Adaptive changes of Influenza virus A/H5N1 Neuraminidase *in vitro* and *in vivo*

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Kellogg College

Submitted to Oxford University for the degree of MSc. Res in the field of Medical Science

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Abstract

H5N1 adaptation process is thought to primarily involve in changes of the 2 main glycoproteins of influenza virus: hemagglutinin HA, responsible for viral entry via binding with host cell receptors, and neuraminidase NA, a receptor destroying enzyme, promoting release of newly formed virions and preventing their self-aggregation. With their opposing roles in viral replication cycle, one rational assumption is that influenza virus needs to maintain a balance between these 2 proteins' activities to ensure productive infection. Because of their impacts on both viral evolution and drug susceptibility, it is necessary to gain insight into the interplay between HA and NA proteins as this will further our understanding of the cross-species A/H5N1 adaptation process, with or without selective pressure.

One of the most suitable systems to study influenza adaptation is cultures of differentiated human epithelial cells; which have been used for more than 30 years in various types of research. This project aimed to set-up that system in our laboratory in Vietnam to passage H5N1 viruses in order to use in later studies monitoring the "adaptive" changes of those viruses, especially in the two major glycoproteins HA and NA. In parallel, H5N1 isolates with different oseltamivir susceptibility profile were investigated to gain clear insight on NA enzymatic properties as well as the underlying mechanism of resistance. Those H5N1 isolates were human clade 1 viruses from the period of 2004 – 2005 in south of Vietnam with different oseltamivir sensitivity. Recombinant variants, with NA derived from H5N1 viruses and other segments from laboratory adapted human seasonal strain WSN33, were generated by reverse genetic techniques and then characterized on NA enzymatic properties. We identified 2 NA

mutations which were suspected to affect NA activity and/or expression. Further investigations are required to conclude whether these 2 substitutions might support or allow the virus(es) to overcome the deficiency caused by oseltamivir resistance mutation, resulting in adaptive evolution of influenza viruses.

Chapter 1

Introduction

I General information

Influenza viruses belong to the family *Orthomyxoviridae*, including five genera of enveloped RNA viruses which are segmented and negative stranded. Three out of these five genera are the influenza viruses A, B and C. The viral genome of influenza virus contains 8 RNA segments (with the exception of Influenza C viruses which only have 7 segments) encoding for total 11-12 proteins (1,2). Influenza A viruses are classified based on their main surface glycoproteins: the hemagglutinin (HA) and the neuraminidase (NA); e.g. H1N1 and H3N2.All influenza viruses follow a standard nomenclature system, consisting of virus type, species from which it was isolated (if non-human), isolate location, number and year, and (for influenza A only) HA and NA subtypes (3). There are 16 HA and 9 NA subtypes identified and maintained in their natural reservoir; waterfowl belonging to the orders *Anseriformes* and *Charadriiformes* (ducks and shorebirds respectively) (4,5). Recently, 2 new distinct subtypes of influenza viruses have been identified in bats, those are H17N10 and H18N11 (6,7). In their natural reservoir of wild waterfowl, influenza A viruses usually cause mild to asymptomatic infections. However, from their natural reservoir they can transmit to new hosts (humans and animals) and adapt up to a level where they are maintained by ongoing cycles of transmission within this new host. Sometimes this results only in mild disease (e.g. in pigs), but in humans this has led to severe epidemics/pandemics with high mortality (5). Among the (known) 18 H's and 11 N's, three HA subtypes (H1, H2 and H3) and two NA subtypes (N1 and N2) have established stable lineages in humans and lead to epidemics/pandemics (8). Influenza B and C viruses almost exclusively infect humans and usually only cause mild disease, primarily in children and are thought

not to have pandemic potential. In contrast, a wide variety of species are susceptible to influenza A virus infection, including birds, human and other wild, domesticated and aquatic mammals as pigs, horses, dogs, whales etc (1) and most have ‘their own’ subtypes maintained in ongoing cycles of transmission without the need for a reservoir (Figure 1.1)

Avian influenza A viruses can be classified into low or non pathogenic and high pathogenic strains (LPAI and HPAI), depending on their pathogenicity in chickens. Viruses of low pathogenicity only cause mild respiratory disease whereas infection with high pathogenic virus is associated with significant mortality rate. HPAI infections in poultry can lead to mortality of up to 100% within 48h post-infection (9). There is evidence that HPAI arose after transmission and adaptation of LPAI subtypes H5 and H7 in new poultry hosts (5).

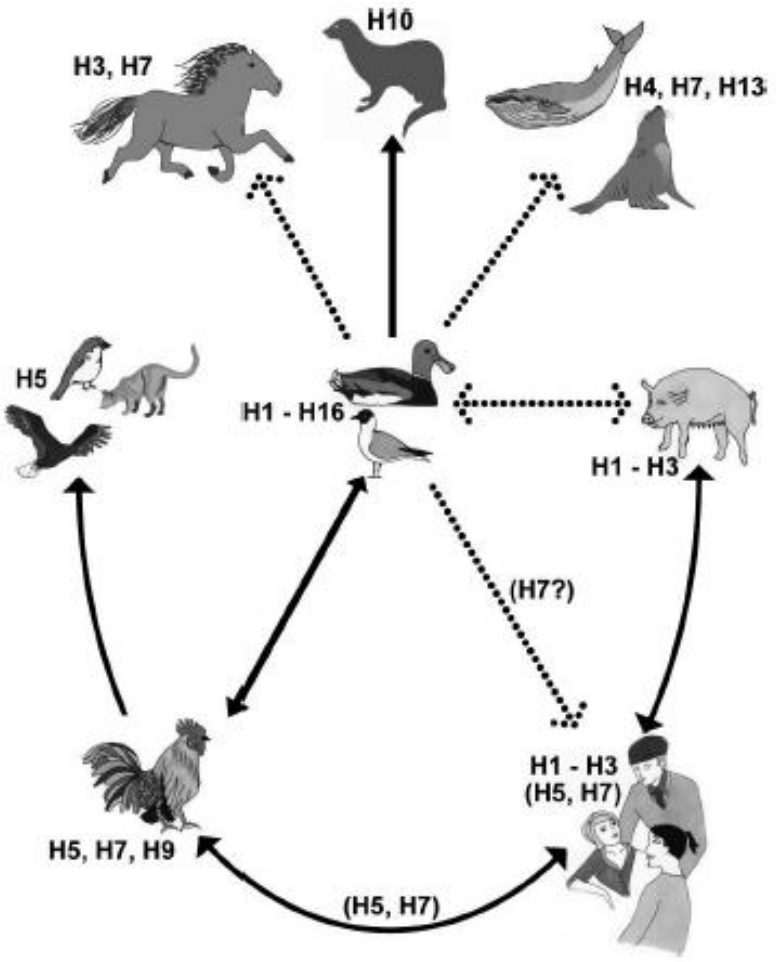


Figure 1.1 **Influenza virus host range** (taken from (10))
Natural reservoir of influenza A virus, accidental hosts and the subtypes identified in different groups.

Avian influenza (AI) viruses can infect a wide range of animals other than aquatic birds. These include terrestrial birds, humans, horses, pigs, cats, dogs and whales. This is an indication for their interspecies transmissibility. However, most of AI virus infection in primates are dead-end infections and are limited to the first recipient and a restricted number of close contacts (9). In these hosts, AI primarily infect upper respiratory tract and result in respiratory disease; whereas in their natural reservoir AI virus often infect and replicate in digestive organs.

Influenza A viruses are evolutionarily very dynamic with high mutation rates, 1×10^{-3} to 8×10^{-3} substitutions per site per year (11). Mutations that occur at the antigenic sites of HA and NA surface proteins might be advantageous for the virus in that they can escape pre-existing immunity, which is specifically against “wild-type” non mutated variants. Such selective events are called antigenic drift. Another important and common event in influenza virus evolution is antigenic shift. Because of their segmented RNA genome, influenza viruses can undergo segment exchange when two different viruses infect the same host (3). This will generate progeny viruses with a new combination of gene segments thus adding in to the evolutionary dynamics of flu viruses. If the human population is immunologically naïve to this newly generated virus, this will result in a pandemic, as was most likely the case in all 4 virologically documented pandemics since 1918.

The virion surface is comprised of a lipid bilayer membrane that is made up of HA, NA and a transmembrane ion channel M2 (1). Among these, HA is the predominant antigen. This protein also plays the main role in viral binding and entry into host cells via membrane fusion. HA protein must be cleaved into its active form of HA1 and HA2 before fusion reaction. Replication of human influenza viruses is usually restricted to the respiratory tract because of the intracellular presence of the enzyme responsible for HA cleavage in that organ. However, HA of HPAI viruses can be cleaved by extracellular proteases produced in many different tissues. This partly explains why HPAI H5N1 infection can lead to multi-organ failure. HA of influenza viruses from different species have different receptor preferences. HA of human influenza viruses prefer to bind to

receptors with a terminal sialic acid linked to galactose through an α2,6 linkage; whereas avian viruses preferentially bind to α2,3 linked sialic acids.

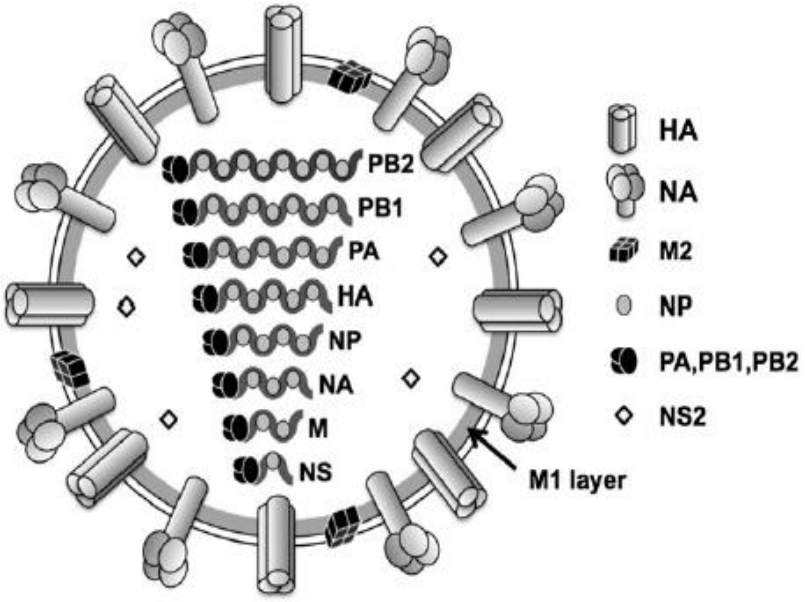


Figure 1.2 **Structure of an Influenza A Virus** (taken from (11)).
Influenza virus surface consists of a lipid bilayer including glycoproteins hemagglutinin HA (majority) and neuraminidase NA (in lesser amount) and a transmembrane ion channel M2 (minority).Matrix M1 protein lies beneath the lipid bilayer, interacting with surface proteins and ribonucleoproteins (RNPs).
Inside the virion are 8 RNA segments, bound by the polymerase complex (PB2, PB1 and PA) at one termini and encapsidated with nucleoprotein (NP) to form the ribonucleoprotein structure.

While HA is responsible for binding, NA has an opposite role. NA cleaves the bond between HA and host cell sialic acid residue, allowing the release of progeny virions from infected cells and therewith completing a successful replication cycle. Consequently, it is reasonable to say that the balance of HA and NA activities is critical for influenza virus infection: changes in activity of only HA or NA could result in no viral entry or self aggregation of viral particles which in turn leads to a nonproductive cycle. HA and NA also play important roles in the continuing evolution of influenza A viruses (1), demonstrated by the number of point mutations accumulated or documented

reassortment events that have generated new variants of circulating strains or novel viruses with high pandemic potential.

II Epidemiology

Because of their high morbidity and mortality, influenza viruses are among the most common and significant causes of human respiratory infections (11). They are responsible for seasonal epidemics and, less frequent but usually more severe, pandemics. In temperate regions, influenza infections have the yearly peak around winter time (different for Northern and Southern hemispheres). However, in tropical countries, infections appear throughout the year with higher incidence during rainy season (CDC website, basic information about influenza). Both influenza A and B viruses can cause epidemics but only influenza A virus can lead to pandemics. For the last 500 years, there have been approximately 13 pandemics, including 4 well-known and well-documented ones in the 20th century and the most recent 2009 pandemic (12). The Spanish influenza pandemic (1918 – 1919), the most devastating one as it killed about 50 million people worldwide, was caused by an avian-origin H1N1 virus. The major cause of death was viral pneumonia complicated by bacterial super infection (12). Sequence data analysis suggest that the 1918 virus was novel to humans at that time and this virus is the progenitor of all influenza A viruses circulating in humans nowadays (13). Sequencing analysis also indicated that this virus had an avian origin, however, the adaptation process of this virus, direct avian-to-human or involving an intermediate host, remains unknown (14). Early 1957, an H2N2 virus emerged and lead to another

pandemic, starting from Southern China then spreading over East Asia to Europe and North America. The 1957 virus was a descendant of the 1918 H1N1 virus with 2 newly acquired segments HA and NA, and an avian-like PB1 gene segment. Again, it is not known in which species that reassortment occurred (12). The following pandemic, Hong Kong flu 1968, was caused an H3N2 strain that had “shifted” from the previous H2N2 virus. New HA and PB1 segments were also acquired by reassortment with other avian viruses. It is believed that this pandemic was relatively mild because of the “old” NA gene of the 1957 virus, to which most of the population was at least partially immunized (13). In 1977, H1N1 virus reappeared and resulted in an outbreak near the Russian – Chinese border and then spread to North America the year after. Unlike the 1968 H3N2 virus which replaced the preceding 1957 H2N2 strain, the “new” H1N1 has been co-circulating with the H3N2 until 2009 (12) (Figure 1.3).

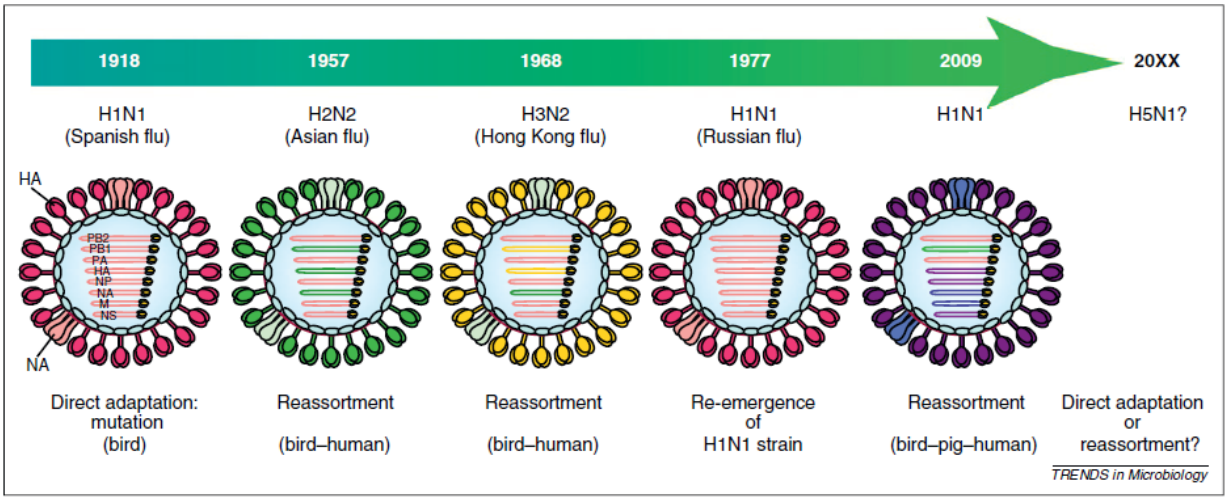


Figure 1.3 **Timeline and evolution of pandemic influenza viruses** (taken from (9))

The most recent influenza pandemic, in 2009, was caused by a novel H1N1 strain (H1N1pdm09). This virus was also a descendent of the 1918 H1N1 although the reassortment process underlying was much more complicated. Since 1918, there have been 2 H1N1 strains circulating in humans (re-emerged in 1977) and pigs ("classical swine virus") both derived from the Spanish pandemic strain (12). In 1979, a distinct Eurasian "avian-like" H1N1 virus emerged in European pigs and co-circulated with the classical swine viruses (2). In late 1990s, a triple-reassortant virus emerged in pigs with genetic materials originated from various sources. Its PB2 and PA derived from an avian virus, PB1 and NA from a human H3N2 and the other 4 segments from classical swine virus (Figure 1.4). The H1N1pdm09 genetic materials originated from this triple-reassortant and the Eurasian avian-like H1N1 strain. The complexity of H1N1pdm09 and the fact that it was derived from viruses which might have been circulating, undetectably, in pig or other populations highlighted the importance of human and animal surveillance to understand as well as to react timely to (re)emergence of zoonotic pathogens (2).

