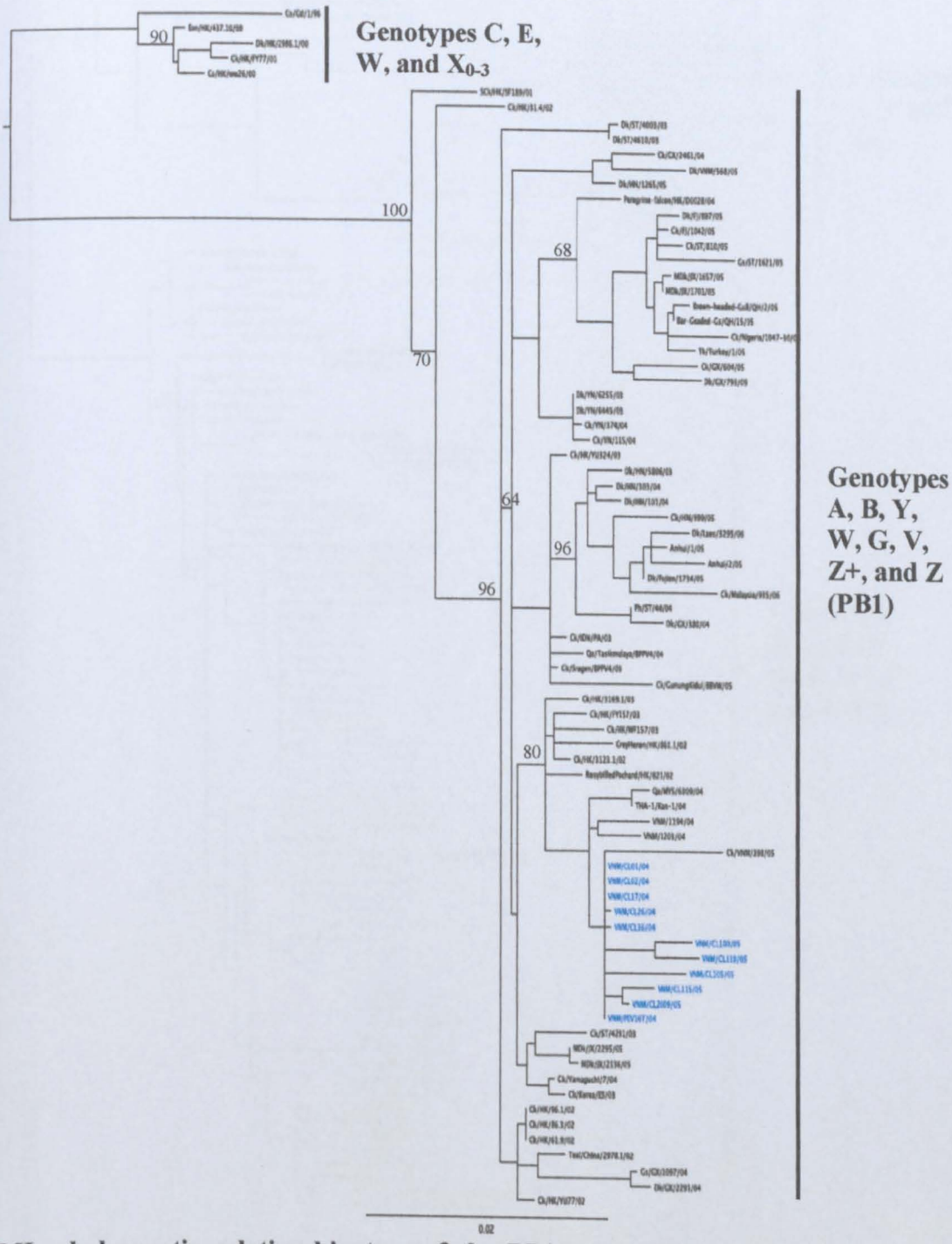
Serial chest radiographs obtained from Patient 16. Panels A, B, and C are radiographs obtained on February 12, 15, and 16, respectively. Patient 16 was the only patient with a normal chest radiograph on admission (Panel A). There were also no abnormalities in his chest radiograph taken on day 5 of illness (3 days after hospitalization) (Panel B). However, a chest radiograph obtained on day six of illness revealed bilateral infiltration (Panel C). The patient died the day after.

6. Appendix 6



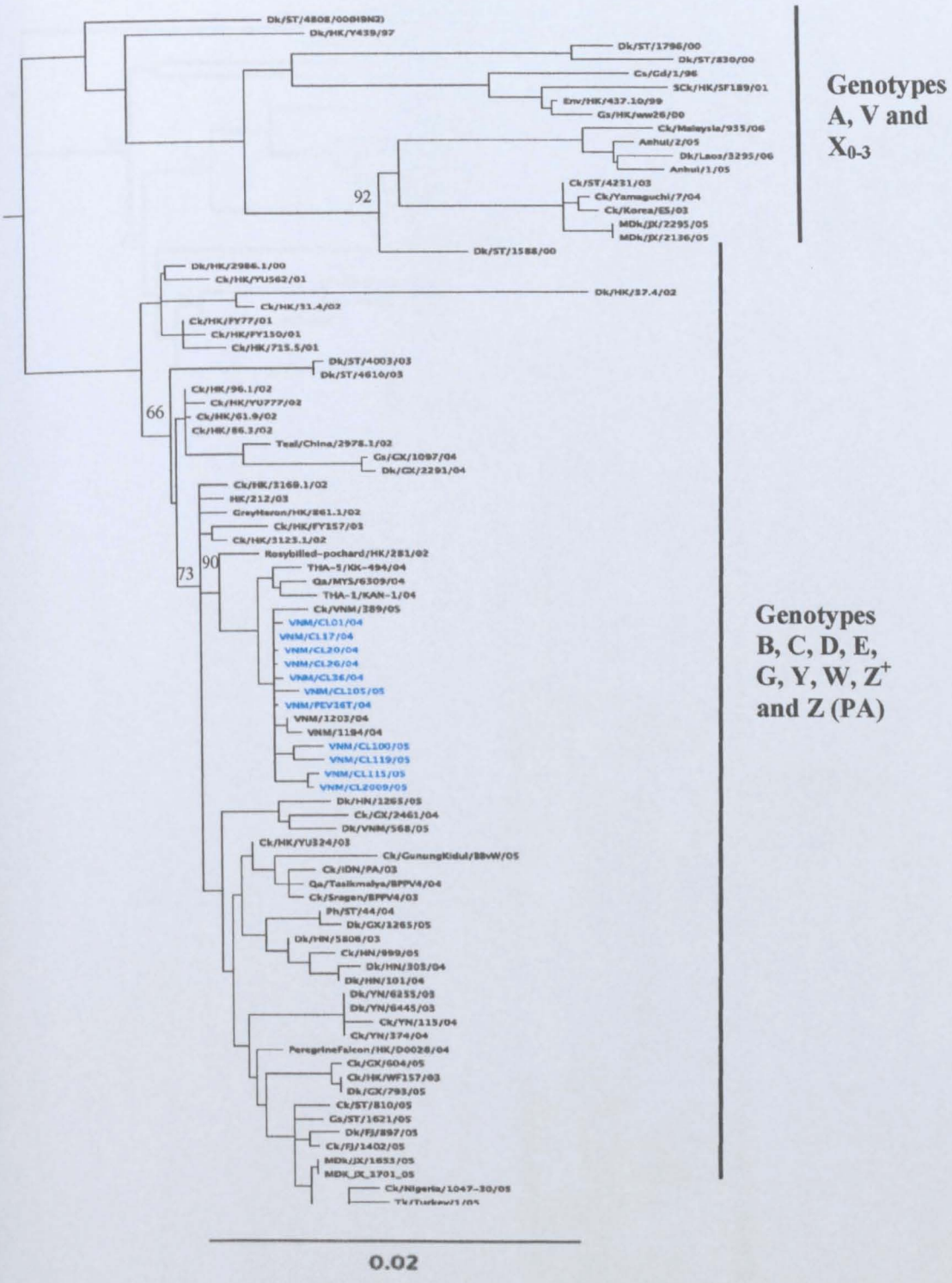
ML phylogenetic relationship tree of the PB2 gene of the 13 virus strains *(in blue).* Number above (or bellow) the branches indicates neighbor joining bootstrap value. Tree was rooted to A/Equine/Prague/1/56(H7N7).

7. Appendix 7



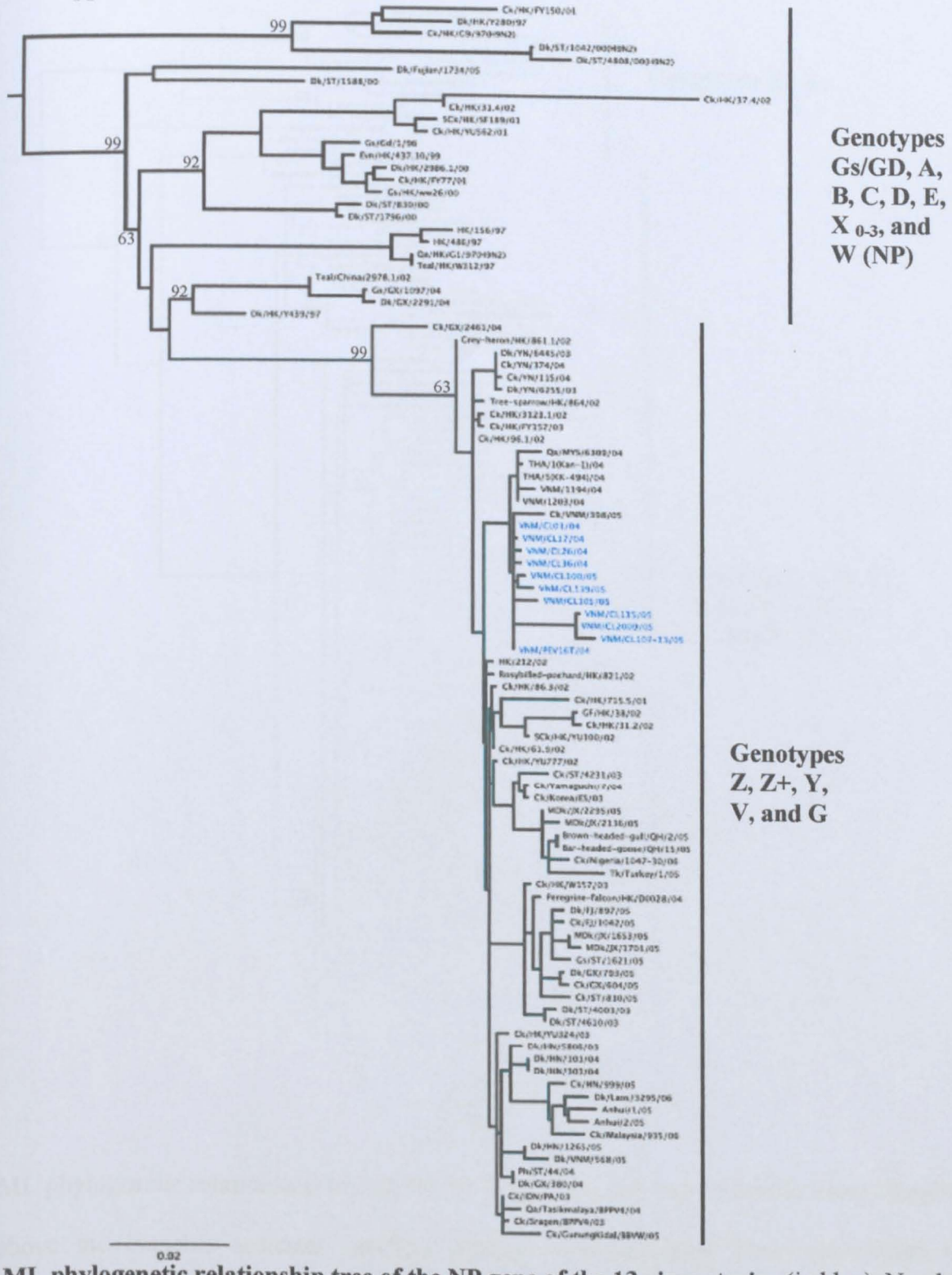
ML phylogenetic relationship tree of the PB1 gene of the 13 virus strains (in blue). Number above (or bellow) the branches indicates neighbor joining bootstrap value. Tree was rooted to A/Equine/Prague/1/56(H7N7).