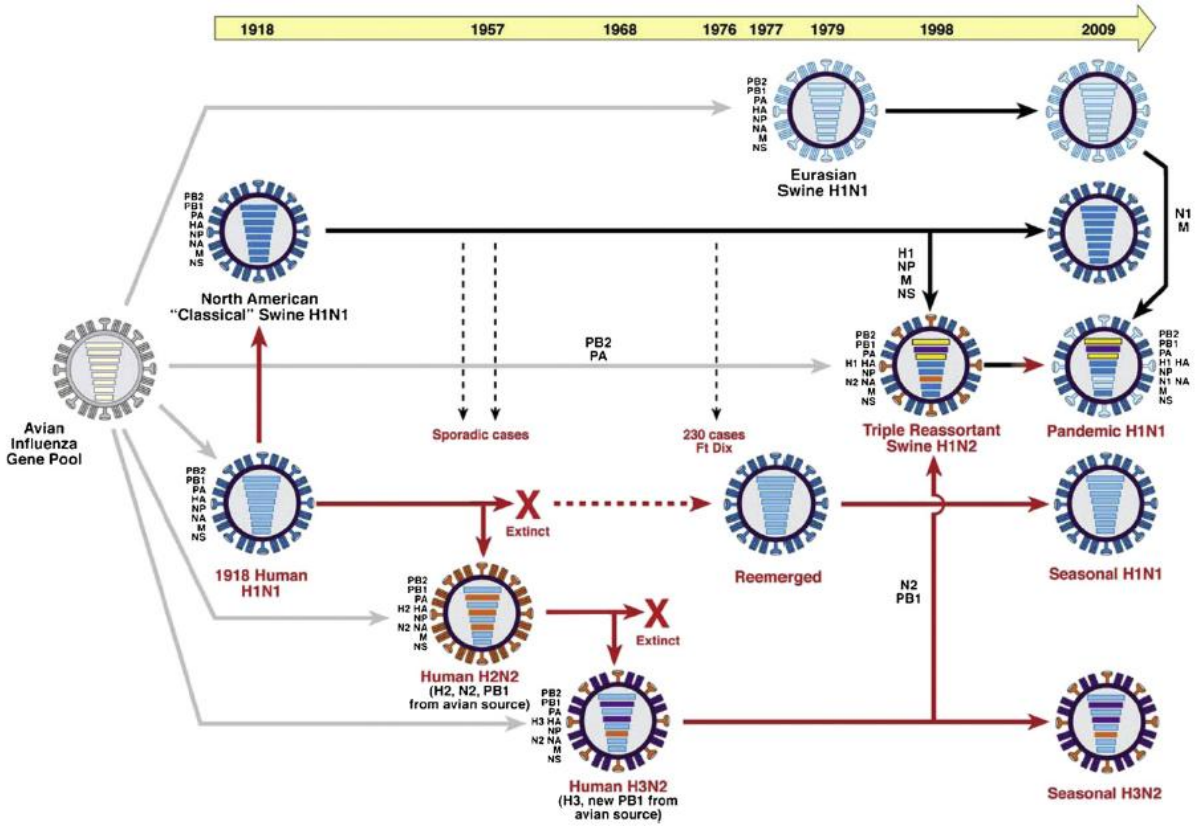


Figure 1.4 **Genetic relationships between human and relevant swine influenza viruses** (adapted from (11)).
Grey arrows indicate exportation of one or more genes from the avian influenza A virus gene pool. The dashed red arrow indicates a period without circulation. Solid red arrows reflect the evolutionary paths of human influenza virus lineages; solid black arrows, of swine influenza virus lineages; and the black-to-red arrow, of a swine-origin H1N1pdm09 influenza virus. The genes of the 1918 human and swine H1N1 and the 1977 H1N1 influenza A viruses were all recently descended from avian influenza A genes, and some have been "donated" to the pandemic human H1N1 strain. The dashed, descending black arrows reflect human zoonotic infections with swine IAVs.

III Receptor binding

In order to initiate an infection, influenza viruses need to enter the cell which is mediated by binding between viral HA and host cell receptors terminating in sialic acids. Upon binding, the virus is internalized; and after fusion of HA and the endosome membrane,

an event that requires the important role of matrix protein, the ribonucleoproteins are released into the cytoplasm. This is followed by transcription and replication of viral genome (15). Budding of newly formed virions is facilitated by neuraminidase protein, which cleaves the linkage between HA and cell surface receptors.

Depending on their origins, influenza viruses recognize different forms of sialic acids (SA). SAs are nine-carbon backbone sugars with various arrangements and modifications, typically found at the end of glycan chains on all cell surfaces and bound to cell-membrane sugars by α2,3, α2,6, α2,8 or α2,9 linkage. With their wide range of variety, their exposed location ubiquitous distribution and negatively charge; sialic acids serve as cell surface receptors for many viral pathogens such as influenza viruses, Newcastle disease virus, hepatitis B virus, rotavirus, polyomavirus and many other viruses (16). The enzyme responsible for linking SA and cell surface galactose is sialyl transferase, which is expressed in a tissue- and species-specific manner (9). Differences in HA receptor binding affinity are believed to be one of the primary determinants of influenza host range and tissue tropism, and are also critically important for pathogenicity.

The most common type of terminal sugars are N-acetylneuraminic acid-α2,3-galactose (NeuAcα2,3Gal) and N-acetylneuraminic acid-α2,6-galactose (NeuAcα2,6Gal). In general, human viruses prefer to bind to oligosaccharide containing NeuAcα2,6Gal whereas most avian viruses have a preference for NeuAcα2,3Gal (17). This corresponds to the predominance of α2,6 type receptor in human upper respiratory tract epithelia and α2,3 type receptor in intestinal epithelia of aquatic birds (e.g. ducks). Receptor distribution differs among various avian species. While aquatic birds such as

ducks and geese mainly express avian-type receptor NeuAcα2,3Gal, some terrestrial birds including chickens, turkeys and quail have both type of receptors in their tracheal epithelial cells. Consequently, terrestrial avian species may play a critical role in host-switching events that involve evolution of HA and receptor specificity (17). Traditionally, pigs have also been considered as key intermediate host facilitating reassortment and emergence of novel human influenza subtypes as pigs express both α2,6 and α2,3 receptors at comparable levels. Recent advances in techniques for measuring receptor distribution have challenged traditional concepts of species differences, and this is an area of active ongoing research (17).

Chandrasekarn *et al*. proposed that it is not the linkage between SA and adjacent sugar moieties itself that determines binding affinity, but rather the distinctive structural topology of these interactions, i.e. cone-like or umbrella-like, that depends in part on the length of the SA chain successful human adaptation of influenza viruses correlates with the ability of HA to bind to long α2,6 sialyl glycans, not the specific α2,3 or α2,6 linkage (18).

IV Anti-influenza drugs and vaccines

Influenza is associated with annual epidemics, with seasonal patterns in temperate areas accounting for more than 500,000 deaths every year (19). This number can increase dramatically in case of pandemics; the highest record is in 1918 when an avian-origin H1N1 killed up to 50 million people worldwide (11). Two subsequent major pandemics in 1957 and 1968 were caused by H2N2 and H3N2 viruses respectively. And then in 2009, a novel H1N1 virus emerged, resulting in the first pandemic of the

21st century. For all of those events, it is worth noting that with the techniques available at the time there was no adequate time to produce sufficient quantities of vaccine against the pandemic virus (20).

For H1N1pdm09 a vaccine was available within 9 months after the first cases and 3 months after the pandemic was declared officially by WHO on June 11 2009; which was although relatively fast but well after the pandemic peak (21). It is expected that mass-production of vaccines against a novel pandemic virus is not likely to become much faster than this is the near future (22), and therefore pre-pandemic stockpiling of vaccines against viruses that are likely to emerge is an option. This would avoid the logistics of developing, manufacturing and distributing a new vaccine prior to the pandemic wave.

For seasonal influenza, vaccination (consisting of the – inactivated - predicted dominant lineages of A/H1N1pdm09, A/H3N2 and B, combined into a single vaccine annually, separately for the Northern and Southern hemisphere) is considered the best approach to prevention, mitigation and control of the annual influenza epidemics especially among high risk groups. However, its efficacy is limited due to several reasons. Firstly, vaccines are almost exclusively used in developed countries and population coverage is low. Secondly, vaccine uptake and immune responses are insufficient in some individuals, especially those at risk (19). Thirdly, due to antigenic drift of influenza virus, seeded viruses in the vaccine need to be updated frequently. Moreover, because of co-circulation of different lineages of the three viruses in the vaccine, sometimes the wrong lineages are predicted to be the dominant lineage in the coming season. This results in

mismatch between vaccine and circulating strain and ultimately leads to inadequate protection

Selection, development and stock-piling of pre-pandemic vaccine against e.g. H5N1 or H7N9 isa high priority for global health. The situation is complicated in case of H5N1 viruses because of antigenically different H5N1 clades, which are not or not entirely cross-reactive, are co-circulating in one (or different) geographic area(s) hence increasing the difficulty to select, and subsequently generate a vaccine strain which provides protection against all those viruses. In addition, H5N1 high infectivity and egg lethality (caused by its multibasic cleavage site) also challenges the current vaccine production method, and would require other methods or generation of recombinant viruses with wild-type HA and NA segments or engineered viruses with removed polybasic cleavage sites.

Because of the reasons above, antiviral drugs are the first choice in managing influenza outbreaks especially of emerging viruses, both for prevention to limit dissemination during an outbreak and for treatment and management of clinical infections.

To date, there are 2 major classes of antiviral drugs approved to use for influenza infections: adamantanes and neuraminidase inhibitors (NAIs). Because of the restricted usage of amandatanes (due to their side effects and rapid emergence of resistance), it will not be review in this introduction section.

Neuraminidase inhibitors (NAIs)

NA enzyme has sialidase activity, and is responsible of cleavage of NeuAc on the α2,3 and α2,6 SA. This enzymatic property is required for virion release, as it prevents self-aggregation of the newly formed virions, and promotes virus penetration and

dissemination through the mucus layer (23). Due to its critical role in the viral replication cycle, NA is the one of most commonly targeted viral proteins for development of novel anti-influenza therapeutics. The NA protein was also one of the first viral proteins to be successfully crystallized for high resolution structural analysis. Based on experimental data from structural and functional studies, several NAIs have been developed utilizing structure-based rational designs. To date, however, only 2 drugs have been approved for clinical use. These are inhaled zanamivir and oral oseltamivir. These drugs target the active site of NA which is highly conserved among influenza viruses, and thus are active against both influenza A and B viruses (20). Another NAI currently in phase III clinical trials in the US is peramivir. Its intravenous form received an emergency use authorization for H1N1pdm09 treatment, and has been approved for use in Japan and South Korea since 2010 (19).

V Drug resistance

Molecular determinants of NAI resistance have been identified from epidemiological meta-analyses, clinical studies, and studies of influenza in vitro evolution. The majority of drug resistance determinants have been localized to sites within the NA catalytic domain, or mutations in HA that alter receptor binding. Several lines of evidence suggest that changes in HA receptor binding will influence NA evolution by either reducing or enhancing requirements for NA enzyme activity. Importantly, however, the only mutations to date, that have been found to be clinically relevant and are used in drug resistance screening, are those located within the NA active site (19). These

mutations involve both framework (E119, R156, W178, S179, D198, I222, E227, N294 and E425) and catalytic residues (R118, D151, R152, R224, E276, 292, R371 and Y406; N2 numbering), and are both drug specific and subtype specific (24). Interestingly, it was demonstrated that 9 NA subtypes, N1 – N9, can be divided into 2 genetically distinct groups based on their ligand-bound conformation (25). Group 1 NAs, (consisting of N1, N4, N5 and N8) adopt an open conformation of the active site loop, the 150-loop, in the absence of inhibitor. This loop will close the adjacent cavity upon drug binding. In contrast, group 2 NA loop (including N2, N3, N6, N7 and N9) always have a closed conformation either in the presence or absence of ligands (26). Therefore, the drug and subtype specific features of NAI resistance might be explained partly by genetic differences of NA family. For instance, H274Y is the most frequently selected mutation in oseltamivir or peramivir resistant H1N1 isolates; whereas R292K and E119V are usually found in oseltamivir resistant H3N2 variants. Resistance to zanamivir is rarely found either in seasonal or pandemic viruses; this might be preliminarily due to low usage of the agent (i.e. relatively low selection pressures) or high level of structural homology to NA natural substrate (24).

Before NAIs were introduced into clinical use, circulating human influenza isolates were found to be naturally susceptible to oseltamivir and zanamivir. During the first years of NAI usage, as high as 0.33% of oseltamivir resistance rate was found in samples collected worldwide. However, during 2007-08, a dramatic increase in oseltamivir resistance was observed in human seasonal H1N1 isolates throughout Europe, with some countries such as Norway exhibiting resistance in as many as 67% of clinical isolates (19). The resistant phenotype was associated with spread of the H274Y

mutation, which appeared to arise independently of NAI use. In the following season, almost 100% of isolates in North America and other regions around the world were H274Y variants. The predominance of the drug resistant H1N1 virus (A/Brisbane/59/2007-like, H274Y) during the 2009 season led to intense clinical as well as scientific concerns. It disappeared with the emergence of H1N1pdm09. The H1N1pdm09 virus is naturally sensitive to NAIs, however intensive use if NAIs during the pandemic, either for prophylaxis or treatment, resulted in rapid emergence of resistant variants. Although there was a few resistant variants isolated from untreated individuals, most of the cases were linked to oseltamivir treatment (19). Surprisingly, there was a significant increase in the percentage of oseltamivir resistant cases, which were not associated with drug usage, in US in the season of 2010-2011, from 11% of the previous season to 74% (24). Among the oseltamivir resistant H1N1pdm09 viruses identified, all contained H274Y, and some viruses had additional NA mutations as well that may have contributed to the resistance phenotype (Table 1.1).

Results of *in vitro* experiments suggest that the impacts of NAI resistance mutations vary depending on the types of mutated residues. Mutations within the catalytic domain such as R292K (in H3N2 viruses), typically lead to significant reduction of NA activity, and consequently, decrease in viral replication when grown in cell culture. In contrast, mutations on framework residues, i.e. H274Y and E119V, tend to be subtype- and strain-specific (27). While H274Y mutation reduced replication capacity of older A/H1N1 viruses, there was no apparent 'fitness cost' associated with the H274Y mutation within the lineage of A/Brisbane/59/2007-like viruses. Indeed, the H274Y A/Brisbane/59/2007-like viruses retained their transmissibility and replaced their wild-type counterpart to

become dominant within the global circulation patterns. The H274Y A/Brisbane/59/2007-like viruses caused a similar spectrum of clinical disease compared to NAI-sensitive viruses. Similarly to old A/Brisbane/59/2007-like viruses, the presence of the H274Y mutation within the NA of H1N1pdm09 was not associated with fitness costs when compared to wild-type H1N1pdm09 in *in vitro* experiments (19). Interestingly, despite the fact that some H274YH1N1pdm09 viruses were isolated from individuals who had not received drug treatment, these mutant variants do not appear to have spread substantially in worldwide circulation, and have not replaced sensitive wild-type H1N1pdm09 (28).

The unexpected dissemination of spontaneously emerging oseltamivir-resistant seasonal H1N1 viruses led to numerous studies to investigate their NA properties as well as fitness and transmissibility (28). Experimental results confirmed that A/Brisbane/59/2007-like viruses with H274Y mutation possessed undiminished fitness and transmissibility and this might be due to changes in NA enzymatic properties of cell surface expression (27). NA of A/Brisbane/59/2007-like viruses had an increased affinity for it substrates, resulting in a restoration of NA function and ultimately led to a better balance of HA-NA activities (29). HA-NA functional balance can also be restored by either “permissive” or “compensatory” mutations, which emerge before or after H274Y mutation occurrence respectively, that enhance the tolerance of resistant viruses with the effect of H274Y on NA protein. In 2010, Bloom and colleagues proposed 2 possible permissive mutations of seasonal H1N1, V234M and R222Q (30). In this study, reduced NA activity caused by H274Y mutation was associated with reduction of NA cell surface expression; which in turn might be because of defects in NA folding of transport to cell

membrane. And V234M and R222Q could increase NA surface expression. Interestingly, these 2 mutations did occur in the evolution of H1N1 strain in the period of 1999 and 2007. The role of permissive mutation R222Q was confirmed by a more recent study of Abed *et al* (31); showing that when reverting back to R, the double mutant H274Y/Q222R displayed remarked reduction in NA activity and affinity consequently leading to a decrease in *in vitro* replication and also replication in lungs of ferrets .

New NAI resistance NA mutations, I222R and S246N, have been identified in H1N1pdm09 background and displayed synergistic effects on the drug susceptibility profile when combined with the H274Y mutation (24). The I222R mutant was less pathogenic in ferrets than the wild-type H1N1pdm09 but demonstrated similar replication ability in vitro and transmissibility in ferrets (27). Additionally, the I222R mutation was reported causing reduced sensitivity to multiple NAIs by itself only. This mutation could also restore the reduced fitness of the H274Y mutation, resulting in an IC50 increase about 10-90 folds compared to H274Y mutation alone (32).

E119V and R292K have been the two most common mutations detected in H3N2 oseltamivir resistant infections in humans (32). While E119V confers resistance to oseltamivir, R292K mutation results in both high level of oseltamivir resistance and decreased zanamivir susceptibility. Earliest data showed that R292K displayed significant reduction in *in vitro* and *in vivo* replication as well as not transmitted among ferrets by direct contact (28,33). In a pediatric study, N294S mutation was reported resulting in oseltamivir resistance by reducing oseltamivir sensitivity be several hundredfold (24).

VI Interplay between HA and NA

Influenza viruses have two main surface glycoproteins, HA and NA, both recognizing the same substrate (SAs) which serves as the primary cellular receptor. These two viral proteins are critical for viral replication, and play antagonizing and complementary roles. Infection starts when multiple HA bind to SAs on glycoproteins or glycolipids on host cell surface (34). After viral genome replication and packaging, virions bud from the surface of the host cell, and are released by the receptor destroying activity of NA that cleaves the sialic bond. SAs are removed from infected cells so that newly formed virions are free to infect new cells.

Phylogenetically, 16 subtypes of HA are divided into 2 groups; group 1 include H1, H2, H5, H6, H8, H9, H12, H13 and H16; group 2 contains H3, H4, H7, H10, H14 and H15. Similarly, all NA subtypes also form 2 groups: N1, N4, N5 and N8 belong to group 1 while N2, N3, N6, N7 and N9 are in group 2. In addition to the important role of HA in receptor binding, HA also mediates fusion between viral and cell membranes, releasing viral genomic materials into the cytoplasm. After endocytosis, HA is activated upon acidification of the endosome. This process involves HA conformational changes that are known to vary by subtype, and that may be inhibited by various subgroup or subtype-specific fusion inhibitors. Similarly, the two major groups of NA also differ in key structural features, namely the conformation of the enzyme active site (34).

Prior to activation, the HA precursor needs to be cleaved into its active form. For all HA subtypes, cleavage occurs extracellularly and is catalyzed by enzymes expressed at the sites of infection, e.g. enzymes present in the human respiratory tract or in the avian intestinal tract. The cleavage site of HA is highly conserved among subtypes, and

usually contains a single arginine residue. However, some H5 and H7 subtypes have acquired a multi-basic cleavage site, through insertional mutation; these cleavage sties may be recognized and cleaved by ubiquitous intra-cellular subtilisin-like enzymes. Consequently, viruses that exhibit a multi-basic cleavage site are highly infectious, with the capacity to spread to many different organs and cause systemic infections. Highly pathogenic H5N1 is an example of such a virus; indeed, presence of the multibasic cleavage site is believed to be the hallmark feature of H5 pathogenesis.

NA is a tetramer with identical subunits, integrated into the viral membrane by a thin stalk that varies in length between different virus strains. NA functions as a receptor destroying enzyme, removing SAs from host cell glycoproteins and glycolipids and also viral membrane proteins (23). This activity supports viral budding process, prevents their self-aggregation and helps the virus to penetrate the mucous layer of respiratory tract. NA is the main target of antiviral drug as well. NAIs, zanamivir and oseltamivir, are active against both group 1 and group 2 NA, and also influenza B virus.

Influenza A viruses of the N1 subtype with reduced susceptibility to neuraminidase inhibitors.

Influenza Subtype	NA mutation[a]	Virus source/NAI used for selection	Phenotype in NA inhibition assays:[b]			Reference
			Oseltamivir	Zanamivir	Peramivir	
A(H1N1)	H274Y	Clinic/oseltamivir	HRI	S	HRI	Mishin et al. (2005)
	Q136K	*In vitro* (clinic?)/none	S	HRI	–	Hurt et al. (2009b)
A(H1N1)pdm09	N294S	Reverse genetics	HRI	S	RI	Pizzorno et al. (2011b)
	H274Y	Clinic/oseltamivir	HRI	S	HRI	Baz et al. (2009)
	S246N/H274Y	Clinic/oseltamivir	HRI	S	HRI	Hurt et al. (2011b)
	I222V/H274Y	Clinic/oseltamivir	HRI	S	–	CDC (2009a)
		Reverse genetics	HRI	S	HRI	Pizzorno et al. (2011b)
	I222R/H274Y	Clinic/oseltamivir	HRI	RI	HRI	Nguyen et al. (2010a)
		Reverse genetics	HRI	RI	HRI	Pizzorno et al. (2012)
	I222R	Clinic/oseltamivir	RI	RI	–	Eshaghi et al. (2011)
		Reverse Genetics	RI	RI	RI	Pizzorno et al. (2012)
	E119G	Reverse genetics	S	HRI	RI	Pizzorno et al. (2011b)
	E119V	Reverse genetics	RI	HRI	RI	Pizzorno et al. (2011b)
A(H5N1)	H274Y	Clinic/oseltamivir	HRI	S	HRI	Le et al. (2005)
	N294S	Clinic/oseltamivir	RI	S	S	Le et al. (2005)
	D198G	*In vitro*/zanamivir	RI	RI	S	Hurt et al. (2009a)
	E119G	*In vitro*/zanamivir	S	HRI	RI/HRI	Hurt et al. (2009a)

[a] Numbers indicate the position of the substituted residue in the NA amino acid sequence (N2 numbering).
[b] S, susceptibility or normal inhibition (<10-fold increase in IC_{50} over WT); RI, reduced inhibition (10–100-fold increase in IC_{50} over WT); HRI, highly reduced inhibition (>100-fold increase in IC_{50} over WT). A(H1N1): seasonal H1N1 viruses, A(H1N1)pdm09: Swine origin H1N1 viruses responsible for the 2009 pandemic.

Table 1.1 **NA mutations that confer NAI resistance in N1 subtype** (taken from (24))
[a] Position of substituted residue in N2 numbering
[b] S, susceptibility or normal inhibition (<10-fold increase in IC50 over WT); RI, reduced inhibition (10–100-fold increase in IC50 over WT); HRI, highly reduced inhibition (>100-fold increase in IC50 over WT).

The presence of the two competitive activities in influenza virus life cycle seems to be contradictory yet very complimentary indeed. HA binding to sialic acids is prerequisite to initiate infection. However, such binding would prevent progeny viruses to infect new cells if not due to NA activity. Moreover, SAs are present on other cell surfaces as well. Thus owing to NA activity, those binding to "decoy" receptors would be significantly diminished (23).

Theoretically, influenza viruses need to maintain a balance between HA and NA to ensure successful replication cycle. Strong HA binding and a low active NA would result in less virus release, hence reduced infectivity; on the contrary, weak HA binding together with a highly active NA could lead to insufficient viral entry and replication. Furthermore, experimental results indicate a correlation between HA and NA activity. Firstly, they share the same substrate/receptor specificity. Misbalance of HA-NA can be restored by changes either on HA and NA. More interestingly, HA mutations can also lead to NAI resistance; explained by decrease in HA affinity reducing the dependency of influenza virus on its NA (35).

There have been numerous studies investigating the interplay of HA and NA, either on seasonal influenza H1N1 and H3N2 or highly pathogenic H5N1. In 2000, Kawaoka *et al* (36) demonstrated that WSN33 mutants with defective NA function could not grow in embryonated chicken eggs but the progeny viruses, after egg passaging, were replication competent. Sequencing data revealed that those mutants contained mutations in the HA gene, which reduced the receptor binding affinity of HA; therefore made the viruses less dependent on NA activity. Using a slightly different approach, Wang *et al* (37) showed that not all HA-NA combinations, of 2 H1N1pdm09 and 1 H5N1

strains, would produce infectious particles. They also noted that some combinations lead to higher infectivity compared to their ancestors, thus indicating a matching pattern between HA and NA. More recently, Fouchier's group has displayed that only one mutation required for an H5N1 HA to acquire human receptor specificity and at the same time remained replication capable in PR8 background, either with NA from the same H5N1 isolate or NA from PR8 (38). Another study performed by Webster and colleagues on avian H5N1 in the presence of NAIs revealed that reduced NA activity, mediated by presence of drug in *in vitro* passaging studies, was essential for viral adaptation to human cell cultures. These findings underscore the complex interplay and co-evolution of HA and NA during the process of switching from avian to human hosts (20). It also underscores the importance of understanding the implications of using antiviral NAI as part of pandemic response strategies.

VII Avian influenza viruses

In addition to the past human pandemics caused by viruses with avian origins, five avian influenza subtypes are known to cause sporadic human infections after direct transmission from avian hosts. They were HPAI viruses H5N1, H7N3 and H7N7; and LPAI viruses H7N2, H7N3, H7N7, H9N2 and H10N7 (9). Among those, the H9N2 subtype has shown pandemic potential and has spread to many countries. They were initially isolated in turkeys and shorebirds, then established in terrestrial poultry and caused sporadic infections in both humans and pigs in Hong Kong. One of the H9N2 lineages, isolated from quail, possesses 6 internal genes similar to the ones of H5N1

human isolates in Hong Kong. Moreover, H9N2 viruses in terrestrial poultry have the ability to spill over to domestic ducks and generate double or triple reassortants with duck viruses (1). Recently, a new influenza subtype has been reported to cause infections in humans in China. The first index case was presented in early 2013 with signs and symptoms of respiratory tract infection and the causative agent was identified as influenza H7N9 virus (39). This virus was shown to be derived from classical avian H7N9 influenza virus and to have the ability to cause infections in mammals. Its genetic materials were derived from at least 4 sources with HA gene from duck and NA gene from migratory birds viruses (39). Geographic and epidemiological analysis suggested that H7N9 and H5N1 high-risk areas for human infections are co-distributed at the bordering provinces Anhui and Zhejiang of China (40). This study also proposed that exposure to contaminated environments or live poultry markets is a possible route of infection.

1. H5N1 emergence and transmission

Zoonotic transmission of influenza viruses contributes significantly to the genetic and antigenic diversity of human seasonal viruses, and H5N1virus with the potential causing novel a pandemic remains a credible threat.

Historically, a number of avian and swine influenza infections in humans have been documented. Among those, the HPAIV H5N1 has the highest number of reported cases; some other smaller epizootics including HPAIV H7N7 in the Netherlands, H7N3 in Canada and LPAIV H9N2 (11). The Asian lineage of HPAIV H5N1 first appeared in geese in Guangdong, China in 1996 (A/goose/Guangdong/1/96).One year later, a reassortant variant of A/goose/Guangdong/1/96 emerged in Hong Kong, causing 18

human cases and 6 deaths. This was the first report of casualties after direct infection with a HPAIV from an avian source (5). Hong Kong government immediately initiated a strategy of culling all poultry, resulting in successfully elimination of A/goose/GD/1/96 genotype; nevertheless, H5N1 reassortants continued to emerge in healthy waterfowl such as geese and domestic ducks in the costal provinces of China in the 1999-2002 period (1). In 2003, H5N1 re-emerged, again causing lethal infections in humans. Since then, H5N1 has spread across South-East Asia, to Europe and even Africa. To date (as of December, 2014), 668 human cases with 393 deaths were reported to WHO (WHO website); among many countries affected, Indonesia, Viet Nam, Egypt, China and Thailand have the highest number of fatal infections (5).

The emergence of HPAIV H5N1 was probably through mutations in the HA cleavage site when a LPAIV was introduced from wild birds into domestic birds (2). Most H5N1 isolated westward showed high genetic and antigenetic similarity, whereas isolates from China and Southeast Asia displayed high levels of genotypic variation (1). There have been more than 40 genotypes identified up to now; among those are genotypes Z and V, the most prevalent ones. Additionally, ten main distinct clades of H5N1 have been recognized, clade 0 to clade 9, and have become endemic in domestic poultry. Recent studies demonstrated that the dominant clade has changed from clade 0 to clade 2.3 (with many different subclades) (41).

The majority of human cases were caused by exposure to sick birds, however, there were a number of confirmed human cases resulting from close contact with H5N1 patient (for example, caretaking of an infected family member). Some studies also showed that H5N1 virus can be transmitted through contact with contaminated water

and sewage (41). Many epidemiological studies have demonstrated that the wide spread of H5N1 viruses, starting from China and Southeast Asia then going westward to Europe and Africa, may result from migration of wild birds, especially long-distance migratory birds. Other key factors in virus dissemination is local poultry industry and international poultry trade (12,41).

2. Molecular determinants of pathogenesis and transmissibility

In their natural reservoir, influenza viruses, including HPAIV H5 and H7, generally cause asymptomatic infections. Nonetheless, after adaptation into a new host, either domestic avian species or mammals, these viruses have also increased their pathogenicity, ranging from mildly to highly pathogenic (1). This process is similar for both mammals and avian species in the extent that the contributing genes are polygenic traits and are usually involved in binding to new host cell receptors, replicating in new environment and overcoming the host defense system. Several molecular determinants of high pathogenicity in mammals have been identified, including PB2 E627K and D701N, HA basic cleavage site insertion and N186K and Q196K mutations and NA stalk deletion (1).

Residue at position 627 of PB2 was first recognized as a major determinant of HPAI H5N1 viruses in 2001. In the subsequent years, Lys was found at this position in a PB2 numerous H5N1 isolated from infected patients. Data from different studies suggest that PB2-K627 is selected during replication in mammals and this amino acid supports efficient virus replication in mammalian species (4). Another important position of PB2 protein is residue 701. Various studies have shown that PB2 D701N also contributes to viral pathogenicity by facilitating recruitment of viral polymerase subunits into the

nucleus, hence enhancing viral transcription and replication in host cells (12). Non-structural gene, segment 8 NS gene, also plays an important role in viral replication and pathogenesis. NS1 protein, encoded by NS gene, can inhibit host innate immune response by reducing interferon productions (42). Single amino acid mutations or short sequence deletion of NS1 protein have been demonstrated to associate with cross species spread and increased virulence of influenza viruses. However, the underlying mechanisms remain to be determined (4,12,41).

Influenza virus infection initiates by binding of HA protein with host cell receptors. HA binding specificity toward its receptor partly determines influenza virus host range and pathogenicity (4,9). Generally, human influenza viruses prefer to bind to receptors with NeuAcα2,6 Gal linkage while avian influenza viruses mainly recognize NeuAcα2,3 Gal moieties. This correlates with the abundance of these 2 types of receptors in different hosts. NeuAcα2,6 Gal moieties are found abundantly on bronchial epithelial cells of human upper respiratory tract, whereas NeuAcα2,3 Gal on epithelial cells of bird intestine and lower respiratory tract of humans (2). However, most of the HPAI H5N1viruses isolated from humans possess avian type receptor specificity, displaying that avian influenza viruses are capable of infecting human. This is supported by recent findings that NeuAcα2,3 Gal oligosaccharides are also detected on ciliated cells and the alveolar epithelia of human respiratory tract. Additionally, the expression levels of either NeuAcα2,3 Gal or NeuAcα2,6 Gal on cell surface vary depending on host age. Taken together, these findings partly explain those human H5N1 cases. Nonetheless, it is still believed that avian viruses need to acquire the ability to recognize human type receptors for sustained replication and transmission in humans (4) and causing a

pandemic (12). This is strengthened by the fact that earliest human isolates of the 1918, 1957 and 1968 pandemics all recognized NeuAcα2,6 Gal receptors in spite of their avian origin. Consequently, the infection of humans with HPAI H5N1 was found to be surprising because viruses isolated from patients in Hong Kong in 1997 preferred to bind to avian type receptors. There have been numerous amino acid substitutions, which affect the receptor specificity of influenza viruses, identified and studied. Most of them are in the receptor binding site of HA protein. Those include residues at positions 138, 190, 194, 225, 226 and 228 (H3 numbering) (single mutations and in combinations) (11,42). For H1 subtype of avian virus, E190D and G225D played the key role in adaptation to humans or swine by increasing NeuAcα2,6 Gal binding. For H2 and H3 subtypes, Q226L and G228S mutations were vital to enhance human type receptor binding (11).