8. Appendix 8



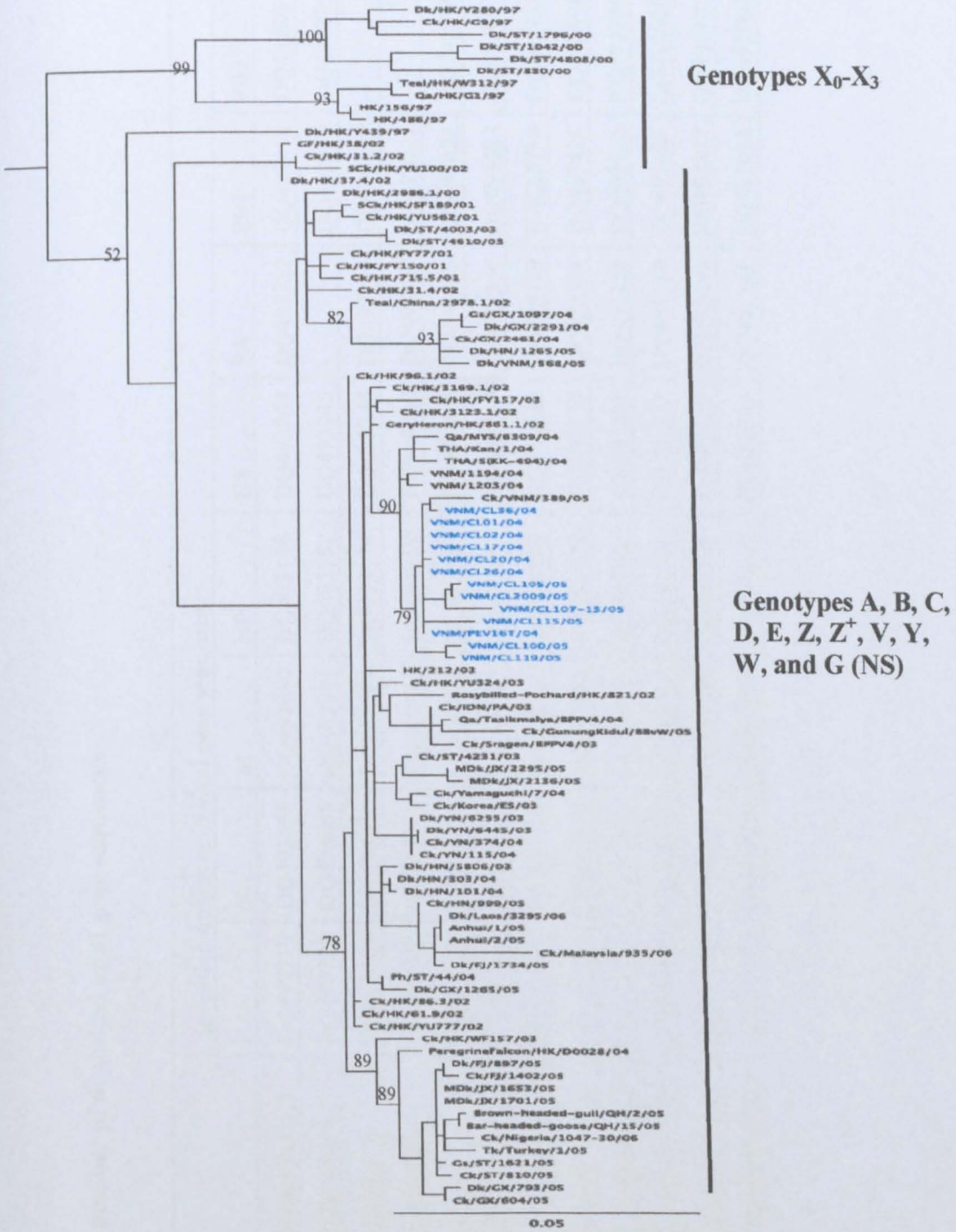
ML phylogenetic relationship tree of the PA gene of the 13 virus strains (in blue). Number above the branches indicates neighbor joining bootstrap value. Tree was rooted to A/Ann/Arbor/6/60(H2N2).

9. Appendix 9



ML phylogenetic relationship tree of the NP gene of the 13 virus strains (in blue). Number above (or bellow) the branches indicates neighbor joining bootstrap value. Tree was rooted to A/Equine/Prague/1/56(H7N7).

10. Appendix 10



ML phylogenetic relationship tree of the NS gene of the 13 virus strains (in blue). Number above the branches indicates neighbor joining bootstrap value. Tree was rooted to A/Equine/Prague/1/56(H7N7).

11. Appendix 11

GenBank accession number of deposited viral gene sequences

Name	Accession numbers of viral gene sequences							
	HA	NA	M	NP	NS	PA	PB1	PB2
A/ Vietnam/CL01/2004(H5N1)	DQ497719	DQ493068	DQ492980	DQ493156	DQ493244	DQ493332	DQ493418	DQ492894
A /Vietnam/CL02/2004(H5N1)	DQ497720	DQ493069	DQ492981	DQ493157	DQ493245	-	DQ493419	DQ492895
A/Vietnam/CL17/2004(H5N1)	DQ497721	DQ493070	DQ492982	DQ493158	DQ493246	DQ493333	DQ493420	-
A/Vietnam/CL20/2004(H5N1)	DQ497722	DQ493071	DQ492983	DQ493159	DQ493247	DQ493334	DQ493421	-
A/Vietnam/CL26/2004(H5N1)	DQ497723	DQ250160	DQ492984	DQ493160	DQ493248	DQ493335	DQ493422	DQ492896
A/Vietnam/CL36/2004(H5N1)	DQ497724	DQ493073	DQ492985	DQ493161	DQ493249	DQ493336	DQ493423	DQ492897
A/Vietnam/PVE16T/2004(H5N1)	DQ535724	DQ535726	DQ535725	DQ535727	DQ535728	DQ535729	DQ535730	DQ535731
A/Vietnam/CL100/2004(H5N1)	DQ497725	DQ493074	DQ492986	DQ493162	DQ493250	DQ493337	DQ493424	DQ492898
A/Vietnam/CL105/2005(H5N1)	DQ497726	DQ493075	DQ492987	DQ493163	DQ493251	DQ493338	DQ493425	DQ492899
A/Vietnam/CL115/2005(H5N1)	DQ497727	DQ493076	DQ492988	DQ493164	DQ493252	DQ493339	DQ493426	DQ492900
A/Vietnam/CL119/2005(H5N1)	DQ497728	DQ493077	DQ492989	DQ493165	DQ493253	DQ493340	DQ493427	DQ492901
A/Vietnam/CL2009/2005(H5N1)	DQ497729	DQ493078	DQ492990	DQ493166	DQ493254	DQ493341	DQ493428	DQ492902

12. Appendix 12 – Unpublished sequences

12.1 Direct HA1 sequence from admission throat swab of Patient 10

GATCAGATTTGCATTGGTTACCACGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAA
AAGAACGTTACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGC
GATCTAGATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGA
AACCCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT
CCAGTCAATGACCTCTGCTACCCAGGGAATTTCAATGACTATGAAGAATTGAAACACCTATTG
AGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGTCATGAA
GCCTCATTGGGGGTGAGTGCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTCAGAAATGTG
GTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTACAATAATACCAAC
CAAGAAGATCTTTTGGTAATGTGGGGGATTCACCATCCTAATGATGCGGCAGAGCAGGCAAAG
CTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACCTCAACACTAAACCAGAGATTGGTA
CCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGAAGGATGGAGTTCTTCTGGACA
ATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAATGGAAATTTCATTGCTCCAGAATAT
GCATACAAAATTGTCAAGAAAGGGGACTCAACAATTATGAAAAGTGAATTGGAATATGGTAAC
TGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTCTAGTATGCCATTCCACAATATA
CACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATCAAACAGATTAGTCCTTGCGACT
GGGCTCAGAAATAGCCCTCAAAGAGAGAGAAGAAGAAAAAAGAGGGGATTATTTGGAGCCATA
GCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGGT

12.2 HA sequences

12.2.1 VNM/CL2009-28/2005

GATCAGATTTGCATTGGTTACCACGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAA
AAGAACGTTACTGTTACACATGCCCAAGACATACTGGAAA-
GACACACAACGGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAG
TGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTC
TTACATAGTGGAGAAGGCCAATCCAGTCAATGACCTCTGCTACCCAGGGAATTTCAATGACTA
TGAAGAATTGAAACACCTATTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAA
AAGTTCTTGGTCCAGTCATGAAGCCTCATTGGGGGTGAGTGCAGCATGTCCATACCAGGGAAA
GTCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAA
GAGGAGCTACAATAATACCAACCAAGAAGATCTTTTGGTAATGTGGGGGATTCACCATCCTAA
TGATGCGGCAGAGCAGGCAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACCTC
AACACTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGG
AAGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAATGG
AAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATTATGAA
AAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTC
TAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATC
AAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAGAGAAGAAGAAAAAA
GAGGGGATTATTTGGAGCCATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGG
TTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAAGAATCCAC
TCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCGATCATTGACAAAATGAACACTCA
GTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAAAGGAGAATAGAGAATTTAAACAAGAA
GATGGAAGACGGGTTCCTAGATGTCTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAA
TGAGAGAACTCTAGACTTCCATGACTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTACA

GCTTAGGGATAATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTATCATAAATGTGATAA
TGAATGTATGGAAAGTGTAAGAAACGGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAG
ATTAAAAAAGAGAGGAAATAAATGGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTC
AATTTATTCTACAGTGGCGAGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATG
GATG

12.2.2 VNM/CL107-13/2005

GATCAGATTTGCATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAA
AAGAACGTTACTGTTACACATGCCCAAGACATACTGGAAA-
GACACACAACGGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTGATTTTGAGAGATTGTAG
TGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTC
TTACATAGTGGAGAAGGCCAATCCAGTCAATGACCTCTGTTACCCAGGGAATTTCAATGACTA
TGAAGAATTGAAACACCTATTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAA
AAGTTCTTGGTCCAGTCATGAAGCCTCATTAGGGGTGAGTGCAGCATGTCCATACCAGGGAAA
GTCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAA
GAGGAGCTACAATAATACCAACCAAGAAGATCTTTTGGTAATGTGGGGGATTCACCATCCTAA
TGATGCGGCAGAGCAGGCAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACCTC
AACACTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGG
AAGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAATGG
AAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATTATGAA
AAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTC
TAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATC
AAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAGAGAAGAAGAAAAAA
GAGGGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGG
TTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAAGAATCCAC
TCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCGATCATTGACAAAATGAACACTCA
GTTTGAGGCCGTTGGAAGGGAATTTAACAATTTAGAAAGGAGAATAGAGAATTTAAACAAGAA
GATGGAAGACGGGTTCCTAGATGTCTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAA
TGAGAGAACTCTAGACTTCCATGACTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTACA
GCTTAGGGATAATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTATCATAAATGTGATAA
TGAATGTATGGAAAGTGTAAGAAACGGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAG
ATTAAAAAAGAGAGGAAATAAATGGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTC
AATTTATTCTACAGTGGCGAGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATG
GATGTGCTCCAATGGGTCGTTACAATGCAGAATTGCATTGAAT

12.2.3 VNM/PEV16B/2004

ACAATAATGGAAAAGAACGTTACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAAC
GGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGA
TGGCTCCTCGGAAACCCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTG
GAGAAGGCCAATCCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTG
AAACACCTATTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGG
TCCAGTCATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTT
TTCAGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC
AATAATACCAACCAAGAAGATCTTTTGGTAATGTGGGGGATTCACCATCCTAATGATGCGGCA
GAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACACTAAAC

CAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGGAGGATGGAG
TTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAATGGAAATTTCATT
GCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATTATGAAAAGTGAATTG
GAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTCTAGTATGCCA
TTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATCAAACAGATTA
GTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAGAGAAGAAGAAAAAAGAGAGGATTA
TTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGGTTGGTATGGG
TACCACCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCA
ATAGATGGAGTCACCAATAAGGTCAACTCGATCATTGACAAAATGAACACTCAGTTTGAGGCC
GTTGGAAGGGAATTTAACAACTTAGAAAGGAGAATAGAGAATTTAAACAAGAAGATGGAAGAC
GGGTTCCTAGATGTCTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACT
CTAGACTTTCATGACTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGAGAT
AATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATG
GAAAGTGTAAGAAACGGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGATTAAAAAGA
GAGGAAATAAGTGGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCT
ACAGTGGCGAGTTCC

12.2.4 VNM/PEV16C/2004

ACAATAATGGAAAAGAACGTTACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAAC
GGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGA
TGGCTCCTCGGAAACCCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTG
GAGAAGGCCAATCCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTG
AAACACCTATTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGG
TCCAGTCATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTT
TTCAGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC
AATAATACCAACCAAGAAGATCTTTTGGTAATGTGGGGGATTCACCATCCTAATGATGCGGCA
GAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACACTAAAC
CAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGGAGGATGGAG
TTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAATGGAAATTTCATT
GCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATTATGAAAAGTGAATTG
GAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTCTAGTATGCCA
TTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATCAAACAGATTA
GTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAGAGAAGAAGAAAAAAGAGAGGATTA
TTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGGTTGGTATGGG
TACCACCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCA
ATAGATGGAGTCACCAATAAGGTCAACTCGATCATTGACAAAATGAACACTCAGTTTGAGGCC
GTTGGAAGGGAATTTAACAACTTAGAAAGGAGAATAGAGAATTTAAACAAGAAGATGGAAGAC
GGGTTCCTAGATGTCTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACT
CTAGACTTTCATGACTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGAGAT
AATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATG
GAAAGTGTAAGAAACGGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGATTAAAAAGA
GAGGAAATAAGTGGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCT
ACAGTGGCGAGTTCC

12.2.5 VNM/PEV16R/2004

ACAATAATGGAAAAGAACGTTACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAAC
GGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGA

TGGCTCCTCGGAAACCCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTG
GAGAAGGCCAATCCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTG
AAACACCTATTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGG
TCCAGTCATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTT
TTCAGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC
AATAATACCAACCAAGAAGATCTTTTGGTAATGTGGGGGATTCACCATCCTAATGATGCGGCA
GAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACACTAAAC
CAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGGAGGATGGAG
TTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAATGGAAATTTCATT
GCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATTATGAAAAGTGAATTG
GAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTCTAGTATGCCA
TTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATCAAACAGATTA
GTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAGAGAAGAAGAAAAAAGAGAGGATTA
TTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGGTTGGTATGGG
TACCACCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCA
ATAGATGGAGTCACCAATAAGGTCAACTCGATCATTGACAAAATGAACACTCAGTTTGAGGCC
GTTGGAAGGGAATTTAACAACTTAGAAAGGAGAATAGAGAATTTAAACAAGAAGATGGAAGAC
GGGTTCCTAGATGTCTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACT
CTAGACTTTCATGACTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGAGAT
AATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATG
GAAAGTGTAAGAAACGGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGATTAAAAAGA
GAGGAAATAAGTGGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCT
ACAGTGGCGAGTTCC