3. Treatment and drug resistance

Because of their highly diverse genetic pool in different geographic areas, developing an effective H5N1 vaccine faces huge challenges. To date, there are more than 20 vaccine candidates that can be used in clinical trials as well as in model systems of pandemic vaccine production (41). High virulence in egg makes it impractical to produce vaccines using wild-type H5N1. Moreover, prototype H5N1 vaccine generated by plasmid-based reverse genetics was found to be poorly immunogenic. Subsequent studies showed that adding of adjuvant could improve the induced protection significantly in animal models (41).

Based on enzymatic inhibition assays, there is a disparity in oseltamivir susceptibility *in vitro* between clade 1 and clade 2 H5N1 viruses (32). Clade 1 viruses, from 2004 from

Vietnam and Cambodia, were as sensitive as human seasonal H1N1 viruses to oseltamivir; whereas some clade 1 2004 Cambodian isolates displayed slight reduction in oseltamivir susceptibility, about 6- to 7-fold. Clade 2 2005 Indonesian viruses showed even higher decrease in oseltamivir sensitivity, between 15 to 30-fold when compared to clade 1 isolates (43). Sequencing analysis together with structural studies suggested that this discrepancy might be caused by H252Y mutation. Clade 1 neuraminidase this is normally H252 but in clade 2 it is all Y252. The amino acid at position 252 can affect reorientation of E276, which is necessary for oseltamivir binding to targeted neuraminidase (43).

Similar to seasonal influenza viruses, and H1N1pdm09 virus, antiviral drugs are the first line of defense for H5N1 infections. Those include the adamantanes and NAIs. Nevertheless, very high amantadine resistance rates were reported in clade 1 viruses in Southeast Asia, along with other clades as well (19), resulting in H5N1 treatment now almost solely relies on NAIs. This explains why the emergence of H5N1 NAI resistant mutants poses serious public health concern (27). H274Y is also the most common mutation isolated from H5N1 oseltamivir-resistance cases, either before or after treatment, together with N294S mutation at lower frequency (28). In some cases, the viral populations contained a mixture of oseltamivir-sensitive and oseltamivir-resistant viruses, with H274Y or N294S. The N294S mutation was also reported to emerge spontaneously in H5N1 viruses in Egypt, causing 57-138 fold decrease in oseltamivir sensitivity (32). Mutations at position I222 have been identified in H5N1 isolates as well. In clade 1 background, the resulting amino acid only reduced oseltamivir sensitivity by a

few fold whilst in clade 2 viruses it was proposed to work synergistically with H252Y change and further increase IC50 values to the range of 40-250nM (32).

4. H5N1 in Vietnam

HPAI H5N1 viruses were first detected in poultry in Vietnam in 2001 and have become enzootic in poultry populations of the country (44). An influenza surveillance program was initiated in 2004 under the collaboration of the Department of Anima Health (DHA), the National Centre for Veterinary Diagnostics (NCVD) and regional DHA laboratories (according to WHO Western Pacific Regional Office website). This program started with identification and characterization of circulating avian H5N1 viruses in Vietnam and using the obtained data in implementing necessary measures to control influenza outbreaks in poultry (44). These extensive biosafety measures, including poultry vaccination and massive culling of infected flocks, have progressively contributed to the decreasing number of poultry H5N1 outbreaks between 2003 and 2011 (from 2388 to 45, FAO website).Nevertheless, the control programs for avian influenza in Vietnam still face many challenges due to rapid evolution of the viruses.

Since the first appearance in 1996 of A/goose/Guangdong/1/96 in China, 12 genotypes (genetic constellations) have evolved/reassorted from this ancestor virus (45). Shortly after this emergence, newly evolved genotypes; namely V, W, X, and Z; have entirely replaced the original ones GD, A, C, D and E. In Vietnam, since 2005, Z and G (a reassortant of W and Z) are the predominant genotypes.

In parallel with the emergence of new genotypes, the antigenic HA protein of the virus has also accumulated mutations and “drifted” into many different clades. Up to now, there are 10 distinct clades, and subclades in first-, second-, third-order generated

(WHO, Western Pacific Regional Office website).The H5N1 virus that was introduced into Vietnam in 2003 belonged to clade 1. This clade was subsequently replaced by clade 2.3.4 after mid- 2005 in northern Vietnam (44). Clade 1, and newly evolved clade 1.1, viruses remained dominant in the Mekong Delta region (southern Vietnam). Phylogenetic studies have shown that these viruses were closely related to those identified in Cambodia and Thailand, strengthening the reports on sustained enzootic circulation of these viruses within the Mekong Delta and its adjacent regions (46). In 2010, newly introduced clade 2.3.2.1 completely replaced clade 2.3.4 in northern and central Vietnam while clade 1.1 has further evolved into 1.1.1. and 1.1.2 in the south (45).

The 2011-2012 period was marked by the formation and spread of new subclades 2.3.2.1a, b and c. Clade 2.3.2.1b disappeared during 2013-2014, while subcladesa and c were in wide circulation throughout the country including the southern parts. Concomitantly, clades 1.1.1 and 1.1.2 were expanding from Mekong Delta to the central region of Vietnam. The antigenic diversification of pre-existing, clade 1.1 in the south, and newly introduced, 2.3.4 or 2.3.2.1 in the north, H5N1 viruses has complicated the enzootic status of Vietnam and posed many challenges for H5N1 control programs of the Vietnamese government. This has been most evidenced in selection of vaccines for poultry. Initially in 2006, Re-1 (clade 0 vaccine) was used for the nationwide vaccination program. During 2007-2010, Re-5 (clade 2.3.4) was introduced due to the emergence of such clade in the North of Vietnam. However, Re-5 is not highly effective against clade 2.3.2.1 viruses so its usage was declined in 2011-2012 period and now the DAH is

planning to import Re-6 vaccine (clade 2.3.2) to use in 2014 (according to DAH website).

Although H5N1 virus was first detected in poultry in Vietnam in 2001, it was not identified as a cause of human cases of severe respiratory disease with high mortality until Jan 2004 (44) (WHO website). From Jan 2004 to Jan 2005, 6 H5N1 human cases were reported. However, at the end of 2005, there were total 65 confirmed H5N1 human cases already. With the successful control program for poultry outbreaks, no more new H5N1 patients reported during the year of 2006. In Jun 2007, the 1st case since Nov 2005 was announced. In about 6 months (to Jan 2008), the number of H5N1 patients increased to more than 100. Since then, sporadic cases have been reported, making the total number of 127 cases (as of Dec 2014) with the case mortality rate approximately 50% for H5N1 infections in Vietnam. As expected, most of the human cases were reported in accordance to the widespread of poultry outbreaks. Details on the timeline of poultry outbreaks, identification of human cases as well as emergence/disappearance of H5N1 clades are shown in Figure 1.5.

Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2014

Country	2003-2009*		2010		2011		2012		2013		2014		Total	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
Azerbaijan	8	5	0	0	0	0	0	0	0	0	0	0	8	5
Bangladesh	1	0	0	0	2	0	3	0	1	1	0	0	7	1
Cambodia	9	7	1	1	8	8	3	3	26	14	9	4	56	37
Canada	0	0	0	0	0	0	0	0	1	1	0	0	1	1
China	38	25	2	1	1	1	2	1	2	2	2	0	47	30
Djibouti	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Egypt	90	27	29	13	39	15	11	5	4	3	12	5	185	68
Indonesia	162	134	9	7	12	10	9	9	3	3	2	2	197	165
Iraq	3	2	0	0	0	0	0	0	0	0	0	0	3	2
Lao People's Democratic Republic	2	2	0	0	0	0	0	0	0	0	0	0	2	2
Myanmar	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Nigeria	1	1	0	0	0	0	0	0	0	0	0	0	1	1
Pakistan	3	1	0	0	0	0	0	0	0	0	0	0	3	1
Thailand	25	17	0	0	0	0	0	0	0	0	0	0	25	17
Turkey	12	4	0	0	0	0	0	0	0	0	0	0	12	4
Viet Nam	112	57	7	2	0	0	4	2	2	1	2	2	127	64
Total	468	282	48	24	62	34	32	20	39	25	27	13	676	398

Table 1.2 **Number of H5N1 cases up to Dec 4th, 2014** (WHO website)

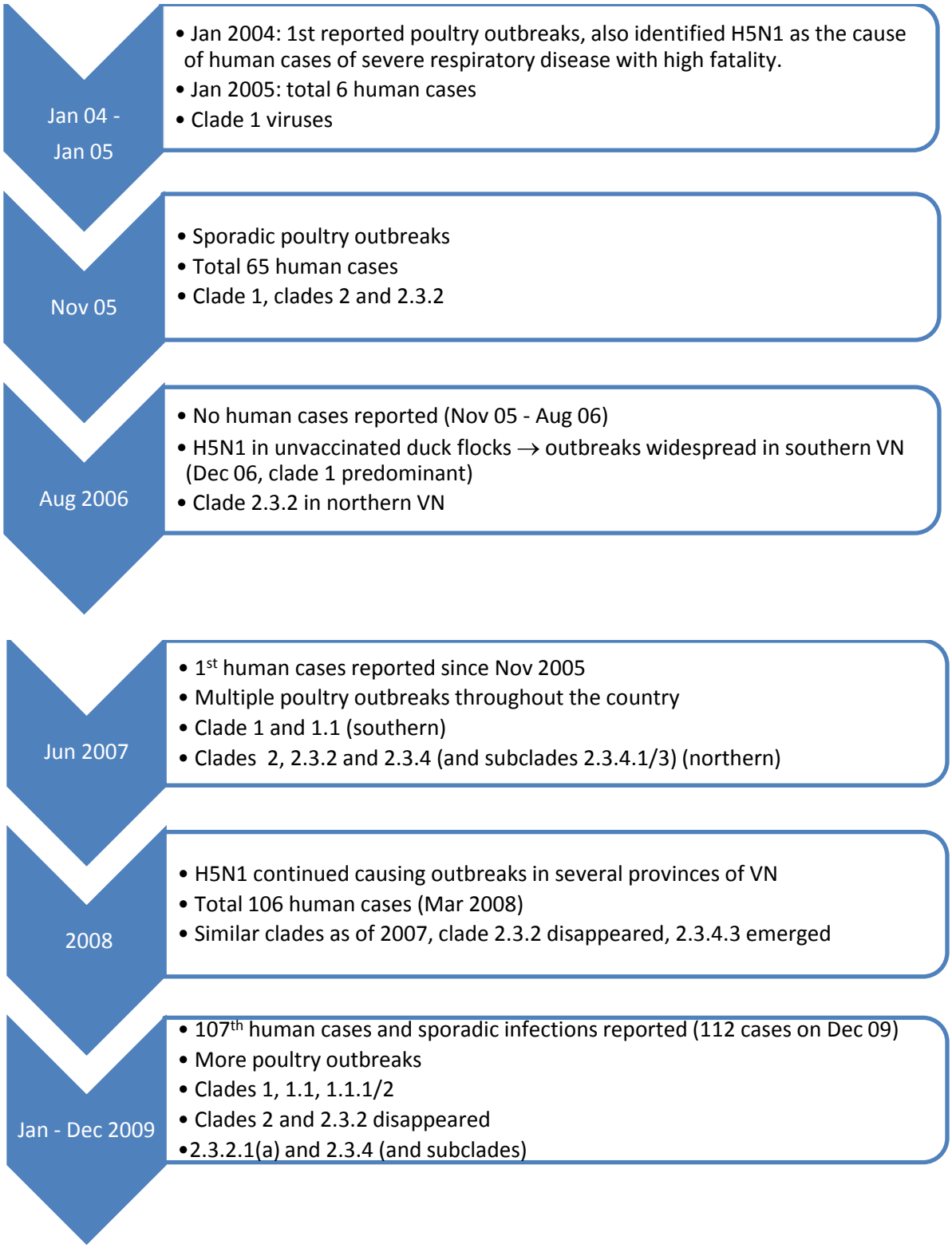


Figure 1.5 **Timeline of H5N1 poultry outbreaks and human infections in Vietnam from 2004 to 2014** (data taken from (45) and WHO website)

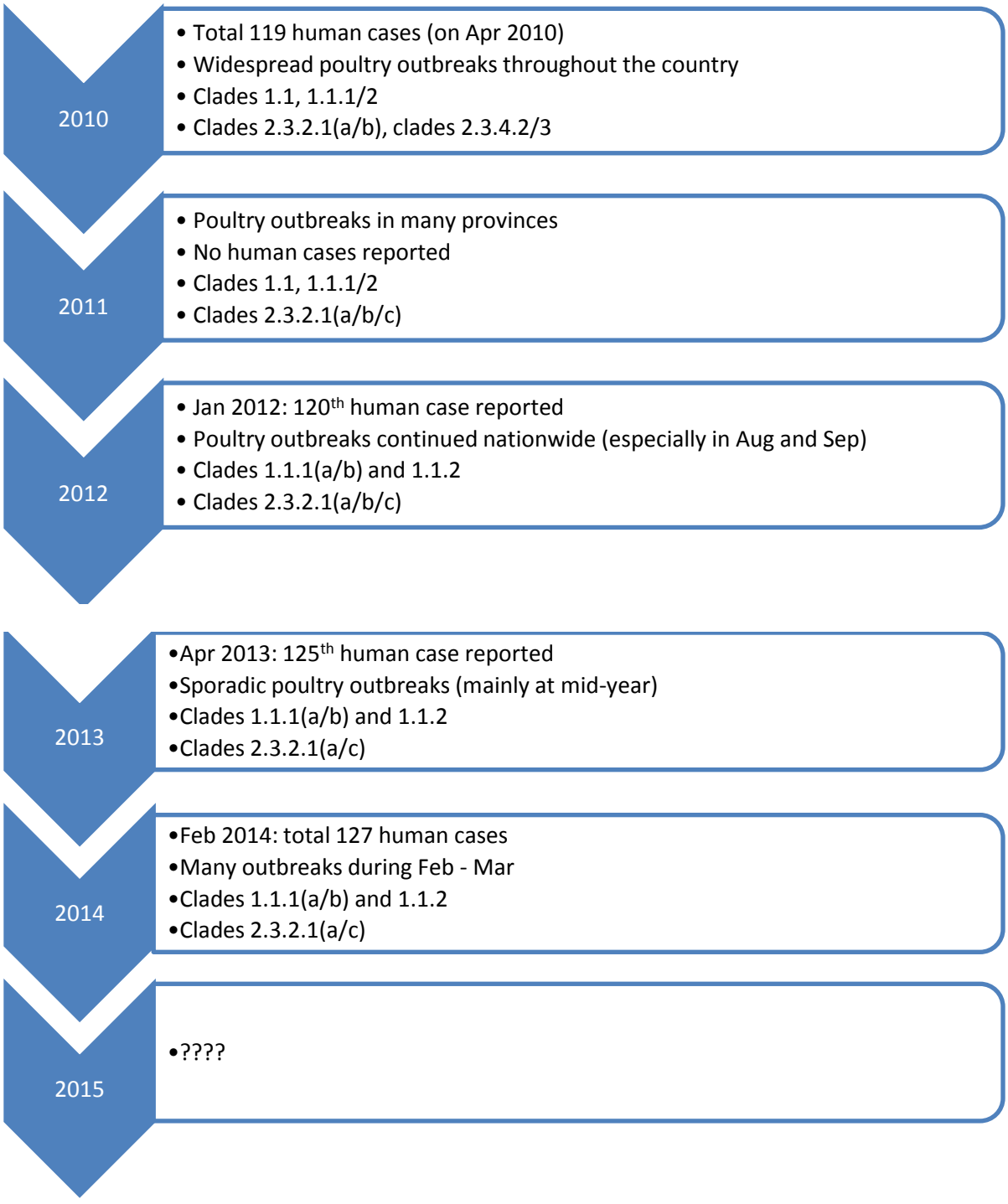


Figure 1.5 **Timeline of H5N1 poultry outbreaks and human infections in Vietnam from 2004 to 2014** (data taken from (45) and WHO website)

VIII Aims of this dissertation

Experimental and field data imply that H5N1 viruses are evolving rapidly although either the direction or the underlying driving force of this evolution remains unclear. There are more and more research investigating the adaptation process of H5N1 when crossing species barrier, switching hosts from wild aquatic birds to domestic poultry, mammals and afterward humans. Two recent, and controversial, studies on H5N1 transmissibility were conducted with either wild-type or recombinant H5N1 viruses. One study (47) serial passaged H5N1 A/Indonesia/5/2005 virus in ferrets and identified a minimal set of mutations that conferred transmissibility via respiratory droplets. Those substitutions were PB2 E627K, HA Q222L, G224S, T156A and H103Y (H5 numbering). The other study (48) also assessed respiratory droplet transmission of H5N1 virus but used recombinant variant containing HA from A/Vietnam/1203/2004 and the rest of the genome derived from H1N1pdm09 A/California/04/2009 virus. They found that 4 mutations on HA; N158D, N224K, Q226L and T318I (H3 numbering) would allow recombinant virus to be transmissible between ferrets. However, they emphasized that whether these mutations would render sustained human-to-human transmission required further investigation. They also implied that the transmissible virus possessed 7 segments from H1N1pdm09 virus, which might play critical roles to its transmissibility.

H5N1 adaptation process is thought to primarily involve in changes of the 2 main glycoproteins of influenza virus: hemagglutinin HA and neuraminidase NA. HA is responsible for viral entry via binding with host cell receptors (α2,3 linkage for avian and α2,6 linkage for humans) while NA is a receptor destroying enzyme, promoting release of newly formed virions and preventing their self-aggregation. With their opposing roles

in viral replication cycle, one rational assumption is that influenza virus needs to maintain a balance between these 2 proteins' activities to ensure productive infection. Moreover, NA is the main target of anti-influenza drugs; therefore NA mutations are clinically relevant. In addition, mutations on HA gene can result in both receptor switching, supporting adaptation in humans, and drug resistance (although not clinically significant). Because of their impacts on both viral evolution and drug susceptibility, it is necessary to gain insight into the interplay between HA and NA proteins as this will further our understanding of the cross-species A/H5N1 adaptation process, with or without selective pressure.

One of the most suitable systems to study influenza adaptation is cultures of differentiated human epithelial cells. These *ex vivo* systems have been used for more than 30 years in various types of research, especially allowing study cell-specific-infection of viruses. This project aimed to set-up that system in our laboratory in Vietnam to passage H5N1 viruses in order to use in later studies monitoring the "adaptive" changes of those viruses, especially in the two major glycoproteins HA and NA. In parallel, H5N1 isolates with different oseltamivir susceptibility profile were investigated to gain clear insight on NA enzymatic properties as well as the underlying mechanism of resistance. Those are H5N1 isolates of human clade 1 viruses from the period of 2004 – 2005 in south of Vietnam. They displayed different oseltamivir sensitivity and possessed suspected mutations that might play a role in NA kinetic properties or expression. Using reverse genetic technique (which was also new and need to be established in our laboratory) to generate recombinant variants with NA derived from H5N1 viruses and other segments from laboratory adapted human

seasonal strain WSN33, we wished to characterize NA enzymatic properties and investigate in impacts of NA mutations on its activity and expression. We speculated that changes in NA genes might support or allow the virus(es) to overcome the deficiency caused by oseltamivir resistance mutation, resulting in adaptive evolution of influenza viruses.

Chapter 2

Materials and methods

I Viruses used in this study

The influenza viruses used in this project were isolated from Vietnamese patients during the outbreak of 2004-2005. These viruses were then passaged twice in embryonated chicken eggs. The allantoic fluid was harvested and stored at -80°C for future experiments. Hereafter, the viruses will be referred as CL105, CL107 and CL2009C, respectively.

Virus	Host	Subtype	Year of isolation	Clade
A/VIETNAM/CL105/2005	Human	H5N1	2005	1
A/VIETNAM/CL107/2005	Human	H5N1	2005	1
A/VIETNAM/CL2009C/2005	Human	H5N1	2005	1
1002	Human	H5N1	2007	2.3.4

Table 2.1 **Human H5N1 isolates used in the study** (apart from virus 1002 which originated from northern of Vietnam, other isolates were from southern of Vietnam)

CL105 was isolated from a 10-year-old boy admitted to hospital 4 days after the onset of illness. Oseltamivir treatment was initiated on admission but the boy died one day later. No clear relation between illness and exposure to sick poultry could be established (49)

CL107 was isolated from an 18-year-old woman. Oseltamivir treatment (75mg twice daily) was started on admission day, which was day6 after the onset of symptoms. The original viral was isolated from a throat swab taken on day 1 after admission. Sequencing analysis revealed that this isolate contained the NAH274Y substitution that

confers resistance to oseltamivir carboxylate. This patient has been previously described as patient 4 (50), she died on day 15 after admission.

CL2009C was isolated from a 13-year-old girl admitted to hospital with fever and coughing symptoms starting two days ago. On the previous day her mother died with confirmed H5N1 infection after two days of oseltamivir treatment (patient 2 (50)).The girl had been exposed to and was suspected to have H5N1 infection so oseltamivir was given at the dose of 75mg, then a second dose of 6 hours after the first one and a third dose 24 hours after admission. Treatment was then continued for four days at the standard dose of 75mg twice daily. The girl died 6 days after admission. The virus was isolated from a throat swab taken on the day of death. It was also shown to harbor the oseltamivir resistance associated NA H274Y mutation.

Another H5N1 virus was used in this project for the purpose of setting up mutagenesis techniques. The virus code was 1002, a clade 2.3.4 virus, isolated in Ha Noi, Vietnam in 2007. Isolate 1002 originated from a 28-year-old female farmer who was involved in chicken husbandry. She was admitted to hospital six days after the onset of illness. Treatment with oseltamivir (150 mg twice daily) started one day after admission. The patient failed to recover and died (49).

All Influenza A/H5N1 viruses were used as part of the partnership between Oxford University Clinical Research Unit and the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam. The study was approved by the Scientific Ethics Committee of the Hospital for Tropical Diseases.

II Continuous cell cultures

Madin-Darby canine kidney (MDCK) and human embryonic kidney (293T) cells were obtained from the American Type Culture Collection. MDCK cells were used for virus stockpiling and titration; 293T cells were used to rescue virus in mutagenesis experiments.

MDCK cells were stably passaged in MEM medium (Invitrogen,Carlsbad, CA, USA), supplemented with L-Glutamine) containing 10% inactivated fetal bovine serum (Gibco, US), 1% non-essential amino acids (NEAA, Gibco), 500µl PenStrep (100x at the concentration of 10000IM/ml Penicillin and 10mg/ml Streptomycin, in-house prepared, stocks from Sigma-Aldrich, St. Louis, MO, USA), 2% 1M HEPES (Sigma-Aldrich) and 2.5% of 7.5% $NaHCO_3$ (Sigma-Aldrich).

293T cells were cultured in DMEM (Invitrogen), supplemented with L-Glutamine and Pyruvate, containing 10% inactivated fetal bovine serum (Gibco), 1% non-essential amino acids (NEAA, Gibco) and 500µl PenStrep (1000x Peniciline-Streptomycine, in-house prepared).

III Virus culture and titration

1. Virus propagation

Monolayers of MDCK cells seeded in T-75cm^2 culture flasks were washed two times with PBS pH 7.2 (Gibco) before inoculating with 5ml of transfection supernatant (from mutagenesis experiments) for passage 0 or 500µl of infection supernatant (from MDCK infection) for subsequent passages. The inoculum was allowed to adsorb to the cell

monolayer for one hour at 37°C. The inoculum was then removed and 15ml of infection medium (similar to growing media but without fetal bovine serum, enriched with 500µl Trypsine (0.5% stock, Gibco)) was added and incubated at 37°C. Cytopatic effect (CPE) was observed daily. The cell culture was harvested when more than 80% of the cell layer showedCPE; infection supernatant was collected and then briefly centrifuged. 7.5% BSA was added to the resulting supernatant to a final concentration of 1%; virus stock was aliquoted to small volume of 500µl and stored at -80°C.

2. $TCID_{50}$ (51)

$TCID_{50}$ (50% tissue culture infectious dose) is a measure of viral titer. It is an endpoint assay to determine the amount of virus needed to produce a cytopathic effect in 50% of inoculated tissue culture cells. In this project the $TCID_{50}$ for influenza A/H5N1 viruses was performed in 96-well plates seeded with monolayers of MDCK cells and infected with 10-fold (or 5-fold, depending on preliminary data on viral infectivity) dilutions of the virus in infection medium, at a final volume 100µl. Dilution started at well 1 of row A to H and was then continued to well 11, no virus was added to well 12 as it served as negative cell control (so total 8 wells for each dilution, 11 dilutions per plate). For virus with suspected high titer, dilution would continue in a second 96-well plate. The plate was incubated at37°C, 5%CO_2 for 3-4 days. The cytopathic effect (CPE) was checked visually under an inverted microscope. The $TCID_{50}$ was calculated using Reed-Münch method (Microsoft Excel template).

Dilutionfactor	5x	from well 2 to well 11)
Input dilution factor	100x	(of the first well of each row)
Testvolume	20ul	(volume added to the first well)

	dilution	# of tests	pos	neg	count pos	count neg	Perc
1	100	8	8	0	15	0	100
2	500	8	4	4	7	4	64
3	2500	8	3	5	3	9	25
4	12500	8	0	8	0	17	0
5	62500	8	0	8	0	25	0
6	312500	8	0	8	0	33	0
7	1562500	8	0	8	0	41	0
8	7812500	8	0	8	0	49	0
9	39062500	8	0	8	0	57	0
10	195312500	8	0	8	0	65	0
11	976562500	8	0	8	0	73	0

Titer/ml according to Reed and Münch	4.75
Titer/ml according to Spearman Kärber	5.36

note: first row should be fully positive

Table 2.2 **Example of a $TCID_{50}$ calculation with Excel template**

IV NA kinetics assay

Enzymatic activity of NA was measured using a fluorogenic analogue of the natural substrate: MUNANA (2' 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate).

NA activity of the viruses of interest was standardized as described below.

1. NA activity standardization

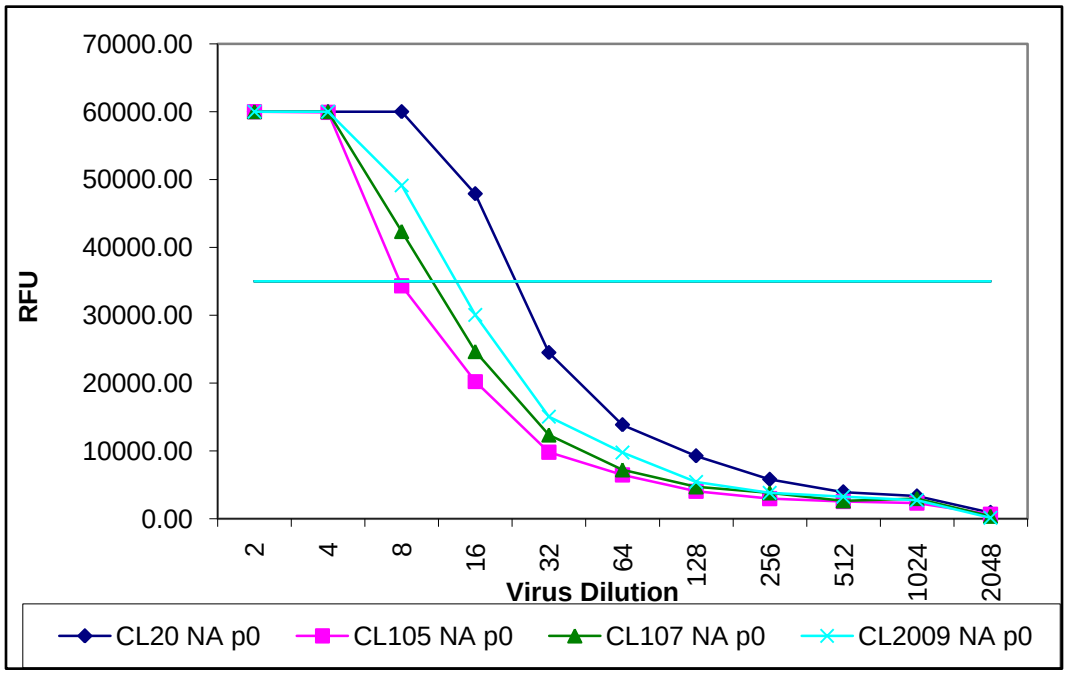


Figure 2.1 **Plotted data obtained in the NA activity determination assay.**
The horizontal line shows the dilution of virus used for subsequent experiment (within linear range of all curves).

Virus stock was diluted in MES assay buffer (2-Morpholinoethanesulfonic acid; Sigma-Aldrich), 20µl of 2-fold dilutions of virus,1/2 to 1/2048,were then incubated with 30µl of 100µM of MUNANA substrate (also prepared in MES buffer) at 37 °C. After 1 hour the reaction is stopped by adding 150µl stop solution, 1M glycine and 25% ethanol. The released fluorescence is read in a FLUOstar Optima microplate fluorescence reader

(BGM Labtech). Fluorescence readings are analyzed using means of a non-linear regression model (Excel, Microsoft) kindly provided by the Virus Reference Division at the Health Protection Agency (HPA, Colindale, London, UK). The plotted data yields a sigmoid dose-response curve. The X axis represents the virus dilution and the Y axis shows the relative fluorescence units (RFU). The concentration of virus needed for following step, IC_{50} determination or NA enzymatic parameter measurement, is calculated from the linear range of the obtained curve.

2. NA kinetics V_{max} and K_m (52)

Similar levels of NA activity are used for all virus of interest (in duplicate). NA enzymatic properties are accessed by testing with different MUNANA concentrations (final concentrations in well: 0,23 µM, 0,69 µM, 2,08 µM, 6,25 µM, 18,75 µM, 56,25 µM, 112,5 µM, 225 µM, 450 µM and 900 µM). Fluorescent intensity is measured every 60 seconds for 60 minutes with an excitation wavelength of 355nm and emission wavelength of 460nm. The readouts were expressed as RFU per minute in each well. These readouts were used to calculate the "slopes" (of the linear range) and then results were fit intoa non-linear regression model (GraphPad Prism 5, GraphPad Software Inc.). The resulting graph demonstrated Michaelis-Menten kinetics where the initial reaction rate (V_0) is related to the substrate concentration [S]. Based on this graph, enzymatic parameters V_{max} and K_m were calculated. V_{max} (maximum velocity of the reaction) is an estimation of enzyme activity, representing the maximum rate of substrate conversion by the enzyme. And K_m, the Michaelis-Menten constant, is the concentration of substrate required to reach half of the V_{max} and usually corresponds to enzyme affinity for the substrate (Figure 2.2)

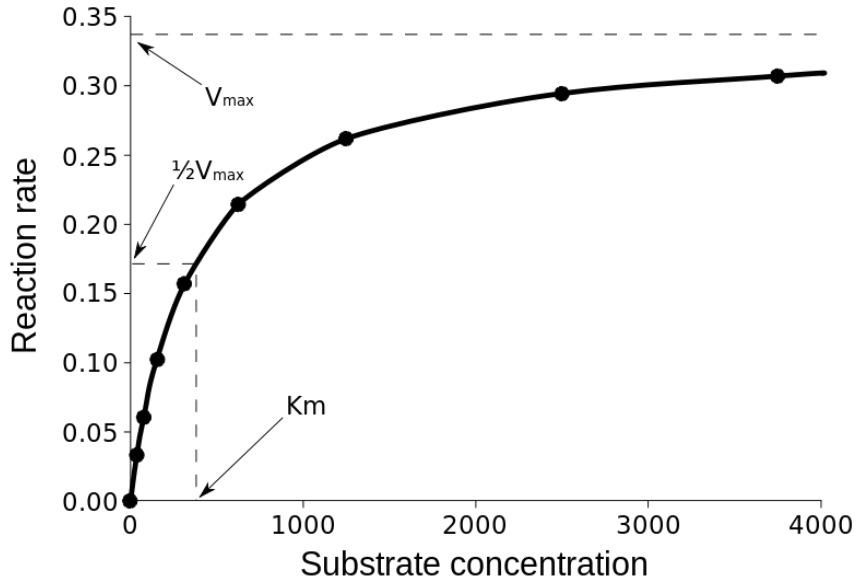


Figure 2.2 **Michaelis-Menten graph demonstrate V_{max} and K_m of an enzymatic reaction.**

3. IC_{50} determination (31,52)

The dilution factor calculated in the standardization step is used to determine the IC_{50} value. The same virus dilution is incubated with equal volumes of five-fold dilutions of the NA inhibitor in MES buffer (12500nM to 0.0064nM). After 30 minutes at 37°C the MUNANA substrate is added at a concentration of 100µM and incubated for another hour in the same conditions as previously described. The reaction was stopped by adding stop solution containing 25% ethanol. The plate is read in the FLUOstar microplate fluorescence reader and the data analyzed by the non-linear regression model. The plotted data yields a sigmoid curve of NA inhibitor concentration (X axis) and RFU (Y axis). The IC_{50} is automatically calculated using a script in Microsoft Excel software.