12.2.6 A/Chicken/VNM/C13/2006(H5N1)

AGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTTACACATGCCCAAGACATACTGGA
AAAGACACATAACGGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTGATTTTGAGAGATTG
TAGTGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGACGAATTCATCAATGTGCCGGAATG
GTCTTACATAGTGGAGAAGGCCAATCCAGTCAATGACCTCTGTTATCCAGGGGATTTCAATGA
CTATGAAGAATTGAAACACCTATTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCC
CAAAAGTTCTTGGTCCAGTCATGAAGCCTCATTGGGGGTGAGCGCAGCATGTCCATACCAGGG
AAAGTCCTCCTTTTTCAGGAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAAT
AAAGAGGAGCTACAATAATACCAACCAAGAAGATCTTTTGGTAATGTGGGGGATCCACCATCC
TAATGATGCGGCAGAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGAC
ATCAACACTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAG
TGGGAGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAA
TGGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATTAT
GAAAAGTGAATTGGAATATGGTAATTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAA
CTCTAGTATGCCATTCCACAATATACATCCTCTCACCATCGGGGAATGCCCCAAATATGTGAA
ATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAGAGAAGAAGAAA
AAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGA
TGGTTGGTATGGGTACCATCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAAGAATC
CACTCAAAAGGCTATAGATGGAGTCACCAATAAGGTCAACTCGATCATTGACAAAATGAACAC
TCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAAAGGAGAATAGAGAATTTAAACAA
GAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTATAATGCTGAACTTCTGGTTCTCATGGA
AAATGAGAGAACTCTAGACTTCCATGACTCAAATGTCAAGAACCTTTACGACAAGGTCCGACT
ACAGCTTAGGGATAATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTATCACAAATGTGA
TAATGAATGTATGGAAAGTGTGAGAAACGGGACGTATGACTACCCGCAGTATTCAGAAGAAGC

AAGATTAAAAAGAGAGGAAATAAGTGGAGTAAAATTGGAATCAATAGGAATTTACCAAATACT
GTCAATTTATTCTACAGTGGCGTAGT

12.2.7 A/Chicken/VNM/C22/2006 (H5N1)

ATGGAGAAAATAGTGCTTCTTTTTGCGATAGTCAGTCTTGTTAAAAGTGATCAGATTTGCATT
GGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTT
ACACATGCCCAAGACATACTGGAAAAGACACATAACGGGAAGCTCTGCGATCTAGATGGAGTG
AAGCCTCTGATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGAC
GAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAATCCAGTCAATGACCTC
TGTTATCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTATTGAGCAGAATAAACCAT
TTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGTCATGAAGCCTCATTGGGGGTG
AGCGCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTCAGGAATGTGGTATGGCTTATCAAA
AAGAACAGTACATACCCAACAATAAAGAGGAGCTACAATAATACCAACCAAGAAGATCTTTTG
GTAATGTGGGGGATCCACCATCCTAATGATGCGGCAGAGCAGACAAAGCTCTATCAAAACCCA
ACCACCTATATTTCCGTTGGGACATCAACACTAAACCAGAGATTGGTACCAAGAATAGCTACT
AGATCCAAAGTAAACGGGCAAAGTGGGAGGATGGAGTTCTTCTGGACAATTTTAAAACCGAAT
GATGCAATCAACTTCGAGAGTAATGGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTC
AAGAAAGGGGACTCAACAATTATGAAAAGTGAATTGGAATATGGTAATTGCAACACCAAGTGT
CAAACTCCAATGGGGGCGATAAACTCTAGTATGCCATTCCACAATATACATCCTCTCACCATC
GGGGAATGCCCCAAATATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGC
CCTCAAAGAGAGAGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAG
GGAGGATGGCAGGGAATGGTAGATGGTTGGTATGGGTACCATCATAGCAATGAGCAGGGGAGT
GGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCTATAGATGGAGTCACCAATAAGGTCAAC
TCGATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA
AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTATAAT
GCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTCCATGACTCAAATGTCAAG
AACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGTAACGGTTGT
TTCGAGTTCTATCACAAATGTGATAATGAATGTATGGAAAGTGTGAGAAACGGGACGTATGAC
TACCCGCAGTATTCAGAAGAAGCAAGATTAAAAAGAGAGGAAATAAGTGGAGTAAAATTGGAA
TCAATAGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCGAGTTCCCTAGCACTGGCA
ATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGGTCGTTACAATGCAAAATTTGC
ATTTAAATTTGTGAGTTCAGATTGTAGTTAAAACACAA

12.2.8 A/VNM/15/2007(H5N1)

ATGGAGAAAATAGTGCTTCTTCTTGCAATAATCAGCCTTGTTAAAAGTGATCAGATTTGCATT
GGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTT
ACACATGCTCAAGATATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTAGATGGAGTG
AAGCCTCTGATTTTAAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGAT
GAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAACCCAGCCAATGACCTC
TGTTACCCAGGGAATTTCAACGACTATGAAGAACTGAAACACCTATTGAGCAGGATAAACCAT
TTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCGATCATGAAGCCTCATCAGGGGTG
AGCTCAGCATGTCCATACCAGGGAGTGCCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAAA
AAGAACAATACATACCCAACAATAAAGAGAAGCTACAATAATACCAACCAGGAAGATCTTTTG
ATACTGTGGGGGATTCATCATTCTAATGATGCAGCAGAGCAGACAAAGCTCTATCAAAACCCA
ACCACCTATATTTCCGTTGGGACATCAACACTGAACCAGAGATTGGTACCAAAAATAGCTACT
AGATCCAAAGTAAACGGGCAAAGTGGAAGGATGGATTTCTTCTGGACAATTTTAAAACCGAAT
GATGCAATCAACTTCGAGAGTAATGGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTC
AAGAAAGGGGACTCGGCAATTATGAAAAGTGAGGTGGAGTATGGTAACTGCAACACCAAGTGT
CAAACTCCAATAGGGGCGATAAACTCTAGTATGCCATTCCACAACATACACCCTCTCACCATC

GGGGAATGCCCCAAATATGTGAAATCAAACAAATTAGTCCTTGCGACTGGGCTCAGAAATAGT
CCTCTAAGAGAAAGAAGAAGAAAAAAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGG

12.2.9 A/VNM/28/2007(H5N1)

ATGGAGAAAATAGTGCTTCTTCTTGCAATAATCAGCCTTGTTAAAAGTGATCAGATTTGCATT
GGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTT
ACACATGCTCAAGATATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTAGATGGAGTG
AAGCCTCTGATTTTAAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGAT
GAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAACCCAGCCAATGACCTC
TGTTACCCAGGGAATTTCAACGACTATGAAGAACTGAAACACCTATTGAGCAGGATAAACCAT
TTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCGATCATGAAGCCTCATCAGGGGTG
AGCTCAGCATGTCCATACCAGGGAGTGCCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAAA
AAGAACAATACATACCCAACAATAAAGAGAAGCTACAATAATACCAACCAGGAAGATCTTTTG
ATACTGTGGGGGATTCATCATTCTAATGATGCAGCAGAGCAGACAAAGCTCTATCAAAACCCA
ACCACCTATATTTCCGTTGGGACATCAACACTGAACCAGAGATTGGTACCAAAAATAGCTACT
AGATCCAAAGTAAACGGGCAAAGTGGAAGGATGGATTTCTTCTGGACAATTTTAAAACCGAAT
GATGCAATCAACTTCGAGAGTAATGGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTC
AAGAAAGGGGACTCGGCAATTATGAAAAGTGAGGTGGAGTATGGTAACTGCAACACCAAGTGT
CAAACTCCAATAGGGGCGATAAACTCTAGTATGCCATTCCACAACATACACCCTCTCACCATC
GGGGAATGCCCCAAATATGTGAAATCAAACAAATTAGTCCTTGCGACTGGGCTCAGAAATAGT
CCTCTAAGAGAAAGAAGAAGAAAAAAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGA
GGAATGGCA

12.2.10 A/VNM/88/2007(H5N1)

ATGGAGAAAATAGTGCTTCTTCTTGCAATAATCAGCCTTGTTAAAAGTGATCAGATTTGCATT
GGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTT
ACACATGCTCAAGATATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTAGATGGAGTG
AAGCCTCTGATTTTAAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGAT
GAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAACCCAGCCAATGACCTC
TGTTACCCAGGAAATTTCAACGACTATGAAGAACTGAAACACCTATTGAGCAGGATAAACCAT
TTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCGATCATGAAGCCTCATCAGGGGTG
AGCTCAGCATGTCCATACCAGGGAGTGCCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAAA
AAGAACAATACATACCCAACAATAAAGAGAAGCTACAATAATACCAACCAGGAAGATCTTTTG
ATACTGTGGGGGATTCATCATTCTAATGATGCAGCAGAGCAGACAAAGCTCTATCAAAACCCA
ACCACCTATATTTCCGTTGGGACATCAACACTGAACCAGAGATTGGTACCAAAAATAGCTACT
AGATCCAAAGTAAACGGGCAAAGCGGAAGGATGGATTTCTTCTGGACAATTTTAAAACCGAAT
GATGCAATCAACTTCGAGAGTAATGGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTC
AAGAAAGGGGACTCGGCAATTATGAAAAGTGAGGTGGAGTATGGTAACTGCAACACCAAGTGT
CAAACTCCAATAGGGGCGATAAACTCTAGTATGCCATTCCACAACATACACCCTCTCACCATC
GGGGAATGCCCCAAATATGTGAAATCAAACAAATTAGTCCTTGCGACTGGACTCAGAAATAGT
CCTCTAAGAGAAAGAAGAAGAAAAAAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGA

12.2.11 A/VNM/123/2007(H5N1)

GTGATCAGATTTGCATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGG
AAAAGAACGTTACTGTTACACATGCTCAAGATATACTGGAAAAGACACACAACGGGAAGCTCT
GCGATCTAGATGGAGTGAAGCCTCTGATTTTAAGAGATTGTAGTGTAGCTGGATGGCTCCTCG
GAAACCCAATGTGTGATGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCA
ACCCAGCCAATGACCTCTGTTACCCAGGGAATTTCAACGACTATGAAGAACTGAAACACCTAT
TGAGCAGGATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCGATCATG
AAGCCTCATCAGGGGTGAGCTCAGCATGTCCATACCAGGGAGTGCCCTCCTTTTTCAGAAATG

TGGTATGGCTTATCAAAAAGAACAATACATACCCAACAATAAAGAGAAGCTACAATAATACCA
ACCAGGAAGATCTTTTGATACTGTGGGGGATTCATCATTCTAATGATGCAGCAGAGCAGACAA
AGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACACTGAACCAGAGATTGG
TACCAAAAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGAAGGATGGATTTCTTCTGGA
CAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAATGGAAATTTCATTGCTCCAGAAT
ATGCATACAAAATTGTCAAGAAAGGGGACTCGGCAATTATGAAAAGTGAGGTGGAGTATGGTA
ACTGCAACACCAAGTGTCAAACTCCAATAGGGGCGATAAACTCTAGTATGCCATTCCACAACA
TACACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATCAAACAAATTAGTCCTTGCGA
CTGGGCTCAGAAATAGTCCTCTAAGAGAAAGAAGAAGAAAAAGAGGACTAT

12.2.12A/Duck/LA404/2006(H4N6)

CATGCTATCAATTGTGATCTTGTTTCTGCTCGTAGCAGAAAATTCCTCCCAAAACTACACAGG
AAATCCTGTGATATGCATGGGACATCATGCTGTTGCCAATGGGACCATGGTGAAGACCCTAAC
CGATGACCAAATAGAAGTGGTCACTGCACAAGAATTGGTAGAATCGCAGAACCTTCCAGAATT
GTGCCCAAGTCCCCTAAGGCTAATTGATGGCCAGACTTGTGACATCATTAATGCAGCCTTAGG
GAGCCCAGGATGTGATCACTTGAATGGTGCAGAATGGGACGTATTCATAGAAAGACCCAATGC
AATAGACACCTGTTACCCGTTTGATGTGCCAGATTACCAAAGCCTGAGAAGCATACTTGCTAA
CAATGGGAAGTTCGAATTCATTGCCGAAGATTTCCAATGGACCACAGTGAAACAAAATGGGAA
ATCTGGAGCCTGCAAGAGAGCGAATGTGAATGATTTCTTTAACAGGCTAAATTGGCTGGTGAA
GTCAGATGGAAATGCATATCCTCTGCAGAACTTGACAAAAGTAAATAACGGTGATTATGCAAG
GCTTTACATTTGGGGAGTTCATCACCCTTCGACAGATACAGAGCAAACTAATCTATATAAAAA
TAACCCTGGAAGAGTCACTGTTTCTACTAAAACTACTCAAACAAGTGTAGTCCCCAACATTGG
TAGTCGACCCTGGGTGAGAGGACAGAGCGGCAGAATAAGTTTCTACTGGACTATTGTAGAGCC
TGGAGACTTAATAGTTTTCAACACAATAGGGAATCTAATAGCCCCAAGAGGGCATTACAAGCT
AAACAACCTGAAAAAGAGCACAATTTTGAACACCGCGGTTCCCATAGGTTCATGTGTCAGCAA
GTGCCACACGGACAAAGGCTCCCTCTCCACGACAAAGCCTTTCCAGAATATCTCAAGAATAGC
AATTGGAGACTGCCCCAAGTATGTCAAACAAGGCTCTTTAAAACTTGCAACAGGAATGAGGAA
CATTCCTGAAAAGGCATCGAGAGGGCTCTTTGGAGCAATAGCCGGGTTCATAGAAAATGGCTG
GCAAGGCCTGATTGATGGTTGGTACGGGTTCAGACATCAGAATGCAGAAGGAACAGGAACAGC
AGCAGACCTCAAGTCCACTCTGGCAGCCATTGATCAGATCAATGGGAAGCTAAATCGTCTTAT
CGAGAAAACAAATGAGAAATATCATCAAATCGAAAAGGAATTCGAACAGGTTGAAGGAAGGAT
TCAAGACCTGGAGAAATATGTTGAGGATACAAAGATTGATTTGTGGTCATATAATGCAGAACT
ATTGGTTGCACTGGAAAATCAGCACACCATAGATGTAACTGACTCGGAAATGAACAAACTTTT
TGAGAGAGTGAGACGCCAACTCAGAGAAAATGCTGAGGACAAAGGGAATGGATGTTTCGAAAT
ATTCCACAAGTGTGATAATAGTTGCATTGAGAGCATTCGAAATGGAACTTATAATCACGACAT
TTATAGAGATGAGGCAATCAACAATCGATTCCAAATCCAGGGAGTTAAATTGACCCAAGGATA
CAAGGACATCATTCTTTGGATTTCATTCTCCATATCATGCTTTTTGCTCGTTGCACTACTTTT
AGCCTTCATTTTGTGGGCTG