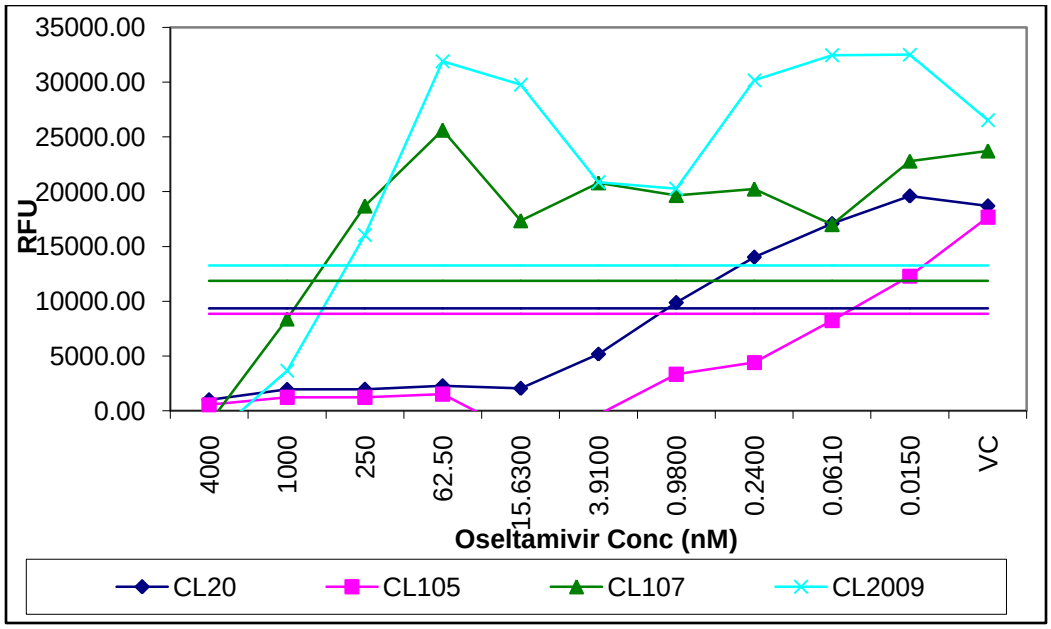


Figure 2.3 **Example of NA inhibition assay.**
The horizontal bars show the calculated IC_{50} for each virus.

V Western Blot

Recombinant viruses were passaged once in MDCK cells. After 3 day incubation at 37°C, 10ml of each culture was harvested. Cell pellets were resuspended in 50µl PBS and 5µl of each sample was used for Western blot. Cellular proteins were separated by electrophoresis into 15% SDS-PAGE gels, and transferred to a PROTRAN BA 85 membrane (10402588, Whatman GmbH) using semi dry transfer cell (Transblot SD). The blots were probed sequentially with a rabbit anti-NA antibody (1:1,000 dilution, MyBioSource MBS432041 against VN1203 NA) and a goat anti-rabbit IRDye 800CW antibody (1:5,000 dilution; 926-32213, LI-COR Biosciences). The blots were scanned using an Odyssey Infrared imaging system, and fluorescence intensity was analysed using Odyssey application software (version 2.1, Li-Cor Biosciences).

VI Sequencing

DNA templates, cloned into backbone plasmids for reverse genetic purposes, were isolated using the Qiagen Miniprep® kit. Apart from universal primers for all influenza gene segments (Uni12 and Uni13), at least 2 internal primer pairs specific to either HA or NA of H5N1 were also utilized to obtain full-length sequences of respective gene. Approximately 300-400ng of plasmid DNA was used in each sequencing reaction containing 0.5µl ABI BigDye v3.1, 20pg of primer, 3.5µl sequencing buffer and water to a final volume of 10µl.

Dye-terminated sequences were precipitated in 95% cold ethanol (–20°C) in the presence of sodium acetate. Purified sequencing products were loaded onto an ABI 3130XL genetic analyzer and results generated were an trimmed and assembled using the Vector NTI ContigExpress (Vector NTI 8.0, Infomax, Inc.)

Primer	Sequence
UNI12G	AGCGAAAGCAGG
UNI12A	AGCAAAAGCAGG
HA-5F	AAAGCAGGGGTHYDATCTGTC
HA-570R	TTGTARCTYCTCTTTATBGTBGG
HA-465F	GRGTRAGCKCAGCATGTCC
HA-917R	GDGTTTGRCACTTGGTGTTGC
HA-803F	AGTAATGGRAATTTCATTGCYCC
HA-1378R	ATTYTCCATKAGAACYAGRAGTTC

HA-1247F	ACTCARTTTGARGCHGTTGG
HA-1789R	AGTAGAAACAAGGGTGTTTT
NA-3F	CRAAAGCAGGAGTTYAAAATG
NA-553R	GCAACAGACTCAAAYCTYGAG
NA-431F	AGCCYTRYTGAATGACAARC
NA-944R	YTGATTGAAAGAYACCCATGG
NA-736F	TTACTGTWATGACWGAYGGACC
NA-1291R	ACCCARAARCAAGGTCTYATGC
NA-959F	AATAGGRTAYATATGCAGYGG
NA-1460R	AGTAGAAACAAGGAGTTTTT

Table 2.3 **Primers used in sequencing reactions of HA and NA genes**

1 cycle	94^0C	1 minute
	94^0C	10 seconds
30 cycles	55^0C	5 seconds
	60^0C	4 minutes
Hold at	15^0C	

Table 2.4 **PCR program for sequencing reactions**

VII Training periods

The two main techniques utilized in this project, human airway epithelial cell cultures and reverse genetics, were new to OUCRU laboratory in Vietnam. Because of this reason, two training periods were conducted so that the student could master the techniques and subsequently transfer them to OUCRU laboratory. The first training was on reverse genetics at the MRC, London with the achievement of successfully removing H5 HA polybasic cleavage site. The second training at the AMC, Amsterdam was for human airway epithelial cell cultures. Since results from those experiments were not used for further analysis, they will be presented as training accomplishments in chapter 3.

Chapter 3

Training achievements

Ex vivo culture system

I Introduction

1. Human airway epithelial cell (HAE)

Ex vivo systems of primary and differentiated human airway epithelial cells have been utilized for nearly 30 years in a variety of different research applications (53). They have been utilized to investigate processes of innate immunity during airway inflammation, mechanisms underlying diseases such as cystic fibrosis or lung cancer (54), and have been widely used in pharmaceutical research for drug efficacy studies (53). Originally, airway tissue fragments were finely minced then explanted and the epithelial cells were harvested. An alternative method is to use proteases to dissociate cells and create a suspension of free epithelial cells. Cell types are identified based on morphologic characteristics and immunostaining for cytokeratin. Primary airway epithelial cells on plastic dishes can be passaged several times. However, the cells differentiate poorly and have a squamous phenotype when cultured on plastic dishes. Only when cultured under appropriate conditions, that enable cellular polarization, freshly harvested or passaged primary airway epithelial cells can obtain remarkable phenotypic changes and closely resemble *in vivo* morphology. This effect can be seen *in vitro* when cells are grown on or within thick collagen gels or when large amounts of cells acquire a three-dimensional spheroid shape. The most widely used method allowing airway epithelial cells undergoing mucociliary differentiation is to grow them on porous surfaces at an air-liquid interface. This type of culture demonstrates

characteristics of normal human airway epithelial cells and allows study of cell-type-specific infection by viruses.

2. Other airway epithelial cell systems

Airway epithelial cells have been successfully isolated and differentiated from various species, including mouse, guinea pig (55) and pig (56,57). Moreover, several groups have succeeded in isolation of primary chicken airway epithelial cells and used these for experiments on avian respiratory virus infection (58). These cultures, especially porcine and avian, are particularly useful in investigating the evolution and host adaptation of influenza viruses.

Human and porcine airway epithelial cell cultures have been successfully employed for influenza virus infection experiments in the AMC, Amsterdam, The Netherlands. The main objective of this training program at AMC is to observe and master these techniques so that they can be transferred to the OUCRU laboratories in Vietnam. If feasible, the avian system, which is still under development by another group in the Netherlands, will be included in this study to gain more insights in adaptation of influenza viruses in different hosts.

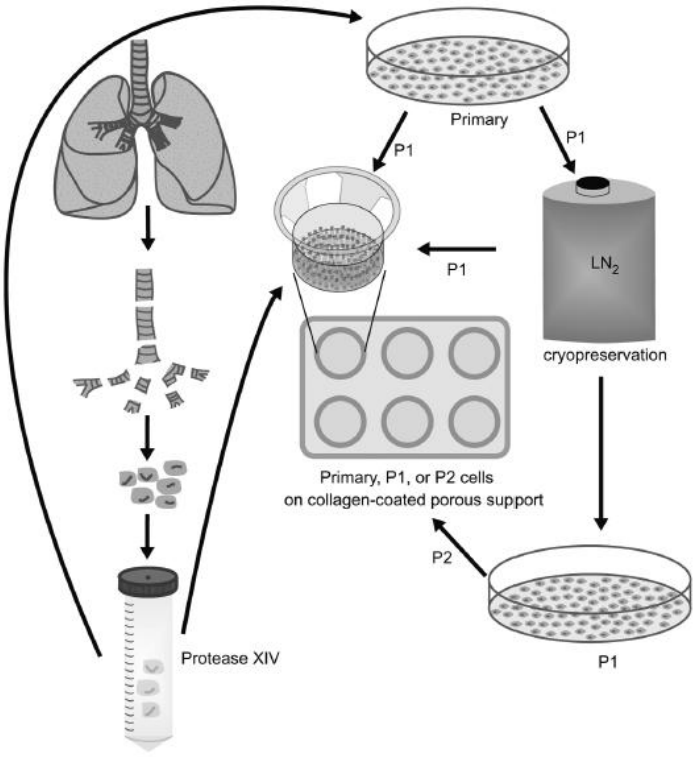


Figure 3.1 **Summary of airway epithelial cell culture process** (taken from (53)).

After isolating from patients materials, primary epithelial cells can be plated directly on porous surface for proliferation and then differentiation. Alternately, primary cells may be seeded on culture dishes for subsequent passaging or cryopreservation.

II Epithelial cell culture isolation

1. Materials and methods

Human airway epithelial cells (53)

Human lung lobectomies were obtained from patients admitted to the Academic Medical Center (AMC), Amsterdam, The Netherlands for surgery of lung cancer. The tissues used for culturing are considered “rest” material and were not linked to any specific studies. Informed consent (to use lung tissue for research purposes) was obtained prior to surgery.

The tissues were examined by pathologists at the pathology department of the AMC to ensure they were free of cancer cells before being used for subsequent experiments. All

excess tissue was removed and the remaining part, airway lobe, was washed with buffer containing antibiotic and antifungal agents to prevent contamination during culture. After 16 – 24h of protease and DNase (Sigma-Aldrich, 1% protease and 0.01% DNase) treatment, epithelial cells were disconnected from underlying tissue and seeded at a density of 2 x 10^6 cells per T75 flask (Corning, NY, USA) coated with type IV collagen (Sigma-Aldrich). At this stage, bronchial epithelial growth medium (BEGM, (53)) is used to allow cell proliferation. During the replication process, medium was changed every 48h until the monolayer reached confluence. Epithelial cells can be put on a porous surface directly in passage 0 or subcultured passages, at a concentration of 1 – 2 x 10^5 cells per insert. When cells are put in transwell inserts, air-liquid interface (ALI, (53)) medium is used to support cell growth and differentiation. An air liquid interface was established after cell layers reached 95% confluency by removing medium from the apical compartment. Thereafter, medium in the baso-lateral compartment was changed every 48h and the apical compartment was gently washed with HBSS (Invitrogen) weekly to remove mucus and secreted debris. The whole process of differentiation, until cilia beating activity is observed, requires 4 – 6 weeks. When the cells are fully differentiated, they are ready for infection. The incubation time for infection usually is 48 to 72h. After 4 weeks, fully differentiated cells start loosing their specific characteristics and cell death occurs.

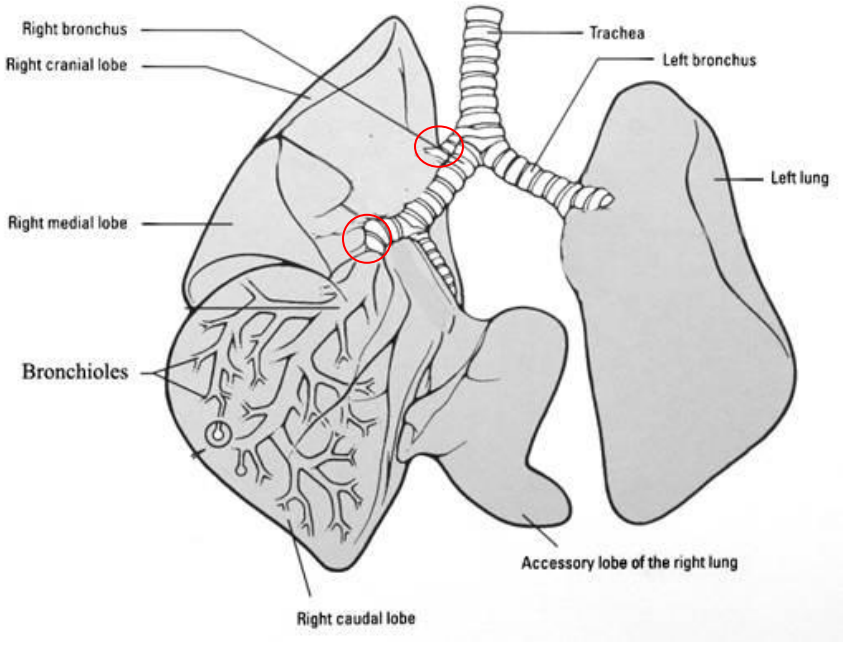


Figure 3.2 **Schematic diagram of human respiratory tract** (source: www.wikipedia.org).
Circles indicate the positions from which materials were taken for human airway epithelial cell cultures, mostly from airways to the lung lobes or smaller branches.

Porcine airway epithelial cells

Fresh porcine lung was acquired from animal unit of the AMC (following AMC rules and regulations on animal materials for research purpose). This type of material was used only once and for training purposes. Isolation procedures were very similar to human airway cells (scaled up to appropriate volume depending on amount of tissues).

2. Results

The first attempt to culture porcine airway epithelial cells was conducted using fresh porcine lung lobe material. Procedures were very similar to human lung lobectomy with slight modifications to scale up to larger volumes of materials. Porcine airway lobe, after dissecting all connective tissues, was cut into 3cm fragments, which is not required in

case of human cell isolation, and then went through all necessary steps as with human lung lobe.

One batch of human airway cells was isolated in parallel with the porcine one. Although both cell types were seeded into transwell inserts at the same concentration, 10^5 cells per insert, porcine primary epithelial cells took more time to render a confluent layer and they also did not show similar replication ability when comparing among inserts. 90% of human cell inserts was transferred to an air-liquid interface after 2 days of proliferation, compared to 70% of porcine inserts. Moreover, about 10% of total porcine inserts were not confluent after 2 weeks. Within 3 – 4 weeks, human airway epithelial cells started to differentiate with cilia beating activities observed in 20 – 30% of inserts. Well-differentiated inserts, either human or porcine, were used for other experiments which was not part of this project.

Although porcine airway epithelial cells have been successfully isolated and differentiated in similar conditions as human cells, the efficiency was significantly lower. One of the important reasons is the difference in physiological environment.

In the second experiment, two batches of fresh human airway epithelial cells were sequentially isolated. In the first batch, all inserts reached confluence at the same time and started to differentiate after 2 weeks in air-liquid interface. However, in the second batch some of the inserts showed sign of overgrowth after one week in air-liquid stage. These were discarded since they were not suitable for any experiments.

3. Discussion

The used system has been specifically optimized for human airway cells; in order to obtain higher yield for porcine cells, new optimization and validation steps are required.

Other research groups have employed porcine airway epithelial cell system for studies on respiratory virus infections, drug transport or host-pathogen interactions (56,57) and each group has developed their own methods, which are all slightly different in terms of medium composition or cell isolation procedures. These protocols will be used as reference to achieve a proper system of isolation and culturing porcine airway epithelial cells in future experiments.

Another *ex vivo* cell system that is very useful in studies of influenza infection is avian airway epithelial cells which is still under development with different veterinary groups. Because of their zoonotic potential, influenza viruses need to be investigated in different cell systems in which they have been stably established. This adds in to the importance of developing appropriate culture systems for influenza virus studies on replication, transmissibility and host cell interaction etc.

Overgrowth of human airway epithelial cell is most often due to the presence of fibroblasts or (mainly) tumour cells in the original culture. In order to exclude fibroblasts, which do not have the ability to differentiate, airway tissue has to be treated with protease and DNase for at least 16h. These two enzymes loosen the linkage between epithelial cells and underlying tissue, so that the epithelial layer can be removed with gently scraping. In some cases, there is a minor population of fibroblasts present but it will be outgrown by epithelial cells. The presence of tumour cells is more problematic. Materials for human epithelial cell isolation are usually obtained from patient with lung cancer. Although they must be checked by pathologists before being use for any research purposes, it is only gross examination. If the dissection is too close to the tumour, one can not totally exclude the chance of contamination. Tumour cells have lost

most of the physiologic functions of the cells we aim to culture and are immortalized and therefore they can easily outgrow other cell types. They can form cell clumps which maybe mistaken for mucus secreted during the differentiation process if not checked carefully under microscope. Epithelial cultures with tumour cells maybe used for virus stockpiling but not for replication kinetics or competition assays.

Attempts at culturing human airway cells from AMC at OUCRU and obtaining human lung tissue from the cardiothoracic surgery department of Cho Ray hospital in Ho Chi Minh City have not been successful, and therefore, no further culture results can be reported.

Reverse genetics

I Introduction

Reverse genetics of negative-sense RNA viruses is defined as the generation of viruses from cloned cDNAs. In order to generate a “synthetic” negative-sense RNA virus, a functional ribonucleoprotein complex is required. This challenge had been successfully overcome for some non-segmented, negative-sense RNA viruses; however, it has proven more difficult for influenza viruses owing to their segmented genome (59). Influenza reverse genetics methods therefore have to effectively deliver both viral genomic RNA and a set of proteins essential for transcription to a susceptible cell (60). Those proteins include the nucleoprotein (NP) and the RNA-dependent RNA polymerase machinery consisting of the two basic polymerases (PB1, PB2) and the acidic polymerase (PA) gene segments. Efforts to generate influenza viruses in the

laboratory were reported as early as 1980s. In these early studies, a helper virus was required to provide the viral ribonucleoproteins, resulting in low efficient generation of desired viruses (the majority were helper viruses) and selection of recombinant viruses was (hands-on) time consuming (61). A main breakthrough in influenza reverse genetics was achieved in 1999 when two independently working groups generated influenza viruses entirely from cloned cDNAs without the need of helper virus. In one system, Neumann *et al* employed a set of 17 plasmids, 8 for RNA synthesis and 9 for protein expression. Shortly after, the number of plasmids was reduced to 12 and this resulted in even higher transfection efficiency. In another system, Fodor *et al* utilized hepatitis delta virus ribozyme to splice the RNA transcripts to appropriate size instead of using RNA polymerase terminator as Neumann's group did (61). Then in 2000, Hoffmann *et al* further improved the system by combining RNA polymerase I and II transcription cassettes in the same plasmid, pHW2000. The newly developed system comprised of RNA I polymerase and terminator and polymerase II promoter from human Cytomegalovirus (CMV) together with a polyadenylation sequence from bovine growth hormone. With this system, negative-sense viral RNA and positive-sense mRNA were produced from the same viral cDNA template in opposing directions (60). Moreover, it required transfection of only 8 plasmids into targeted cells, hence increasing its efficiency and decreasing total time needed. The eight plasmid system has been used widely since its establishment.

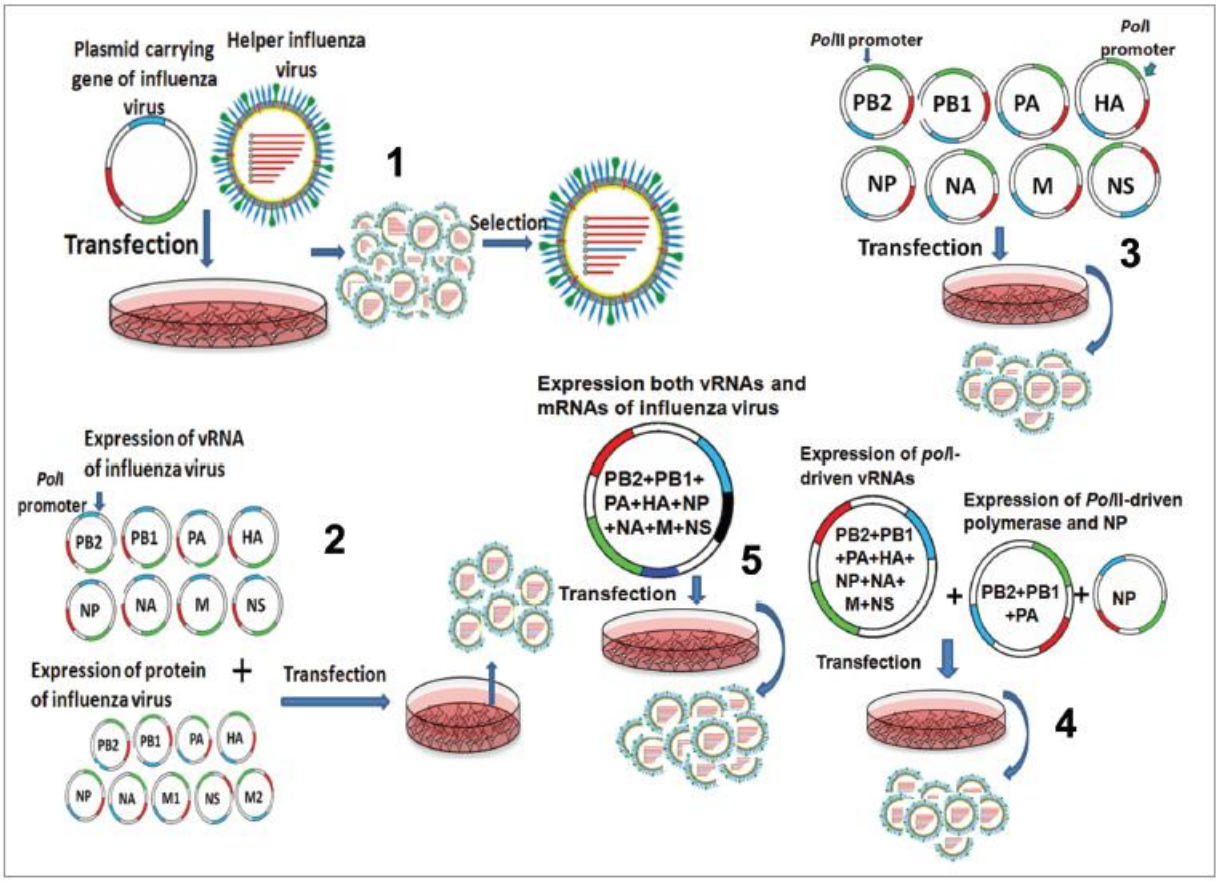


Figure 3.3 **Influenza reverse genetics technique evolution** (adapted from (61))

(1) Reverse genetics system with helper virus
(2) 17-plasmid system, 8 for RNA synthesis and 9 for protein expression
(3) 8-plasmid system using polymerase I/II promoter
(4) 3-plasmid system with one carrying all 8 viral RNA transcription units
(5) One plasmid system for chicken cells

After 2000, several groups have tried reducing the number of plasmids as well as boosting the transfection efficiency. One of the systems contained only 3 plasmids; one carries 8 polymerase I promoter-driven viral RNA transcribing units, the second plasmid encodes 3 polymerase proteins under the control of the polymerase II promoter and the last plasmid carries the NP gene (61). In 2009, a novel system for chicken cells consisted of only 1 plasmid was introduced by Zhang *et al*. In this plasmid, four

transcription units under the control of a chicken polymerase I promoter were combined with four bidirectional polymerase I/II units (60). These low plasmid number systems are very advantageous in influenza vaccine development and production, especially when the influenza vaccine has to be revised annually due to antigenic drift (and shift) (61).

The 8-plasmid system was utilized in this study because of its well established and widely used. In the first experiment, the HA gene of clade 2.3.4 H5N1 virus was cloned into a pHW2000 plasmid and then transfected with other segments of the PR8 strain. The purpose of this was to set up the removal of the HA polybasic cleavage site, so that it could be applied for other H5N1 viruses.

In the second reverse genetics experiment, a different plasmid was employed to perform single-reaction amplification of the influenza genome. This method (62) takes advantage of the conserved and complementary 12 and 13 nucleotide sequences at the 3’ and 5’ termini of all influenza A viruses, respectively. Base pairing between these conserved residues forms a “panhandle”’ and this RNA structure is required for all genetic processes including transcription, replication and packaging of the genome.

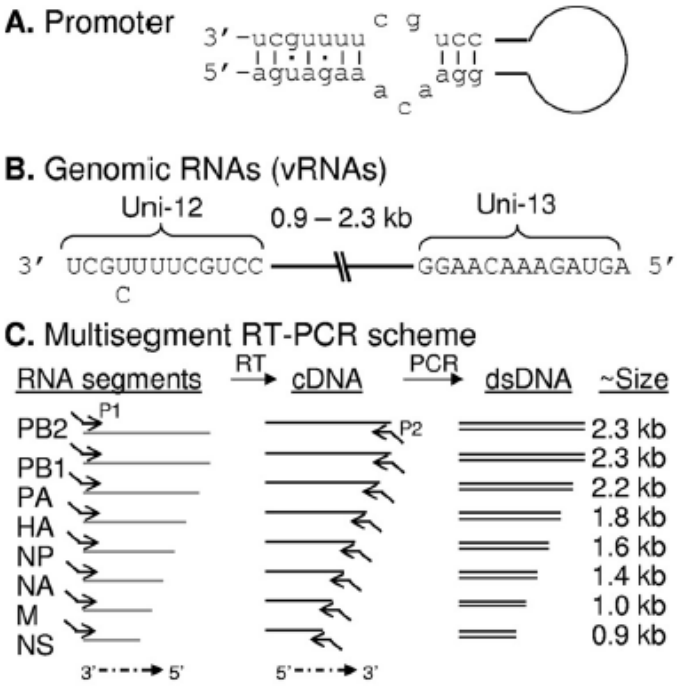


Figure 3.4 **Basis of single-reaction amplification of influenza genome** (adapted from (62))

A. Classical panhandle of base pairing between 3' and 5' termini in each gene segment

B. Illustration of influenza viral RNA segments

C. Multisegment reverse transcription PCR scheme demonstrating the reverse primer P1 complementary to Uni12 sequence and the forward P2 primer complementary to Uni13 sequence

II Materials and methods

1. HA poly basic cleavage site removal

Viral RNA isolated from sample 1002 served as template for cDNA synthesis. The entire HA gene was amplified using specific primers for cloning into a pHW2000 plasmid with *Bsm*BI restriction sites (63).

For optimal amplification, the HA gene was amplified into two overlapping segments using H5 HA specific primers then combined to a full-length sequence by another PCR reaction using primers for the *Bsm*BI cloning site. The PCR reaction contained 4µl cDNA, 2µM (each) primer and necessary reagents for Platinum Pfx DNA Polymerase (Invitrogen) according to the manufacturer's instructions. Thermocycling conditions are indicated in Table 3.1.

After cloning, the polybasic cleavage site was removed using the site directed mutagenesis kit (Stratagene) according to the manufacturers instructions with primers specifically designed for the clade 2.3.4 HA (Table 3.4). The engineered HA was tested by transfection with other genes of PR8 virus as previously described (64) and recombinant virus viability was tested by infecting MDCK cells.

1 cycle	94^0C	10 minutes
42 cycles	94^0C	30 seconds
	55^0C	30 seconds
	68^0C	2 minutes
1 cycle	68^0C	10 minutes
Hold at	15^0C	

Table 3.1 **PCR program used for HA amplification**

Primer name	**Sequence**
BsmBIHAF1	TAT TCG TCT CAG GGA GCA AAA GCA GGG G
BsmBIHAR1	ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT GTT TT
H5HAF1	AGC AAA AGC AGG GGT ATA ATC
H5HAF898	ATG GTA ACT GCA ACA CCA AGT GTC
H5HAR1	AAG GGT GTT TTT AAC TAA CAA TCT
H5HAR1265	ACG GCC TCA AAC TGA GTG TTC ATT

Table 3.2 **Primer sequences used for HA amplification, overlapping PCR and cloning**

2. 8-segment cloning

For one-step 8-segment cloning of influenza A viruses, MDCK culture of H1N1pdm09 was used as template (the use of H1N1pdm09 was only for training purposes). The viral genome was amplified using primers designed for cloning into specific reverse genetic plasmids, which were kindly provided by the Wentworth group (the State University of New York) (62). Amplicons were generated by one-step RT-PCR (Invitrogen) using the Uni12Inf1/Uni12Inf3 primers in combination with Uni13Inf1 primers (reagent concentrations and PCR programs according to the manufacturers instructions). PCR products were cloned into two similar cloning vectors, both were derived from pG26A12 and different in only 1 nucleotide for higher yield of polymerase genes (62). In-Fusion cloning kit (Clontech®) was employed for fast and directional cloning for more than one DNA fragments. All cloning (ligation) reactions were prepared according to the manufacturer's instruction.

After cloning, colony PCR with primers specific for all segments of influenza genome (Table 3.3) was performed to select appropriate plasmids (based on size). Selected plasmids were confirmed by sequencing using primers specific for individual gene of influenza virus (sequencing protocol in section VI, chapter 2). In 500µl transfection reaction, for one well of a 6-well plate, 500ng plasmid DNA of each segment was mixed with 10µl Lipofectamine (Invitrogen) and 410µl OptiMEM (Invitrogen), incubated at room temperature for 20 minutes then applied onto a 60% confluent layer of 293T cells. Recombined virus was rescued in 293T cells as previously described (65) then inoculated in MDCK cells to increase viral titre.

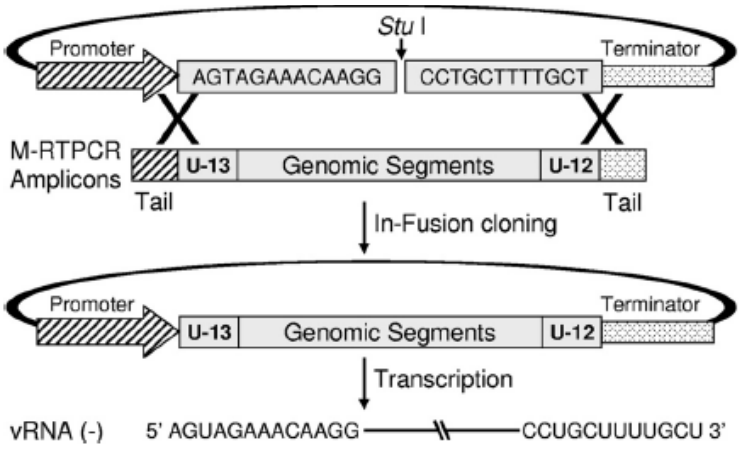


Figure 3.5 **Schematic diagram of 8-segment cloning for whole genome of Influenza A virus** (adapted from (62)).

Reverse genetic plasmid was modified by StuI restriction enzyme, genome amplicons with appropriate flanking sequences were cloned into vector backbone in Infusion reaction. Expression plasmids were then transformed into TOP10 competent cells (Invitrogen) to generate clones with influenza virus genes.

Primer name	Sequence
Uni12Inf1	GGGGGGAGCAAAAGCAGG
Uni13Inf1	CGGGTTATTAGTAGAAACAAGG
Uni12Inf3	GGGGGGAGCGAAAGCAGG

Table 3.3 **Primers used in 8-segment cloning reactions**

3. Generation of recombinant viruses

Restriction site cloning (66) used to clone H5 HA and NA genes into the appropriate plasmid backbone utilized a similar protocol as in the HA polybasic cleavage site removal section. In the first PCR, NA specific primers were used to generate full-length NA. In the second PCR, NA gene bearing appropriate restriction sites were produced using *Bsm*BI primers (Table 3.4). This product was then ligated into pHW2000 which was already "open" by *Bsm*BI enzyme. Other segments of the influenza genome come from laboratory adapted strain of human seasonal H1N1/1977 virus A/WSN/33 (WSN33), which was kindly provided by Dr Robert Webster, St. Jude Children's

Research Hospital, Memphis, TN, USA. Recombinant viruses were generated using the Lipofectamine protocol (similar as in section for H1N1pdm09 virus).

Primer name	Sequence
BsmBINAF1	TATTGGTCTCAGGGAGCAAAAGCAGGAGT
BsmBINAR1	ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT
N1NA3F	CRAAAGCAGGAGTTYAAAATG
N1NA1460R	AGTAGAAACAAGGAGTTTTT

Table 3.4 **Primers used NA amplification and cloning**

III Results

1. HA polybasic cleavage site removal

The polybasic cleavage site of the HA gene of a clade 2.3.4 H5N1 virus was removed and a recombined virus with the 7 remaining genes of PR8 virus was successfullyrescued. 10 clones were sequenced, of about 30 in total, and 2 contained the desired sequences (the remainder of the clones either had deletions or insertions in other parts of HA).