12.2.13A Duck/LA409/2006(H4N6)

ATGCTATCAATTGTGATCTTGTTTCTGCTCGTAGCAGAAAATTCCTCCCAAAACTACACAGGA
AATCCTGTGATATGCATGGGACATCATGCTGTTGCCAATGGGACCATGGTGAAGACCCTAACC
GATGACCAAATAGAAGTGGTCACTGCACAAGAATTGGTAGAATCGCAGAACCTTCCAGAATTG
TGCCCAAGTCCCCTAAGGCTAATTGATGGCCAGACTTGTGACATCATTAATGCAGCCTTAGGG
AGCCCAGGATGTGATCACTTGAATGGTGCAGAATGGGACGTATTCATAGAAAGACCCAATGCA
ATAGACACCTGTTACCCGTTTGATGTGCCAGATTACCAAAGCCTGAGAAGCATACTTGCTAAC
AATGGGAAGTTCGAATTCATTGCCGAAGATTTCCAATGGACCACAGTGAAACAAAATGGGAAA
TCTGGAGCCTGCAAGAGAGCGAATGTGAATGATTTCTTTAACAGGCTAAATTGGCTGGTGAAG
TCAGATGGAAATGCATATCCTCTGCAGAACTTGACAAAAGTAAATAACGGTGATTATGCAAGG

CTTTACATTTGGGGAGTTCATCACCCTTCGACAGATACAGAGCAAACTAATCTATATAAAAAT
AACCCTGGAAGAGTCACTGTTTCTACTAAAACTACTCAAACAAGTGTAGTCCCCAACATTGGT
AGTCGACCCTGGGTGAGAGGACAGAGCGGCAGAATAAGTTTCTACTGGACTATTGTAGAGCCT
GGAGACTTAATAGTTTTCAACACAATAGGGAATCTAATAGCCCCAAGAGGGCATTACAAGCTA
AACAACCTGAAAAAGAGCACAATTTTGAACACCGCGGTTCCCATAGGTTCATGTGTCAGCAAG
TGCCACACGGACAAAGGCTCCCTCTCCACGACAAAGCCTTTCCAGAATATCTCAAGAATAGCA
ATTGGAGACTGCCCCAAGTATGTCAAACAAGGCTCTTTAAAACTTGCAACAGGAATGAGGAAC
ATTCCTGAAAAGGCATCGAGAGGGCTCTTTGGAGCAATAGCCGGGTTCATAGAAAATGGCTGG
CAAGGCCTGATTGATGGTTGGTACGGGTTCAGACATCAGAATGCAGAAGGAACAGGAACAGCA
ACAGATCTCAAGTCCACTCAGGCAGCCATTGATCAGATCAATGGGAAGCTAAATCGTCTTATC
GAGAAAACAAATGAGAAATATCATCAAATCGAAAAGGAATTCGAACAGGTTGAAGGAAGGATT
CAAAACCTAGAAGTGGTCCTTGAGGATACAAAGATTGATTTATGGTCATATAAAGCAGAACTA
TTGGTTGCACTGGAAAATCAGCACACCATAGATGTAACTGACTCGGAAATGAACAAACTTTTT
GAGAGAGTGAGACGCCAACTCAGAGAAAATGCTGAGGACAAAGGGAATGGATGTTTCGAAATA
TTCCACAAGTGTGATAATAGTTGCATTGAGAGCATTCGAAATGGAACTTATAATCACGACATT
TATAGAGATGAGGCAATCAACAATCGATTCCAAATCCAGGGAGTTAAATTGACCCAAGGATAC
AAGGACATCATTCTTTGGATTTCATTCTCCATATCATGCTTTTTGCTCGTTGCCTTACTTTTA
GCCTTCATTTTGTGGGC

12.2.14A/Duck/LA427/2006(H4N6)

TATCAATTGTGATCTTGTTTCTGCTCGTAGCAGAAAATTCCTCCCAAAACTACACAGGAAATC
CTGTGATATGCATGGGACATCATGCTGTTGCCAATGGGACCATGGTGAAGACCCTAACCGATG
ACCAAATAGAAGTGGTCACTGCACAAGAATTGGTAGAATCGCAGAACCTTCCAGAATTGTGCC
CAAGTCCCCTAAGGCTAATTGATGGCCAGACTTGTGACATCATTAATGCAGCCTTAGGGAGCC
CAGGATGTGATCACTTGAATGGTGCAGAATGGGACGTATTCATAGAAAGACCCAATGCAATAG
ACACCTGTTACCCGTTTGATGTGCCAGATTACCAAAGCCTGAGAAGCATACTTGCTAACAATG
GGAAGTTCGAATTCATTGCCGAAGATTTCCAATGGACCACAGTGAAACAAAATGGGAAATCTG
GAGCCTGCAAGAGAGCGAATGTGAATGATTTCTTTAACAGGCTAAATTGGCTGGTGAAGTCAG
ATGGAAATGCATATCCTCTGCAGAACTTGACAAAAGTAAATAACGGTGATTATGCAAGGCTTT
ACATTTGGGGAGTTCATCACCCTTCGACAGATACAGAGCAAACTAATCTATATAAAAATAACC
CTGGAAGAGTCACTGTTTCTACTAAAACTACTCAAACAAGTGTAGTCCCCAACATTGGTAGTC
GACCCTGGGTGAGAGGACAGAGCGGCAGAATAAGTTTCTACTGGACTATTGTAGAGCCTGGAG
ACTTAATAGTTTTCAACACAATAGGGAATCTAATAGCCCCAAGAGGGCATTACAAGCTAAACA
ACCTGAAAAAGAGCACAATTTTGAACACCGCGGTTCCCATAGGTTCATGTGTCAGCAAGTGCC
ACACGGACAAAGGCTCCCTCTCCACGACAAAGCCTTTCCAGAATATCTCCCGAATAGCAATTG
GAGACTGCCCCAAGTATGTCAAACAAGGCTCTTTAAAACTTGCAACAGGAATGAGGAACATTC
CTGAAAAGGCATCGAGAGGGCTCTTTGGAGCAATAGCCGGGTTCATAGAAAATGGCTGGCAAG
GCCTGATTGATGGTTGGTACGGGTTCAGACATCAGAATGCAGAAGGAACAGGAACAGCAGCAG
ACCTCAAGTCCACTCAGGCAGCCATTGATCAGATCAATGGGAAGCTAAATCGTCTTATCGAGA
AAACAAATGAGAAATATCATCAAATCGAAAAGGAATTCGAACAGGTTGAAGGAAGGATTCAAG
ACCTGGAGAAATATGTTGAGGATACAAAGATTGATTTGTGGTCATATAATGCAGAACTATTGG
TTGCACTGGAAAATCAGCACACCATAGATGTAACTGACTCGGAAATGAACAAACTTTTTGAGA
GAGTGAGACGCCAACTCAGAGAAAATGCTGAGGACAAAGGGAATGGATGTTTCGAAATATTCC
ACAAGTGTGATAATAGTTGCATTGAGAGCATTCGAAATGGAACTTATAATCACGACATTTATA
GAGATGAGGCAATCAACAATCGATTCCAAATCCAGGGAGTTAAATTGACCCAAGGATACAAGG
ACATCATTCTTTGGATTTCATTCTCCATATCATGCTTTTTGCTCGTTGCACTACTTTTAGCCT
TCATTTTGTGGGCTG

12.2.15A/Duck/DT416/2006(H3N8)

CAGCAAAAGCAGGGGATACTTTCATTAACCATGAAGGCCATTATTGCTTTAAGCTGCATTTTC
TGTCTGGCTTTCAGTCAGGACCTTCCAGGGAATGACAACAATACAGCAACACTGTGTTTGGGA
CATCATGCAGTACAGAATGGGACACTAGTGAAGACAATCACAGATGATCAGATTGAGGTGACT
AATGCTACCGAATTGGTCCAAAGCTCTTCAACAGGGAAAATATGCAACAATCCTCACAAGATT
CTTGATGGAAGGGATTGCACACTAGTAGATGCCCTGTTAGGGGATCCTCACTGTGATGTCTTT
CAAGATGAAACATGGGACCTTTTTGTGGAACGAAGCAATGCTTTCAGCAATTGTTACCCATAT
GATGTACCAGATTACGCATCCCTTCGATCCCTGATTGCATCATCAGGCACATTAGAGTTCATT
ACAGAGGGTTTCACTTGGACCGGAGTGACTCAGAATGGAGGGAGCGGTGCTTGCAAAAGGGGA
CCTGCTAATGGCTTCTTCAGTAGATTGAATTGGCTGACTAAATCAGGAAGTGCATACCCAGTG
CTTAACGTGACTATGCCAAACAATGACAACTTTGACAAGCTGTACATCTGGGGAGTTCACCAT
CCAAGTACAAATAGAGAACAAACTGACCTGTATGTCCAAGCATCAGGAAGAGTAACAGTTTCT
ACCAGAAAAAGCCAACAGACCATAATCCCGAATATTGGATCTAGACCCTGGGTAAGGGGTCAG
TCTGGCAGAATAAGCATCTATTGGACAATAGTCAAACCTGGGGATGTTCTGGTAATCAATAGT
AATGGAAACCTAATAGCCCCTCGAGGTTATTTCAAGATGCGCATTGGGAAAAGCTCAATAATG
AAATCAGATGCACCTATTGACACCTGCATTTCTGAATGCATCACTCCAAATGGGAGCATTCCC
AATGACAAGCCCTTTCAAAATGTAAACAAGATAACATATGGAGCATGTCCCAAATATGTGAAG
CAAAACACCCTGAAGTTGGCAACAGGRATGCGRAATATGCCTGAGAAACAAACCAGGGGCCTA
TTCGGTGCAATAGCAGGTTTTATAGAAAATGGATGGGAAGGAATGATAGATGGTTGGTATGGA
TTCCGGCATCAAAATTCTGAAGGCACAGGACAAGCAGCAGATCTAAAAAGCACTCAAGCGGCC
ATTGACCAAATCAATGGGAAACTGAACAGAGTAATTGAAAAGACAAATGAAAAGTTCCACCAA
ATAGAAAAGGAATTCTCCGAAGTAGAAGGGAGGATCCAGGACCTTGAGAAGTACGTTGAAGAC
ACAAAGATAGATCTCTGGTCTTACAATGCAGAGCTTCTTGTTGCCCTGGAAAATCAGCACACA
ATTGATTTGACTGATTCAGAAATGAACAAGCTGTTTGAAAAGACCAGGAGACAATTGAGGGAA
AATGCTGAAGATATGGGCAATGGTTGTTTCAAAATATACCACAAATGTGACAATGCTTGCATA
GAATCAATTAGAAACGGGACTTATGACCACGATATATATCGAGATGAGGCATTGAACAATCGG
TTCCAGATCAAGGGTGTGGAGCTGAAATCTGGATATAAGGACTGGATCCTGTGGA

12.2.16A/Duck/DT516/2006(H6N1)

CAGGAGCGAAAGCAGGGGAAAATGATTGCAATCATTGTAATAGCAATACTGGCTTCAGCCGGA
AAATCAGACAAAATCTGTATTGGGTATCATGCCAACAACTCGACAACACAAGTAGATACAATA
CTTGAGAAGAATATCACCGTCACACACTCAGTTGAATTGCTGGAAAATCAGAAGGAAGAAAGA
TTCTGCAAGATTTTGAACAAGGCCCCTCTAGACTTGAGGGGATGCACCATAGAGGGTTGGATT
TTAGGAAATCCTCAATGTGACCTACTGCTTGGTGACCAAAGCTGGTCATATATAGTGGAGAGG
CCCACTGCTCAAAATGGGATTTGCTATCCAGGGGCTTTGAATGAAGTGGAAGAACTGAAGGCA
CTTATTGGGTCAGGAGAAAGAGTAGAAAGATTTGAGATGTTCCCCAAAAGCACATGGGCAGGA
GTAGACACGAATAGTGGGGTGACAAAGGCTTGCCCTTATAATAGTGGCTCATCTTTCTATAGA
AACCTCCTATGGATAATAAAGACCAAGTCAGCAGCATATCCTGTAATTAAAGGAACTTACAAC
AACACTGGAAATCAGCCAATTCTTTATTTCTGGGGTGTGCACCACCCTCCTGACACCAATGAA
CAGAATACCTTGTATGGCTCTGGTGATAGATACGTTAGAATGGGAACCGAAAGCATGAATTTT
GCTAAGGGTCCGGAAATTGCGGCAAGACCTGCTGTGAACGGTCAGAGAGGCAGGATTGATTAT
TATTGGTCTGTTTTAAAACCAGGGGAAACCCTGAATGTGGAGTCTAATGGAAATTTAATTGCC
CCTTGGTATGCATACAAATTTGTCAGCACAAATAACAAAGGAGCCGTCTTCAAATCAAATTTG
CCAATAGAGAACTGTGATGCCACATGCCAGACTGTTGCAGGAGTCCT

12.3 NA sequences

12.3.1 VNM/CL2009-28/2005

GAGATTAAAATGAATCCAAATCAGAAGATAATAACCATGTATGGTAACCGGAATAGTTAGCTT
AATGTTACAAGTTGGGAACATGATCTCAATATGGGTCAGTCATTCAATTAACACAGGGAATCA

ACACCAAGCTGAACCAATCAGCAATGCTAATTTTCTTACTGAGAAAGCTGTGGCTTCAGTAAA
ATTAGCGGGCAATTCATCTCTTTGCCCCATTAACGGATGGGCTGTATACAGTAAGGACAACAG
TATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAGAGCCGTTCATCTCCTGCTCCCA
CTTGGAATGCAGAACTTTCTTTTTGACTCAGGGAGCCTTGCTGAATGACAAACACTCCAATGG
GACTGTCAAAGACAGAAGCCCTCACAGAACACTAATGAGTTGTCCTGTGGGTGAGGCTCCCTC
CCCATATAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAAGTGCTTGCCATGATGGCACCAG
TTGGTTGACAATTGGAATTTCTGGCCCAGACAATGGGGCTGTAGCTGTATTGAAATACAATGG
CATAATAACAGACACTATCAAGAGTTGGAGGAACAACATACTGAGAACTCAAGAGTCTGAATG
TGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGACGGACCAAGTAATGGTCAGGCATC
ACATAAGATCTTCAAAATGGAAAAAGGGAAAGTGGTTAAATCAGTCGAATTGGATGCTCCTAA
TTATTACTATGAGGAATGCTCCTGTTATCCTGATGCCGGCGAAATCACATGTGTGTGCAGGGA
TAATTGGCATGGTTCAAATCGGCCGTGGGTATCTTTCAATCAAAACTTGGAGTATCAAATAGG
ATATATATGCAGTGGAGTTTTCGGAGACACTCCACGCCCCAATGATGGAACAGGTAGTTGTGG
TCCGGTGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAATGGTGT
CTGGATCGGGAGAACCAAAAGCACTAATTCCAGGAGCGGCTTTGAAATGATTTGGGATCCAAA
TGGGTGGACTGAAACGGACAGTAGCTTTTCAGTGAAACAAGATATCGTAGCAATAACTGATTG
GTCAGGATATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGACTAGATTGCATAAGACC
TTGTTTCTGGGTTGAGTTAATCAGAGGGCGGCCCAAAGAGAGCACAATTTGGACTAGTGGGAG
CAGCATATCTTTTTGTGGTGTAAATAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGA
GTTGCCATTCACCATTGACAAGTAGTTGTTCCAAAAACTC

12.3.2 VNM/CL107-13/2005

GAGATTAAAATGAATCCAAATCAGAAGATAATAACCATGTATGGTAACTGGAATAGTTAGCTT
AATGTTACAAGTTGGGAACATGATCTCAATATGGGTCAGTCATTCAATTCACACAGGGAATCA
ACACCAAGTTGAACCAATCAGCAATGCTAATTTTCTTACTGAGAAAGCTGTGGCTTCAGTAAA
ATTAGCGGGCAATTCATCTCTTTGCCCCATTAACGGATGGGCTGTATACAGTAAGGACAACAG
TATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAGAGCCGTTCATCTCATGCTCCCA
CTTGGAATGCAGAACTTTCTTTTTGACTCAGGGAGCCTTGCTGAATGACAAACACTCCAATGG
GACTGTCAAAGACAGAAGCCCTCACAGAGCACTAATGAGTTGTCCTGTGGGTGAGGCTCCCTC
CCCATATAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAAGTGCTTGCCATGATGGCACCAG
TTGGTTGACAATTGGAATTTCTGGCCCAGACAATGGGGCTGTAGCTGTATTGAAATACAATGG
CATAATAACAGACACTATCAAGAGTTGGAGGAACAACATACTGAGAACTCAAGAGTCTGAATG
TGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGACGGACCAAGTAATGGTCAGGCATC
ACATAAGATCTTCAAAATGGAAAAAGGGAAAGTGGTTAAATCAGTCGAATTGGATGCTCCTAA
TTATTACTATGAGGAATGCTCCTGTTATCCTGATGCCGGCGAAATCACATGTGTGTGCAGGGA
TAATTGGCATGGTTCAAATCGGCCGTGGGTATCTTTCAATCAAAACTTGGAGTATCAAATAGG
ATATATATGCAGTGGAGTTTTCGGAGACACTCCACGCCCCAATGATGGAACAGGTAGTTGTGG
TCCGGTGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAATGGTGT
CTGGATCGGGAGAACCAAAAGCACTAATTCCAGGAGCGGCTTTGAAATGATTTGGGATCCAAA
TGGGTGGACTGAAACGGACAGTAGCTTTTCAGTGAAACAAGATATCGTAGCAATAACTGATTG
GTCAGGATATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGACTAGATTGCATAAGACC
TTGTTTCTGGGTTGAGTTAATCAGAGGGCGGCCCAAAGAGAGCACAATTTGGACTAGTGGGAG
CAGCATATCTTTTTGTGGTGTAAATAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGA
GTTGCCATTCACCATTGACAAGTAGTTGTTCCAAAAACTCCT

12.3.3 VNM/PEV16B/2004

GGTAACTGGAATAGTTAGCTTAATGTTACAAGTTGGGAACATGATCTCAATATGGGTCAGTCA
TTCAATTCACACAGGGAGTCAACACCAAGCTGAACCAATCAGCAATACTAATTTTCTTACTGA
GAAAGCTGTGGCTTCAGTAAAATTAGCGGGCAATTCATCTCTTTGCCCCATTAACGGATGGGC
TGTATACAGTAAGGACAACAGTATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAGA
GCCGTTCATCTCATGCTCCCACTTGGAATGCAGAACTTTCTTTTTGACTCAGGGAGCCTTGCT
GAATGACAAACACTCCAATGGGACTGTCAAAGACAGAAGCCCTCACAGAACATTAATGAGTTG
TCCTGTGGGTGAGGCTCCCTCCCCATATAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAAG
TGCTTGCCATGATGGCACCAGTTGGTTGACAATTGGAATTTCTGGCCCAGACAATGGGGCTGT
GGCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGAGTTGGAGGAACAACATACT
GAGAACTCAAGAGTCTGAATGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGACGG
ACCAAGTAATGGTCAGGCATCACATAAGATCTTCAAAATGGAAAAAGGGAAAGTGGTTAAATC
AGTCGAATTGGATGCTCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTGATGCCGGCGA
AATCACATGTGTGTGCAGGGATAATTGGCATGGCTCAAATCGGCCATGGGTATCTTTCAATCA
AAACTTGGAGTATCAAATAGGATATATATGCAGTGGAGTTTTCGGAGACAATCCACGCCCCAA
TGATGGAACAGGTAGTTGTGGTCCGGTGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTC
ATTTAAATACGGCAATGGTGTCTGGATCGGGAGAACCAAAAGCACTAATTCCAGGAGCGGCTT
TGAAATGATTTGGGATCCAAATGGGTGGACTGAAACGGACAGTAGCTTTTCAGTGAAACAAGA
TATCGTAGCAATAACTGATTGGTCAGGATATAGCGGGAGTTTTGTCCAGCATCCAGAACTGAC
AGGACTAGATTGCATAAGACCTTGTTTCTGGGTTGAGTTGATCAGAGGGCGGCCCAAAGAGAG
CACAATTTGGACTAGTGGGAGCAGCATATCTTTTTGTGGTGTAAATAGTGACACTGTGGGTTG
GTCTTGGCCAGACGGTGCTGA

12.3.4 VNM/PEV16C/2004

GGTAACTGGAATAGTTAGCTTAATGTTACAAGTTGGGAACATGATCTCAATATGGGTCAGTCA
TTCAATTCACACAGGGAGTCAACACCAAGCTGAACCAATCAGCAATACTAATTTTCTTACTGA
GAAAGCTGTGGCTTCAGTAAAATTAGCGGGCAATTCATCTCTTTGCCCCATTAACGGATGGGC
TGTATACAGTAAGGACAACAGTATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAGA
GCCGTTCATCTCATGCTCCCACTTGGAATGCAGAACTTTCTTTTTGACTCAGGGAGCCTTGCT
GAATGACAAACACTCCAATGGGACTGTCAAAGACAGAAGCCCTCACAGAACATTAATGAGTTG
TCCTGTGGGTGAGGCTCCCTCCCCATATAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAAG
TGCTTGCCATGATGGCACCAGTTGGTTGACAATTGGAATTTCTGGCCCAGACAATGGGGCTGT
GGCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGAGTTGGAGGAACAACATACT
GAGAACTCAAGAGTCTGAATGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGACGG
ACCAAGTAATGGTCAGGCATCACATAAGATCTTCAAAATGGAAAAAGGGAAAGTGGTTAAATC
AGTCGAATTGGATGCTCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTGATGCCGGCGA
AATCACATGTGTGTGCAGGGATAATTGGCATGGCTCAAATCGGCCATGGGTATCTTTCAATCA
AAACTTGGAGTATCAAATAGGATATATATGCAGTGGAGTTTTCGGAGACAATCCACGCCCCAA
TGATGGAACAGGTAGTTGTGGTCCGGTGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTC
ATTTAAATACGGCAATGGTGTCTGGATCGGGAGAACCAAAAGCACTAATTCCAGGAGCGGCTT
TGAAATGATTTGGGATCCAAATGGGTGGACTGAAACGGACAGTAGCTTTTCAGTGAAACAAGA
TATCGTAGCAATAACTGATTGGTCAGGATATAGCGGGAGTTTTGTCCAGCATCCAGAACTGAC
AGGACTAGATTGCATAAGACCTTGTTTCTGGGTTGAGTTGATCAGAGGGCGGCCCAAAGAGAG
CACAATTTGGACTAGTGGGAGCAGCATATCTTTTTGTGGTGTAAATAGTGACACTGTGGGTTG
GTCTTGGCCAGACGGTGCTGA

12.3.5 VNM/PEV16R/2004

GGTAACTGGAATAGTTAGCTTAATGTTACAAGTTGGGAACATGATCTCAATATGGGTCAGTCA
TTCAATTCACACAGGGAGTCAACACCAAGCTGAACCAATCAGCAATACTAATTTTCTTACTGA
GAAAGCTGTGGCTTCAGTAAAATTAGCGGGCAATTCATCTCTTTGCCCCATTAACGGATGGGC
TGTATACAGTAAGGACAACAGTATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAGA
GCCGTTCATCTCATGCTCCCACTTGGAATGCAGAACTTTCTTTTTGACTCAGGGAGCCTTGCT
GAATGACAAACACTCCAATGGGACTGTCAAAGACAGAAGCCCTCACAGAACATTAATGAGTTG
TCCTGTGGGTGAGGCTCCCTCCCCATATAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAAG
TGCTTGCCATGATGGCACCAGTTGGTTGACAATTGGAATTTCTGGCCCAGACAATGGGGCTGT
GGCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGAGTTGGAGGAACAACATACT
GAGAACTCAAGAGTCTGAATGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGACGG
ACCAAGTAATGGTCAGGCATCACATAAGATCTTCAAAATGGAAAAAGGGAAAGTGGTTAAATC
AGTCGAATTGGATGCTCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTGATGCCGGCGA
AATCACATGTGTGTGCAGGGATAATTGGCATGGCTCAAATCGGCCATGGGTATCTTTCAATCA
AAACTTGGAGTATCAAATAGGATATATATGCAGTGGAGTTTTCGGAGACAATCCACGCCCCAA
TGATGGAACAGGTAGTTGTGGTCCGGTGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTC
ATTTAAATACGGCAATGGTGTCTGGATCGGGAGAACCAAAAGCACTAATTCCAGGAGCGGCTT
TGAAATGATTTGGGATCCAAATGGGTGGACTGAAACGGACAGTAGCTTTTCAGTGAAACAAGA
TATCGTAGCAATAACTGATTGGTCAGGATATAGCGGGAGTTTTGTCCAGCATCCAGAACTGAC
AGGACTAGATTGCATAAGACCTTGTTTCTGGGTTGAGTTGATCAGAGGGCGGCCCAAAGAGAG
CACAATTTGGACTAGTGGGAGCAGCATATCTTTTTGTGGTGTAAATAGTGACACTGTGGGTTG
GTCTTGGCCAGACGGTGCTGA

12.4 PB2 sequences

12.4.1 VNM/CL2009-28/2005

AGTGCATTTCATTATCCAAAGGTTTACAAAACATACTTTGAGAAGGTTGAAAGGTTAAAACAT
GGAACCTTCGGTCCCGTCCATTTCCGAAACCAAGTTAAAATACGCCGCCGAGTGGATATAAAT
CCTGGCCATGCAGATCTCAGTGCTAAAGAAGCACAAGATGTCATCATGGAAGTCGTTTTCCCA
AATGAAGTGGGAGCTAGAATATTGACATCAGAGTCGCAATTGACAATAACGAAAGAGAAGAAA
GAAGAGCTCCAAGATTGTAAGATTGCTCCCTTAATGGTTGCATACATGTTGGAAAGGGAACTG
GTCCGCAAAACCAGATTCCTACCGGTAGCAGGCGGAACAAGCAGTGTGTACATTGAGGTATTG
CATTTGACTCAAGGGACCTGCTGGGAACAGATGTACACTCCAGGCGGAGAAGTGAGAAATGAC
GATGTTGACCAGAGTTTGATCATCGCTGCCAGAAACATTGTTAGGAGGGCAACGGTATCAGCG
GATCCACTGGCATCGCTGCTGGAGATGTGTCACAGCACACAAATTGGTGGGATAAGGATGGTG
GACATCCTTAGGCAAAATCCAACTGAGGAACAAGCTGTGGATATATGCAAAGCAGCAATGGGT
CTGAGGATCAGTTCTTCCTTTAGCTTTGGAGGCTTCACTTTCAAAAGAACAAGTGGATCATCC
GTCAAGAAGGAAGAGGAAGTACTTACAGGCAACCTCCAAACATTGAAAATAAAAGTACATGAG
GGGTATGAGGAATTCACAATGGTTGGGCGGAGGGCAACAGCTATCCTGAGGAAAGCAACTAGA
AGGCTGATTCAGTTGATAGTAAGTGGAAGAGACGAACAATCAATCGCTGAGGCAATCATTGTA
GCAATGGTGTTCTCACAGGAGGATTGCATGATAAAGGCAGTCCGAGGCGATCTGAATTTCGTA
AACAGAGCAAACCAAAGATTAAACACCATGCATCAACTCCTGAGACATTTTCAAAAGGATGCA
AAAGTGTTATTTCAGAATTGGGGAATTGAACCCATTGATAATGTCATGGGGATGATCGGAATA
TTACCTGACATGACTCCCAGCACAGAAATGTCACTGAGAGGAGTAAGAGTTAGTAAAATGGGA
GTGGATGAATATTCCAGCACTGAGAGAGTAGTTGTAAGTATTGACCGTTTCTTAAGGGTTCGA
GATCAGCGGGGGAACGTACTCTTATCTCCCGAAGAGGTCAGCGAAACCCAGGGAACAGAGAAA
TTGACAATAACATATTCATCATCAATGATGTGGGAAATCAACGGTCCTGAGTCAGTGCTTGTT
AACACCTATCAGTGGATCATCAGAAACTGGGAGACTGTGAAGATTCAATGGTCTCAAGACCCC

ACGATGCTGTACAATAAGATGGAGTTTGAACCGTTCCAATCCTTGGTACCCAAAGCTGCCAGA
GGTCAATACAGTGGATTTGTGAGAACATTATTCCAACAAATGCGTGACGTACTGGGGACATTT
GATACTGTCCAGATAATAAAGCTGCTACCATTTGCAGCAGCCCCACCGAAGCAGAGCAGAATG
CAGTTTTCTTCTCTAACTGTGAATGTGAGAGGCTCAGGAATGAGAATACTCGTAAGGGGCAAT
TCCCCTGTGTTCAACTACAATAAGGCAACCAAAAGGCTTACCGTTCTTGGAAAGGACGCAGGT
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CTTGCGAAGGGGGAGAAAGCTAATGTGCTGATAGGGCAAGGAGACGTGGTGTTGGTAATGAAA
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12.4.2 VNM/CL107-13/2005

ACGAGATCTTACGAGATCTAATGTCACAGTCCCGCACTCGCGAGATACTAACAAAAACCACTG
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TTCATTATCCAAAGGTTTACAAAACATACTTTGAGAAGGTTGAAAGGTTAAAACATGGAACCT
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TGGCATCGCTGCTGGAGATGTGTCACAGCACACAAATTGGTGGGATAAGGATGGTGGACATCC
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AGTGTCGAATTGTTTAAAAA

12.4.3 VNM/PEV16B/2004

TCGCGAGATACTAACAAAAACCACTGTGGACCATATGGCCATAATCAAGAAATACACATCAGG
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GGTTGAAAGGTTAAAACATGGAACCTTCGGTCCCGTCCATTTCCGAAACCAAGTTAAAATACG
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AATAACGAAAGAGAAGAAAGAAGAGCTCCAAGATTGTAAGATTGCTCCCTTAATGGTTGCATA
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CATCAATGAACTGAGCAATCTTGCGAAGGGGGAGAAAGCTAATGTGCTGATAGGGCAAGGAGA
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12.4.4 VNM/PEV16C/2004

TCGCGAGATACTAACAAAAACCACTGTGGACCATATGGCCATAATCAAGAAATACACATCAGG
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12.4.5 VNM/PEV16R/2004

TCGCGAGATACTAACAAAAACCACTGTGGACCATATGGCCATAATCAAGAAATACACATCAGG
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12.5 PB1 sequences

12.5.1 VNM/PEV16B/2004

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GATACTGAACTCTCCTTTACAATTACTGGAGACAATACCAAATGGAATGAGAATCAGAATCCT
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GCTCTTCAGTTATTCATCAAGGACTACAGATACACATACCGATGCCACAGAGGGGATACGCAA
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12.5.2 VNM/PEV16C/2004

ATGGATGTCAATCCGACTTTACTTTTCTTGAAAGTACCAGTGCAAAATGCTATAAGTACCACA
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TGCTTAAAATGGGAATTGATGGATGAAGATTACCAGGGCAGACTGTGTAATCCTCTGAATCCA
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12.5.3 VNM/PEV16R/2004

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12.6 PA sequences

12.6.1 VNM/PEV16B/2004

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AATGGCCACCAAAGCGGACTACACCCTTGATGAAGAGAGCAGGGCAAGAATTAAAACCAGGCT
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CGTGAAACCACATGAAAAAGGTATAAACCCCAATTACCTCCTGGCTTGGAAGCAAGTGCTGGC
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CCAGTTGAAGTGGGCACTCGGTGAGAACATGGCACCAGAGAAAGTAGACTTTGAGGACTGCAA
AGATGTTAGCGATCTAAGACAGTATGACAGTGATGAACCAGAGTCTAGATCACTAGCAAGCTG
GATTCAGAGTGAATTCAACAAGGCATGTGAATTGACAGATTCGAGTTGGATTGAACTTGATGA
GATAGGAGAAGACGTAGCTCCAATTGAGCACATTGCAAGTATGAGAAGGAACTATTTTACAGC
GGAAGTATCCCATTGCAGGGCCACTGAATACATAATGAAGGGGGTGTACATAAACACAGCCCT
GTTGAATGCATCCTGTGCAGCCATGGATGACTTTCAACTGATTCCAATGATAAGCAAATGCAG
AACCAAAGAAGGAAGACGGAAAACTAATCTGTATGGATTCATTATAAAAGGGAGATCCCACTT
GAGGAATGATACCGATGTGGTAAATTTTGTGAGTATGGAATTCTCTCTTACTGATCCGAGGCT
GGAGCCACACAAGTGGGAAAAGTACTGTGTCCTCGAGATAGGAGACATGCTCCTCCGGACTGC
AGTAGGCCAAGTTTCAAGGCCCATGTTCCTGTATGTAAGAACCAATGGAACCTCCAAGATCAA
AATGAAATGGGGCATGGAAATGAGGCGATGCCTTCTTCAATCCCTTCAACAAATTGAAAGCAT
GATTGAAGCCGAGTCTTCTGTCAAAGAGAAGGACATGACCAAAGAATTCTTTGAAAACAAATC
AGAAACATGGCCGATTGGAGAGTCCCCCAAGGGAGTGGAGGAAGGCTCCATCGGAAAGGTGTG
CAGAACCTTGCTGGCGAAGTCTGTGTTCAACAGTTTATATGCATCTCCACAACTCGAGGGGTT
TTCAGCTGAATCAAGAAAATTGCTTCTCATTGCTCAGGCACTTAGGGACAACCTGGAACCTGG
GACCTTCGATCTTGGAGGGCTATATGAAGCAATTGAGGAGTGCCTGATTAACGATCCCTGGGT
TTTGCTTAATGCGTCTTGGTTCAACTCCTTCCTCGCACATGCACTGAAATAGTTGTGGCAATG
CTACTATTTGCTATCCATACTGTC

12.6.2 VNM/PEV16C/2004

ATGCTTCAATCCAATGATTGTCGAGCTTGCAGAAAAGGCAATGAAAGAATATGGGGAAGATCC
GAAAATCGAAACGAACAAGTTTGCTGCAATATGCACACACTTGGAGGTCTGTTTCATGTATTC
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ATTATTGAAACACCGATTTGAAATAATTGAAGGAAGAGACCGAACGATGGCCTGGACTGTGGT
GAATAGTATCTGCAACACCACAGGAGTTGAGAAACCTAAATTTCTCCCAGATTTGTATGACTA
CAAAGAGAACCGATTCATCGAAATTGGAGTGACACGGAGGGAAGTTCATACATACTATCTGGA
GAAAGCCAACAAGATAAAATCCGAGAAGACACATATTCACATATTCTCATTCACAGGGGAGGA
AATGGCCACCAAAGCGGACTACACCCTTGATGAAGAGAGCAGGGCAAGAATTAAAACCAGGCT
GTTCACCATAAGGCAGGAAATGGCCAGTAGGGGTCTATGGGATTCCTTTCGTCAATCCGAGAG
AGGCGAAGAGACAATTGAAGAAAAATTTGAAATCACTGGAACCATGCGCAGACTTGCAGACCA
AAGTCTCCCACCGAACTTCTCCAGCCTTGAAAACTTTAGAGCCTATGTGGATGGATTCGAACC
GAACGGCTGCATTGAGGGCAAGCTTTCTCAAATGTCAAAAGAAGTGAATGCTAGAATTGAGCC
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GAAGTTCTTGCTGATGGATGCCCTTAAATTAAGCATCGAAGACCCGAGTCATGAGGGGGAGGG
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GATTCAGAGTGAATTCAACAAGGCATGTGAATTGACAGATTCGAGTTGGATTGAACTTGATGA
GATAGGAGAAGACGTAGCTCCAATTGAGCACATTGCAAGTATGAGAAGGAACTATTTTACAGC
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GTTGAATGCATCCTGTGCAGCCATGGATGACTTTCAACTGATTCCAATGATAAGCAAATGCAG
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AGTAGGCCAAGTTTCAAGGCCCATGTTCCTGTATGTAAGAACCAATGGAACCTCCAAGATCAA
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GATTGAAGCCGAGTCTTCTGTCAAAGAGAAGGACATGACCAAAGAATTCTTTGAAAACAAATC
AGAAACATGGCCGATTGGAGAGTCCCCCAAGGGAGTGGAGGAAGGCTCCATCGGAAAGGTGTG
CAGAACCTTGCTGGCGAAGTCTGTGTTCAACAGTTTATATGCATCTCCACAACTCGAGGGGTT
TTCAGCTGAATCAAGAAAATTGCTTCTCATTGCTCAGGCACTTAGGGACAACCTGGAACCTGG
GACCTTCGATCTTGGAGGGCTATATGAAGCAATTGAGGAGTGCCTGATTAACGATCCCTGGGT
TTTGCTTAATGCGTCTTGGTTCAACTCCTTCCTCGCACATGCACTGAAATAGTTGTGGCAATG
CTACTATTTGCTATCCATACTGTC

12.6.3 VNM/PEV16R/2004

ATGCTTCAATCCAATGATTGTCGAGCTTGCAGAAAAGGCAATGAAAGAATATGGGGAAGATCC
GAAAATCGAAACGAACAAGTTTGCTGCAATATGCACACACTTGGAGGTCTGTTTCATGTATTC
GGATTTTCACTTTATTGATGAACGGAGTGAATCAATAATTGTAGAATCTGGAGATCCGAATGC
ATTATTGAAACACCGATTTGAAATAATTGAAGGAAGAGACCGAACGATGGCCTGGACTGTGGT
GAATAGTATCTGCAACACCACAGGAGTTGAGAAACCTAAATTTCTCCCAGATTTGTATGACTA
CAAAGAGAACCGATTCATCGAAATTGGAGTGACACGGAGGGAAGTTCATACATACTATCTGGA
GAAAGCCAACAAGATAAAATCCGAGAAGACACATATTCACATATTCTCATTCACAGGGGAGGA
AATGGCCACCAAAGCGGACTACACCCTTGATGAAGAGAGCAGGGCAAGAATTAAAACCAGGCT
GTTCACCATAAGGCAGGAAATGGCCAGTAGGGGTCTATGGGATTCCTTTCGTCAATCCGAGAG
AGGCGAAGAGACAATTGAAGAAAAATTTGAAATCACTGGAACCATGCGCAGACTTGCAGACCA
AAGTCTCCCACCGAACTTCTCCAGCCTTGAAAACTTTAGAGCCTATGTGGATGGATTCGAACC
GAACGGCTGCATTGAGGGCAAGCTTTCTCAAATGTCAAAAGAAGTGAATGCTAGAATTGAGCC
ATTTTTGAAGACAACGCCACGCCCTCTCAGACTACCTGATGGGCCTCCTTGCTCTCAGCGGTC
GAAGTTCTTGCTGATGGATGCCCTTAAATTAAGCATCGAAGACCCGAGTCATGAGGGGGAGGG
GATACCACTATACGATGCAATCAAATGCATGAAGACATTTTTCGGCTGGAAAGAACCCAACAT
CGTGAAACCACATGAAAAAGGTATAAACCCCAATTACCTCCTGGCTTGGAAGCAAGTGCTGGC
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CCAGTTGAAGTGGGCACTCGGTGAGAACATGGCACCAGAGAAAGTAGACTTTGAGGACTGCAA
AGATGTTAGCGATCTAAGACAGTATGACAGTGATGAACCAGAGTCTAGATCACTAGCAAGCTG
GATTCAGAGTGAATTCAACAAGGCATGTGAATTGACAGATTCGAGTTGGATTGAACTTGATGA
GATAGGAGAAGACGTAGCTCCAATTGAGCACATTGCAAGTATGAGAAGGAACTATTTTACAGC
GGAAGTATCCCATTGCAGGGCCACTGAATACATAATGAAGGGGGTGTACATAAACACAGCCCT
GTTGAATGCATCCTGTGCAGCCATGGATGACTTTCAACTGATTCCAATGATAAGCAAATGCAG
AACCAAAGAAGGAAGACGGAAAACTAATCTGTATGGATTCATTATAAAAGGGAGATCCCACTT
GAGGAATGATACCGATGTGGTAAATTTTGTGAGTATGGAATTCTCTCTTACTGATCCGAGGCT
GGAGCCACACAAGTGGGAAAAGTACTGTGTCCTCGAGATAGGAGACATGCTCCTCCGGACTGC
AGTAGGCCAAGTTTCAAGGCCCATGTTCCTGTATGTAAGAACCAATGGAACCTCCAAGATCAA
AATGAAATGGGGCATGGAAATGAGGCGATGCCTTCTTCAATCCCTTCAACAAATTGAAAGCAT
GATTGAAGCCGAGTCTTCTGTCAAAGAGAAGGACATGACCAAAGAATTCTTTGAAAACAAATC
AGAAACATGGCCGATTGGAGAGTCCCCCAAGGGAGTGGAGGAAGGCTCCATCGGAAAGGTGTG
CAGAACCTTGCTGGCGAAGTCTGTGTTCAACAGTTTATATGCATCTCCACAACTCGAGGGGTT
TTCAGCTGAATCAAGAAAATTGCTTCTCATTGCTCAGGCACTTAGGGACAACCTGGAACCTGG
GACCTTCGATCTTGGAGGGCTATATGAAGCAATTGAGGAGTGCCTGATTAACGATCCCTGGGT
TTTGCTTAATGCGTCTTGGTTCAACTCCTTCCTCGCACATGCACTGAAATAGTTGTGGCAATG
CTACTATTTGCTATCCATACTGTC

12.7 NP sequences

12.7.1 VNM/CL107-13/2005

ACCGAGTGACATCAACATCATGGCGCGATCTTATGAACAGATGGAAACTGGTGGGGAACGCCA
GAATGCTACTGAGATCAGGGCATCTGTTGGAAGAATGGTTAGTGGCATTGGGAGGTTCTACAT
ACAGATGTGCACAGAACTCAAACTCAGTGACTATGAAGGGAGGCTGATCCAGAACAGCATAAC
AATAGAGAGAATGGTACTCTCTGCATTTGATGAAAGAAGGAACAGATACCTGGAAGAACACCC
CAGTGCGGGAAAGGACCCGAAGAAGACTGGAGGTCCAATTTATCGGAGGAGAGACGGAAAGTG
GGTGAGAGAGCTAATTCTGTACGACAAAGAGGAGATCAGGAGGATTTGGCGTCAAGCGAACAA
TGGAGAGGACGCAACTGCTGGTCTTACCCACCTGATGATATGGCATTCCAATCTAAATGATGC
CACATATCAGAGAACGAGAGCTCTCGTGCGTACTGGAATGGACCCAAGGATGTGCTCTCTGAT
GCAAGGGTCAACTCTCCCGAGGAGATCTGGAGCTGCTGGTGCAGCAGTAAAGGGGGTAGGGAC
AATGGTGATGGAGCTGATTCGGATGATAAAACGAGGGATCAACGACCGGAATTTCTGGAGAGG
CGAAAATGGAAGAAGAACAAGGATTGCATATGAGAGAATGTGCAACATCCTCAAAGGGAAATT
CCAAACAGCAGCACAAAGAGCAATGATGGATCAAGTGCGAGAGAGCAGAAATCCTGGGAATGC
TGAAATTGAAGATCTCATTTTTCTGGCACGGTCTGCACTCATCCTGAGAGGATCAGTGGCCCA
TAAGTCCTGTTTGCCTGCTTGTGTGTACGGACTTGCAGTGGCCAGTGGATATGACTTTGAGAG
AGAAGGGTACTCTCTGGTTGGAATAGATCCTTTCCGCCTGCTTCAAAACAGCCAGGTCTTTAG
TCTCATTAGACCAAATGAGAATCCAGCACATAAGAGTCAATTAGTGTGGATGGCATGCCACTC
TGCAGCATTTGAGGACCTTAGAGTCTCAAGTTTCATCAGAGGGACAAGAGTGGTCCCAAGAGG
ACAGCTATCCACCAGAGGGGTTCAAATTGCTTCAAATGAGAACATGGAGGCAATGGACTCCAA
CACTCTTGAACTGAGAAGCAGATATTGGGCTATAAGAACCAGAAGCGGAGGAAACACCAACCA
GCAGAGGGCATCTGCAGGACAGATCAGCGTTCAGCCCACTTTCTCGGTACAGAGAAACCTTCC
CTTCGAAAGAGCGACCATTATGGCAGCATTTACAGGAAATACTGAGGGCAGAACGTCTGACAT
GAGAACTGAAATCATAAGAATGATGGAAAGTGCCAGACCAGAAGATGTGTCATTCCAGGGGCG
GGGAGTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAA
TAATGAAGGATCTTATTTCTTCGGAGACAATGCA

12.7.2 VNM/PEV16B/2004

TCTTATGAACAGATGGAAACTGGTGGGGAACGCCAGAATGCTACTGAGATCAGGGCATCTGTT
GGAAGAATGGTTAGTGGCATTGGGAGGTTCTACATACAGATGTGCACAGAACTCAAACTCAGT
GACTATGAAGGGAGGCTGATCCAGAACAGCATAACAATAGAGAGAATGGTACTCTCTGCATTT
GATGAAAGAAGGAACAGATACCTGGAAGAACACCCCAGTGCGGGAAAGGACCCGAAGAAGACT
GGAGGTCCAATTTATCGGAGGAGAGACGGGAAATGGGTGAGAGAGCTAATTCTGTACGACAAA
GAGGAGATCAGGAGGATTTGGCGTCAAGCGAACAATGGAGAGGACGCAACTGCTGGTCTTACC
CACCTGATGATATGGCATTCCAATCTAAATGATGCCACATATCAGAGAACGAGAGCTCTCGTG
CGTACTGGAATGGACCCAAGGATGTGCTCTCTGATGCAAGGGTCAACTCTCCCGAGGAGATCT
GGAGCTGCTGGTGCAGCAGTAAAGGGGGTAGGGACAATGGTGATGGAGCTGATTCGGATGATA
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TATGAGAGAATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACAAAGAGCAATGATG
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CGGTCTGCACTCATCCTGAGAGGATCAGTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTAC
GGACTTGCAGTGGCCAGTGGATATGACTTTGAGAGAGAAGGGTACTCTCTGGTTGGAATAGAT
CCTTTCCGCCTGCTTCAAAACAGCCAGGTCTTTAGTCTCATTAGACCAAATGAGAATCCAGCA
CATAAGAGTCAATTAGTGTGGATGGCATGCCACTCTGCAGCATTTGAGGACCTTAGAGTCTCA
AGTTTCATCAGAGGGACAAGAGTGGTCCCAAGAGGACAGCTATCCACCAGAGGGGTTCAAATT
GCTTCAAATGAGAACATGGAGGCAATGGACTCCAACACTCTTGAACTGAGAAGCAGATATTGG
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GTTCAGCCCACTTTCTCGGTACAGAGAAACCTTCCCTTCGAAAGAGCGACCATTATGGCAGCA
TTTACAGGAAATACTGAGGGCAGAACGTCTGACATGAGGACTGAAATCATAAGAATGATGGAA
AGTGCCAGACCAGAAGATGTGTCATTCCAGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAG
GCAACGAACCCGATCGTGCCTTCCTTTGACATGAATAATGAAGGATCTTATTTCTTCGGAGAC
AATGCAGAGGA

12.7.3 VNM/PEV16C/2004

TCTTATGAACAGATGGAAACTGGTGGGGAACGCCAGAATGCTACTGAGATCAGGGCATCTGTT
GGAAGAATGGTTAGTGGCATTGGGAGGTTCTACATACAGATGTGCACAGAACTCAAACTCAGT
GACTATGAAGGGAGGCTGATCCAGAACAGCATAACAATAGAGAGAATGGTACTCTCTGCATTT
GATGAAAGAAGGAACAGATACCTGGAAGAACACCCCAGTGCGGGAAAGGACCCGAAGAAGACT
GGAGGTCCAATTTATCGGAGGAGAGACGGGAAATGGGTGAGAGAGCTAATTCTGTACGACAAA
GAGGAGATCAGGAGGATTTGGCGTCAAGCGAACAATGGAGAGGACGCAACTGCTGGTCTTACC
CACCTGATGATATGGCATTCCAATCTAAATGATGCCACATATCAGAGAACGAGAGCTCTCGTG
CGTACTGGAATGGACCCAAGGATGTGCTCTCTGATGCAAGGGTCAACTCTCCCGAGGAGATCT
GGAGCTGCTGGTGCAGCAGTAAAGGGGGTAGGGACAATGGTGATGGAGCTGATTCGGATGATA
AAACGAGGGATCAACGACCGGAATTTCTGGAGAGGCGAAAATGGAAGAAGAACAAGGATTGCA
TATGAGAGAATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACAAAGAGCAATGATG
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CGGTCTGCACTCATCCTGAGAGGATCAGTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTAC
GGACTTGCAGTGGCCAGTGGATATGACTTTGAGAGAGAAGGGTACTCTCTGGTTGGAATAGAT
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AGTTTCATCAGAGGGACAAGAGTGGTCCCAAGAGGACAGCTATCCACCAGAGGGGTTCAAATT
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TTTACAGGAAATACTGAGGGCAGAACGTCTGACATGAGGACTGAAATCATAAGAATGATGGAA
AGTGCCAGACCAGAAGATGTGTCATTCCAGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAG
GCAACGAACCCGATCGTGCCTTCCTTTGACATGAATAATGAAGGATCTTATTTCTTCGGAGAC
AATGCAGAGGA

12.7.4 VNM/PEV16R/2004

TCTTATGAACAGATGGAAACTGGTGGGGAACGCCAGAATGCTACTGAGATCAGGGCATCTGTT
GGAAGAATGGTTAGTGGCATTGGGAGGTTCTACATACAGATGTGCACAGAACTCAAACTCAGT
GACTATGAAGGGAGGCTGATCCAGAACAGCATAACAATAGAGAGAATGGTACTCTCTGCATTT
GATGAAAGAAGGAACAGATACCTGGAAGAACACCCCAGTGCGGGAAAGGACCCGAAGAAGACT
GGAGGTCCAATTTATCGGAGGAGAGACGGGAAATGGGTGAGAGAGCTAATTCTGTACGACAAA
GAGGAGATCAGGAGGATTTGGCGTCAAGCGAACAATGGAGAGGACGCAACTGCTGGTCTTACC
CACCTGATGATATGGCATTCCAATCTAAATGATGCCACATATCAGAGAACGAGAGCTCTCGTG
CGTACTGGAATGGACCCAAGGATGTGCTCTCTGATGCAAGGGTCAACTCTCCCGAGGAGATCT
GGAGCTGCTGGTGCAGCAGTAAAGGGGGTAGGGACAATGGTGATGGAGCTGATTCGGATGATA
AAACGAGGGATCAACGACCGGAATTTCTGGAGAGGCGAAAATGGAAGAAGAACAAGGATTGCA
TATGAGAGAATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACAAAGAGCAATGATG
GATCAAGTGCGAGAGAGCAGAAATCCTGGGAATGCTGAAATTGAAGATCTCATTTTTCTGGCA
CGGTCTGCACTCATCCTGAGAGGATCAGTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTAC
GGACTTGCAGTGGCCAGTGGATATGACTTTGAGAGAGAAGGGTACTCTCTGGTTGGAATAGAT

CCTTTCCGCCTGCTTCAAAACAGCCAGGTCTTTAGTCTCATTAGACCAAATGAGAATCCAGCA
CATAAGAGTCAATTAGTGTGGATGGCATGCCACTCTGCAGCATTTGAGGACCTTAGAGTCTCA
AGTTTCATCAGAGGGACAAGAGTGGTCCCAAGAGGACAGCTATCCACCAGAGGGGTTCAAATT
GCTTCAAATGAGAACATGGAGGCAATGGACTCCAACACTCTTGAACTGAGAAGCAGATATTGG
GCTATAAGAACCAGAAGCGGAGGAAACACCAACCAGCAGAGGGCATCTGCAGGACAGATCAGC
GTTCAGCCCACTTTCTCGGTACAGAGAAACCTTCCCTTCGAAAGAGCGACCATTATGGCAGCA
TTTACAGGAAATACTGAGGGCAGAACGTCTGACATGAGGACTGAAATCATAAGAATGATGGAA
AGTGCCAGACCAGAAGATGTGTCATTCCAGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAG
GCAACGAACCCGATCGTGCCTTCCTTTGACATGAATAATGAAGGATCTTATTTCTTCGGAGAC
AATGCAGAGGA

12.8 MP sequences

12.8.1 VNM/CL2009-28/2005

AAAGATGAGTCTTCTAACCGAGGTCGATCATCCCGTCAGGCCCCCTCAAAGCCGAGATCGCGC
GGAAACTTGAAGATGTCTTTGCAGGAAAGAACACCGATCTCGAGGCTCTCATGGAGTGGCTAA
AGACAAGACCAATCCTGTCACCTCTGACTAAAGGGATTTTGGGATTTGTATTCACGCTCACCG
TGCCCAGTGAGCGAGGACTGCAGCGTAGACGCTTTGTCCAGAACGCCCTAAATGGAAATGGAG
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ATGGGGCTAAGGAGGTCGCACTCAGCTACTCAACCGGTGCACTTGCCAGTTGCATGGGTCTCA
TATACAACAGGATGGGAACGGTGACTACGGAAGTGGCTTTTGGCCTAGTGTGTGCCACTTGTG
AGCAGATTGCAGATTCACAGCATCGGTCTCACAGACAGATGGCAACTATCACCAACCCACTAA
TCAGACATGAGAACAGAATGGTGCTGGCCAGCACTACAGCTAAGGCTATGGAGCAGATGGCGG
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CAATGAGGACAATTGGGACTCATCCTAACTCTAGTGCTGGTCTGAGAGATAATCTTCTTGAAA
ATTTGCAGGCCTACCAGAAACGAATGGGAGTGCAGATGCAGCGATTCAAGTGACCCTATTGTT
GTTGCCGCAAATATCATTGGGATCTTGCACTTGATATTGTGGATTCTTGATCGTCTTTTCTTC
AAATGCATTTATCGTCGCCTTAAATACGGTTTGAAAAGAGGGCCTGCTATGGCAGGGGTACCT
GAGTCTATGAGGGAAGAGTACCGGCAGGAACAGCAGAGTGCTGTGGATGTTGACGATGGTCAT
TTTGTCAACATAGAATTGGAGTAAAAA

12.8.2 VNM/CL107-13/2005

AAAGATGAGTCTTCTAACCGAGGTCGATCATCCCGTCAGGCCCCCTCAAAGCCGAGATCGCGC
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TGCCCAGTGAGCGAGGACTGCAGCGTAGACGCTTTGTCCAGAACGCCCTAAATGGAAATGGAG
ATCCAAATAATATGGATAGGGCAGTTAAGCTATATAAGAAGCTGAAAAGAGAAATAACATTCC
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TCAGACATGAGAACAGAATGGTGCTGGCCAGCACTACAGCTAAGGCTATGGAGCAGATGGCGG
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CAATGAGGACAATTGGGACTCATCCTAACTCTAGTGCTGGTCTGAGAGATAATCTTCTTGAAA
ATTTGCAGGCCTACCAGAAACGAATGGGAGTGCAGATGCAGCGATTCAAGTGATCCTATTGTT
GTTGCCGCAAATATCATTGGGATCTTGCACTTGATATTGTGGATTCTTGATCGTCTTTTCTTC
AAATGCATTTATCGTCGCCTTAAATACGGTTTGAAAAGAGGGCCTGCTATGGCAGGGGTACCT

GAGTCTATGAGGGAAGAGTACCGGCAGGAACAGCAGAGTGCTGTGGATGTTGACGATGGTCAT
TTTGTCAACATAGAATTGGAGTAAAAAT

12.8.3 VNM/PEV16B/2004

AGGCCCCCTCAAAGCCGAGATCGCGCAGAAACTTGAAGATGTCTTTGCAGGAAAGAACACCGA
TCTCGAGGCTCTCATGGAGTGGCTAAAGACAAGACCAATCCTGTCACCTCTGACTAAAGGGAT
TTTGGGATTTGTATTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCGTAGACGCTTTGT
CCAGAACGCCCTAAATGGAAATGGAGATCCAAATAATATGGATAGGGCAGTTAAGCTATATAA
GAAGCTGAAAAGAGAAATAACATTCCATGGGGCTAAGGAGGTCGCACTCAGCTACTCAACCGG
TGCACTTGCCAGTTGCATGGGTCTCATATACAACAGGATGGGAACGGTGACTACGGAAGTGGC
TTTTGGCCTAGTGTGTGCCACTTGTGAGCAGATTGCAGATTCACAGCATCGGTCTCACAGACA
GATGGCAACTATCACCAACCCACTAATCAGACATGAGAACAGAATGGTGCTGGCCAGCACTAC
AGCTAAGGCTATGGAGCAGATGGCGGGATCAAGTGAGCAGGCAGCGGAAGCCATGGAGATCGC
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TGGTCTGAGAGATAATCTTCTTGAAAATTTGCAGACCTACCAGAAACGAATGGGAGTGCAGAT
GCAGCGATTCAAGTGATCCTATTGTTGTTGCCGCAAATATCATTGGGATCTTGCACTTGATAT
TGTGGATTCTTGATCGTCTTTTCTTCAAATGCATTTATCGTCGCCTTAAATACGGTTTGAAAA
GAGGGCC

12.8.4 VNM/PEV16C/2004

AGGCCCCCTCAAAGCCGAGATCGCGCAGAAACTTGAAGATGTCTTTGCAGGAAAGAACACCGA
TCTCGAGGCTCTCATGGAGTGGCTAAAGACAAGACCAATCCTGTCACCTCTGACTAAAGGGAT
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CCAGAACGCCCTAAATGGAAATGGAGATCCAAATAATATGGATAGGGCAGTTAAGCTATATAA
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TGCACTTGCCAGTTGCATGGGTCTCATATACAACAGGATGGGAACGGTGACTACGGAAGTGGC
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GATGGCAACTATCACCAACCCACTAATCAGACATGAGAACAGAATGGTGCTGGCCAGCACTAC
AGCTAAGGCTATGGAGCAGATGGCGGGATCAAGTGAGCAGGCAGCGGAAGCCATGGAGATCGC
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TGGTCTGAGAGATAATCTTCTTGAAAATTTGCAGACCTACCAGAAACGAATGGGAGTGCAGAT
GCAGCGATTCAAGTGATCCTATTGTTGTTGCCGCAAATATCATTGGGATCTTGCACTTGATAT
TGTGGATTCTTGATCGTCTTTTCTTCAAATGCATTTATCGTCGCCTTAAATACGGTTTGAAAA
GAGGGCC

12.8.5 VNM/PEV16R/2004

AGGCCCCCTCAAAGCCGAGATCGCGCAGAAACTTGAAGATGTCTTTGCAGGAAAGAACACCGA
TCTCGAGGCTCTCATGGAGTGGCTAAAGACAAGACCAATCCTGTCACCTCTGACTAAAGGGAT
TTTGGGATTTGTATTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCGTAGACGCTTTGT
CCAGAACGCCCTAAATGGAAATGGAGATCCAAATAATATGGATAGGGCAGTTAAGCTATATAA
GAAGCTGAAAAGAGAAATAACATTCCATGGGGCTAAGGAGGTCGCACTCAGCTACTCAACCGG
TGCACTTGCCAGTTGCATGGGTCTCATATACAACAGGATGGGAACGGTGACTACGGAAGTGGC
TTTTGGCCTAGTGTGTGCCACTTGTGAGCAGATTGCAGATTCACAGCATCGGTCTCACAGACA
GATGGCAACTATCACCAACCCACTAATCAGACATGAGAACAGAATGGTGCTGGCCAGCACTAC
AGCTAAGGCTATGGAGCAGATGGCGGGATCAAGTGAGCAGGCAGCGGAAGCCATGGAGATCGC
TAATCAGGCTAGGCAGATGGTGCAGGCAATGAGGACAATTGGGACTCATCCTAACTCTAGTGC
TGGTCTGAGAGATAATCTTCTTGAAAATTTGCAGACCTACCAGAAACGAATGGGAGTGCAGAT

GCAGCGATTCAAGTGATCCTATTGTTGTTGCCGCAAATATCATTGGGATCTTGCACTTGATAT
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GAGGGCC

12.9 NS sequences

12.9.1 VNM/CL2009-28/2005

CTGCTTTCTTTGGCATGTCCGCAAACGATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCT
TGACCGGCTTCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAACACTCTTGGTCTGGACAT
TGAAACAGCTACTCGCGCAGGAAAGCAGATAGTGGAGCGGATTCTGGAGGAGGAGTCTGATAA
GGCACTTAAAATGCCGGCTTCATGCTACCTAACTGACATGACTCTCGAAGAAATGTCAAGGGA
CTGGTTCATGCTCATGCCCAAGCAGAAAGTGGCAGGTTCCCTTTGCATCAAAATGGACCAGGC
AATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGACTTTTGACCGGTTGGAAAC
CCTAATACTACTTAGAGCTTTCACAGAAGGAGGAGCAATCGTGGGAGAAATCTCACCATTACC
TTCTCTTCCAGGACATACTGGTGAGGATGTCAAAAATGCAATTGGCGTCCTCATCGGAGGACT
TGAATGGAATGATAACACAGTTCGAGTCACTGAAACTATACAGAGATTCGCTTGGAGAAGCAG
TGATGAGGATGGGAGACTTCCACTCCCTCCAAATCAGAAACGGAAAATGGCGAGAACAATTGA
GTCAGAAGTTTGAAGAAATAAGGTGGCTGATTGAAGAATTAAGACATAGATTGAAAATTACAG
AAAACAGCTTCGAACAAATAACGTTTATGCAAGCCTTACAACTACTGCTTGAAGTGGAGCAAG
AGATAAGAAC

12.9.2 VNM/CL107-13/2005

ACATAATGGATCCCAACACTGTGTCACTGCTTTCTTTGGCATGTCCGCAAACGATTTGCAGAC
CAAGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGATCAGAAGTCCCTAAGAGGA
AGAGGCAACACTCTTGGTCTGGACATCGAAACAGCCACTCGCGCAGGAAAGCAGACAGTGGAG
CGGATTCTGGAGGAGGAGTCCGATAAGGCACTTAAAATGCCGGCTTCATGCTACCTAACTGAC
ATGACTCTCGAAGAAATGTCAAGGGACTGGTTCATGCTCATGCCCAAGCAGAAAGTGGCAGGT
TCCCTTTGCATCAAAATGGACCAGGCAATAATGGATAAAACCATCATATTGAAAGCAAACTTC
AGTGTGACTTTTGACCGGTTGGAAACCCTAATACTACTTAGAGCTTTCACAGAAGGAGGAGCA
ATCGTGGGAGAAATCTCACCATTACCTTCTCTTCCAGGACATACTGGTGAGGATGTCAAAAAT
GCAATTGGCGTCCTCATCGGAGGACTTGAATGGAATGATAACACAGTTCAAGTCACTGAAACT
ATACAGAGATTCGCTTGGAGAAGCAGTGATGAGGATGGGAGACTTCCACTCCCTCCAAATCAG
AAACGGAAAATGGCGAGAACAATTGAGTCAGAAGTTTGAAGAAATAAGGTGGCTGATTGAAGA
ATTAAGACATAGATTGAAAATTACAGAAAACAGCTTCGAACAAATAACGTTTATGCAAGCCTT
ACAACTACTGCTTGAAGTGGAGCAAGAGATAAGAAC

12.9.3 VNM/PEV16B/2004

CAAAAACATAATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGTTTCTTTGGCATGTC
CGCAAACGATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGAT
CAGAAGTCCCTAAGAGGAAGAGGCAACACTCTTGGTCTGGACATCGAAACAGCTACTCGCGCA
GGAAAGCAGATAGTGGAGCGGATTCTGGAGGAGGAGTCTGATAAGGCACTTAAAATGCCGGCT
TCACGCTACCTAACTGACATGACTCTCGAAGAAATGTCAAGGGACTGGTTCATGCTCATGCCC
AAGCAGAAAGTGGCAGGTTCCCTTTGCATCAAAATGGACCAGGCAATAATGGATAAAACCATC
ATATTGAAAGCAAACTTCAGTGTGACTTTTGACCGGTTGGAAACCCTAATACTACTTAGAGCT

TTCACAGAAGGAGGAGCAATCGTGGGAGAAATCTCACCATTACCTTCTCTTCCAGGACATACT
GGTGAGGATGTCAAAAATGCAATTGGCGTCCTCATCGGAGGACTTGAATGGAATGATAACACA
GTTCGAGTCACTGAAACTATACAGAGATTCGCTTGGAGAAGCAGTGATGAGGATGGGAGACTT
CCACTCCCTCCAAATCAGAAACGGAAAATGGCGAGAACAATTGAGTCAGAAGTTTGAAGAAAT
AAGGTGGCTGATTGAAGAAGTAAGACATAGATTGAAAATTACAGAAAACAGCTTCGAACAGAT
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TCAGCTTATTTAATGATAAA

12.9.4 VNM/PEV16C/2004

CAAAAACATAATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGTTTCTTTGGCATGTC
CGCAAACGATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGAT
CAGAAGTCCCTAAGAGGAAGAGGCAACACTCTTGGTCTGGACATCGAAACAGCTACTCGCGCA
GGAAAGCAGATAGTGGAGCGGATTCTGGAGGAGGAGTCTGATAAGGCACTTAAAATGCCGGCT
TCACGCTACCTAACTGACATGACTCTCGAAGAAATGTCAAGGGACTGGTTCATGCTCATGCCC
AAGCAGAAAGTGGCAGGTTCCCTTTGCATCAAAATGGACCAGGCAATAATGGATAAAACCATC
ATATTGAAAGCAAACTTCAGTGTGACTTTTGACCGGTTGGAAACCCTAATACTACTTAGAGCT
TTCACAGAAGGAGGAGCAATCGTGGGAGAAATCTCACCATTACCTTCTCTTCCAGGACATACT
GGTGAGGATGTCAAAAATGCAATTGGCGTCCTCATCGGAGGACTTGAATGGAATGATAACACA
GTTCGAGTCACTGAAACTATACAGAGATTCGCTTGGAGAAGCAGTGATGAGGATGGGAGACTT
CCACTCCCTCCAAATCAGAAACGGAAAATGGCGAGAACAATTGAGTCAGAAGTTTGAAGAAAT
AAGGTGGCTGATTGAAGAAGTAAGACATAGATTGAAAATTACAGAAAACAGCTTCGAACAGAT
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TCAGCTTATTTAATGATAAA

12.9.5 VNM/PEV16R/2004

CAAAAACATAATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGTTTCTTTGGCATGTC
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CAGAAGTCCCTAAGAGGAAGAGGCAACACTCTTGGTCTGGACATCGAAACAGCTACTCGCGCA
GGAAAGCAGATAGTGGAGCGGATTCTGGAGGAGGAGTCTGATAAGGCACTTAAAATGCCGGCT
TCACGCTACCTAACTGACATGACTCTCGAAGAAATGTCAAGGGACTGGTTCATGCTCATGCCC
AAGCAGAAAGTGGCAGGTTCCCTTTGCATCAAAATGGACCAGGCAATAATGGATAAAACCATC
ATATTGAAAGCAAACTTCAGTGTGACTTTTGACCGGTTGGAAACCCTAATACTACTTAGAGCT
TTCACAGAAGGAGGAGCAATCGTGGGAGAAATCTCACCATTACCTTCTCTTCCAGGACATACT
GGTGAGGATGTCAAAAATGCAATTGGCGTCCTCATCGGAGGACTTGAATGGAATGATAACACA
GTTCGAGTCACTGAAACTATACAGAGATTCGCTTGGAGAAGCAGTGATGAGGATGGGAGACTT
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AAGGTGGCTGATTGAAGAAGTAAGACATAGATTGAAAATTACAGAAAACAGCTTCGAACAGAT
AACGTTTATGCAAGCCTTACAACTACTGCTTGAAGTGGAGCAAGAGATAAGAACCTTCTCGTT
TCAGCTTATTTAATGATAAA