To improve the efficiency of HA site directed mutagenesis, shorter primers were employed (as longer ones can have secondary structures and reduce the amount of PCR products). DMSO was added to prevent oligonucleotides from forming secondary structures in some of the PCR reactions to compare product yield. Surprisingly, neither

shorter primers nor DMSO significantly increased the efficiency of mutagenesis PCR. There were similar numbers of colonies in all experiments and 2 out of 8 clones generated from shorter primers had the desired sequences (data not shown).

First primer pair	
H5-COF	CAGAAATAGTCCTCTAAGAGAAAGAGGACTATTTGGAGCTATAGC
H5-COR	GCTATAGCTCCAAATAGTCCTCTTTCTCTTAGAGGACTATTTCTG
Second primer pair	
H5-COFm	ATAGTCCTCTAAGAGAAAGAGGACTATTTGGAGC
H5-CORm	GCTCCAAATAGTCCTCTTTCTCTTAGAGGACTAT

Table 3.5 **Primer sequences used in HA polybasic cleavage site removal experiment**

```
Before          NKLVLATGLRNSPLRERRXXKRGLFGAIAGFIEGGWQG
                ||||||||||||||||  ||||||||||||||||
After    clone 2 NKLVLATGLRNSPLRE.....RGLFGAIAGFIEGGWQG
         clone 7 NKLVLATGLRNSPLRE.....RGLFGAIAGFIEGGWQG
```

Figure 3.6 **Amino acid alignment of HA sequences of a clade 2.3.4 H5N1 virus before and after polybasic cleavage site removal**

2. 8-segment cloning of H1N1pdm09

MDCK culture of H1N1pdm09 was used as template for RT-PCR with vRNA extracted following the Boom method (67). Products of a one-step RT-PCR with specific primers (Table 3.3) were purified and served as inserts for cloning. The vector backbone (pG26A12 provided by Wentworth group) was also modified as previously described

(62). The amount of insert and vector used in the Infusion cloning reaction was calculated according to the manufacturer's protocol.

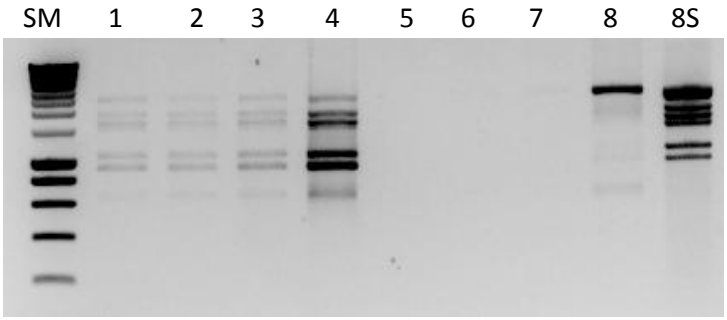
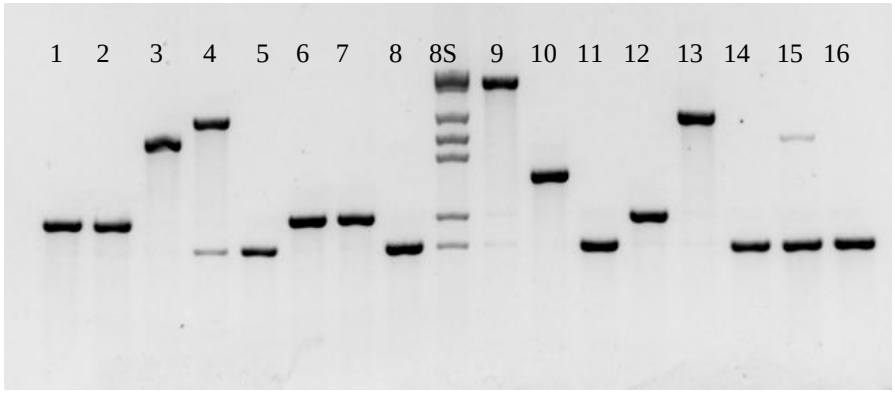


Figure 3.7 **One-step RT-PCR of an H1N1 isolate with different dilutions of templates.**
SM, Smart ladder (Eurogentec); 8S, 8 segment ladder of H1N1 virus. vRNA was reverse-transcribed using Uni12Inf1/3, lane 4 (for HA, NP, NA, M and NS genes); and Uni12Inf3, lane 8 (for polymerase genes PB2, PB1 and PA).

Two Infusion reactions were performed: one with sample 8 (according to figure 3.4) for the polymerase genes and one with sample 4 for the rest of the influenza virus genome. After transformation, colony PCR was performed with universal primers for all influenza viruses (Uni12 and Uni13). Clones were chosen based on the size of PCR products, which should correspond to the known size of the 8 segments of the influenza virus genome. For screening of the polymerase genes (due to their similar gene length), specific primer pairs were utilized to distinguish between PB2, PB1 and PA.



8S ladder (size based on H1N1 virus)	
PB2 – PB1	2.3kb
PA	2.2kb
HA	1.8kb
NP	1.6kb
NA	1.4kb
M	1.0kb
NS	0.9kb

Figure 3.8 **Colony PCR of the first screening and 8S ladder reference.**
3 clones of each segment were randomly picked up for further characterization.

Three different clones of each segment were selected for reverse genetics experiments. 100ng of each segment was used for transfection into 293T cells (24-well plate format). Each clone was tested individually with the rest of the influenza genome derived from a WSN33 recombinant strain. Three combinations of only H1N1 clones were also included in transfection. After 3 days incubation, the transfection supernatant was used to infect MDCK cells (6-well plate format). CPE was scored for all wells, including a positive control of WSN33 and a negative control of WSN33 without the PB2 gene. Only one set of H1N1 alone produced distinguishable CPE compared to the negative control well. For individual clone testing, some represented high CPE scores while some did not induce cell death.

All 24 clones were sequenced to confirm colony PCR and reverse genetics results. One nucleoprotein NP clone was the matrix M gene, and one NA clone was non-structural protein NS. This explains why 2 out of 3 combinations did not show CPE in MDCK infection. Sequences of other clones were similar to the template virus, with some synonymous and non-synonymous mutations in most of the clones. Further investigations to obtain conclusive data on the function of each recombinant H1N1pdm09 gene will be required if these clones are used in later experiments

One of the interesting observations is that although each H1N1pdm09 cloned segment worked well in combination with WSN33 genes, as demonstrated by early appearance of marked CPE, the wholly recombinant H1N1pdm09 did not have that feature. The only working set induced much less distinguishable CPE compared to other positive infections.

In summary, to date polybasic cleavage site removal of H5N1 HA and full reverse engineering of one H1N1pdm09 virus has been successfully completed. This virus can be used as a representative of human-adapted influenza virus in future *in vitro* experiments to test specific hypotheses regarding genetic basis of host adaptations. The same cloning and transfection strategies will be employed to engineer H5N1 viruses used in this project.

Chapter 4

Mutations in H5N1 NA that restore NA activity

I Abstract

The re-emergence and widespread occurrence of HPAI H5N1 viruses in poultry and wild birds, and resulting sporadic spill-over infections in humans, have lead to increased interests in pandemic prediction and preparedness. Antiviral drugs, especially oseltamivir, are currently the most effective treatment against H5N1. Oseltamivir resistant variants have been isolated from H5N1 patients during and after treatment and H274Y is the predominant resistance associated mutation among subtypes and strains of the same subtype. 2 H5N1 oseltamivir resistant and 1 sensitive isolates from patients were selected for this investigation. Both of the resistant isolates harbor the H274Y mutation but differ in NA activity. Sequencing analysis revealed that one resistant strain with normal NA activity and increased replication contains 2 possibly permissive/compensatory mutations. Each mutation was then characterized to demonstrate the mechanism underlying changes in NA kinetic properties.

II Introduction

Avian influenza remains a credible threat for pandemic emergence. These viruses are stably maintained in their natural hosts, wild waterfowl, and show very high antigenic divergence (5). Based on their virulence in chickens, avian influenza viruses are classified into low pathogenic avian influenza or high pathogenic avian influenza (LPAI and HPAI respectively) (47). All of the HPAI viruses belong to subtypes H5 and H7, which are responsible for all outbreaks until now (5). NAIs play an important role in influenza (post-exposure) prophylaxis and treatment, both in the severe phenotype of

seasonal influenza and in sporadic cases of avian influenza. In general, NAI resistant rates are low, with the notable exception of the emergence and spread of naturally oseltamivir resistant seasonal H1N1 viruses of the Brisbane lineage during 2007 – 09 that was subsequently replaced by the H1N1pdm09 virus. Notably, spontaneously emerged or drug-induced resistant variants have been reported in humans for both seasonal influenza and H5N1 viruses (52). Resistance to NAIs can result from mutations in the NA active site or near the receptor binding site of HA. This may be explained by changes in receptor binding, hence reducing the need of a highly active NA. However, only mutations on NA gene are clinically relevant (19). Many research groups have been working on NA mutations, especially H274Y, and demonstrated that those mutations would attenuate influenza virus unless there are other permissive or compensatory mutations that “restore” the viral NA protein to normal activity level (30). In one study, Simon and colleagues suggested that the NA I222V mutation, in an H3N2 clinical isolate, partially restored the viral fitness of the E119V mutant whereas oseltamivir resistance was maintained and this was probably due to an increase in NA activity (68). The H274Y mutation has been shown to have a different impact on different context of old and recent H1N1 isolates depending on secondary NA mutations (31). In another study, Boivin’s group, using the recombinant wild-type Bris07 strain in both *in vitro* and ferret experiments, demonstrated that NA R222Q mutation was the main permissive mutation that allowed the widespread dissemination of the oseltamivir-resistant H274Y mutant during the 2007–09 influenza seasons (31).

Influenza A/H5N1 viruses are endemic among birds in Vietnam, and both HPAI and LPAI viruses have been reported in the northern and southern regions (4). Although

viral pneumonia is thought to be the primary cause of death, H5N1 virus can spread to different organs beyond the respiratory tract (52), resulting in multi-organ failure. There have been several “out-break” time points for human cases of H5N1 infections in Vietnam. Based on preliminary data on the drug resistance profile and NA kinetics, we selected 3 H5N1 strains isolated from patients in 2005, which display differences in oseltamivir susceptibility, to characterize their NA activities. Those included 1 oseltamivir sensitive strain A/Vietnam/CL105/2005 and 2 resistant strains, showing different NA kinetic parameters, A/Vietnam/CL107/2005 and A/Vietnam/CL2009/2005. Recombinant variants, with the NA gene from selected strains and the other segments from A/WSN/33 (WSN33), were engineered to investigate the differences in NA kinetic properties. We hypothesized that, apart from H274Y oseltamivir resistant mutation, the two resistant strains contained additional amino acid change(s) that effected NA enzymatic properties. These changes could influence NA kinetic parameters by direct impacts on NA enzyme itself or by indirect effect on protein expression. Subsequently, the specific underlying mechanisms would be investigated by exchanging individual mutations.

III Materials and methods

1. Cells, viruses, and compounds

Madin-Darby canine kidney (MDCK) and human embryonic kidney (293T) cells were obtained from the American Type Culture Collection. All cell cultures were maintained as previously described in chapter 2.

H5N1 viruses were obtained from the Hospital for Tropical Diseases, Ho Chi Minh city, Vietnam. After 2 passages in 10-day-old embryonated chicken eggs, viral RNA was extracted. NA genes of those isolates were reverse-transcribed and then cloned into the pIN3000 backbone (62). Other segments of H5N1 recombinant viruses came from the lab-adapted WSN33 strain (in the pHW2000 backbone (64)). Recombinant viruses were generated by DNA transfection using 293T cells.

2. Infectivity of H5N1influenza viruses

The infectivity of H5N1viruses was determined by 50% tissue culture infectious dose ($TCID_{50}$). Briefly, confluent MDCK-SIAT1 cells were inoculated with 10-fold serial dilutions of virus. After 3 days of incubation at 37°C, CPE was scored and TCID50 values were calculated using the Reed and Munch method (details in chapter 2).

3. NA enzyme activity and kinetics (52)

A fluorometric assay was used to determine the NA activity of the recombinant H5N1 viruses. We measured the NA enzyme kinetics at pH 6.5 with 32.5 mM 2-(*N*-morpholino) ethanesulfonic acid hydrate (MES; Sigma-Aldrich, St. Louis, MO, USA), 4 mM $CaCl_2$, and the fluorogenic substrate 2'-(4-methylumbelliferyl)--D-*N*-acetylneuraminic acid [MUNANA] at a final substrate concentration of 0 to 900 µM (Sigma-Aldrich, St. Louis, MO, USA). All H5N1viruses were standardized to an equivalent infectious dose of $1.25x10^8$ infectious particles per ml. The reaction was conducted at 37°C in a total volume of 50 µl, and the fluorescent intensity of released product 4-methylumbelliferone was measured every 60s for 1 hour in a Fluostar instrument (Optima) using excitation and emission wavelengths of 355 nm and 460 nm,

respectively. To measure the inhibitory effect of oseltamivir on NA activity, H5N1 viruses were pre-incubated for 30 min at 37°C in the presence of various concentrations of the drugs (0 to 4000 nM). The kinetic parameters, Michaelis-Menten constant (K_m) and maximum velocity of substrate conversion (V_{max}), of the NAs were calculated by fitting the data to the appropriate Michaelis-Menten equations and using nonlinear regression in the commercially available GraphPad Prism, version 5, software (GraphPad Software, La Jolla, CA, USA).

4. Quantification of NA protein by western blot analysis (69)

Recombinant viruses were passaged once in MDCK cells. After 3 day incubation at 37ºC, 10ml of infection supernatant was harvested. These supernatants were also the one used in enzymatic assay. In the first attempt of Western blot, virus stocks were diluted to the same TCID as in the MUNANA assay, then mixed at a ratio of 1:1 with protein loading buffer and 15µl was used per sample. In the concentrated experiment, virus stocks were ultra-centrifuged to concentrate approximately 30 times. Virus pellets were resuspended in 50µl PBS and 5µl of each sample was used for Western blot.

Cellular proteins were separated by electrophoresis into 15% SDS-PAGE gels, and transferred to a PROTRAN BA 85 membrane (10402588, Whatman GmbH, Germany) using semi dry transfer cell (Transblot SD). The blots were probed sequentially with a rabbit anti-NA antibody (1:1,000 dilution; MyBioSource MBS432041) against VN1203 NA and a goat anti-rabbit IRDye 800CW antibody (1:5,000 dilution; 926-32213, LI-COR Biosciences). The blots were scanned using an Odyssey Infrared imaging system, and fluorescence intensity was analyzed using Odyssey application software (version 2.1, Li-Cor Biosciences).

IV Results

1. Generation of H5N1 recombinant (mutant) viruses

Preliminary work on H5N1 was performed by Martin Crusat as part of his PhD thesis (49). These works included determination of IC_{50} value against oseltamivir and kinetic characterization of the NA enzyme (which was based on total NA activity standardization). Notably, all results were duplicates of one experiment only.

To study the differences in NA activities of H5N1 isolates (Table 4.1) we used the reverse genetics technique to generate recombinant viruses with NA originating from 3 different H5N1 viruses, including A/Vietnam/CL105/2005, A/Vietnam/CL107/2005 and A/Vietnam/CL2009/2005 (all egg passage 2), and the other 7 segments derived from the lab-adapted strain A/WSN/33.

Virus	OST susceptibility	IC_{50} (nM)
A/Vietnam/CL105/2005	S	0.077
A/Vietnam/CL107/2005	R	113.550
A/Vietnam/CL2009/2005	R	333.471

Table 4.1 **Oseltamivir resistance and IC_{50} values of selected H5N1 isolates** (experiment was performed using egg passage 5 isolates) (reproduced from (49))

Virus	Mean Km (µM)	Mean Vmax (RFU)
A/VN/CL105/05	1.9	1677
A/VN/CL107/05	11.0	1673
A/VN/CL2009c/05	110.0	1095

Table 4.2 **NA enzyme kinetics of clade 1 H5N1 isolates** (reproduced from(49))

All recombinant viruses (referred as CL105, CL107 and CL2009) were successfully rescued from transfected 293T cells. Viral stocks were prepared using MDCK cells and sequence analysis data confirmed that those viruses retained the NA sequences from the original strains. Recombinant viruses were then used in oseltamivir susceptibility tests to demonstrate their drug resistance profile. CL107 and CL2009 maintained their resistant phenotype with very high IC_{50} values (626.02 and 341.07 nM, respectively); while CL105 was sensitive to oseltamivir (IC_{50} 0.05 nM) (Figure 4.1 and Table 4.3).

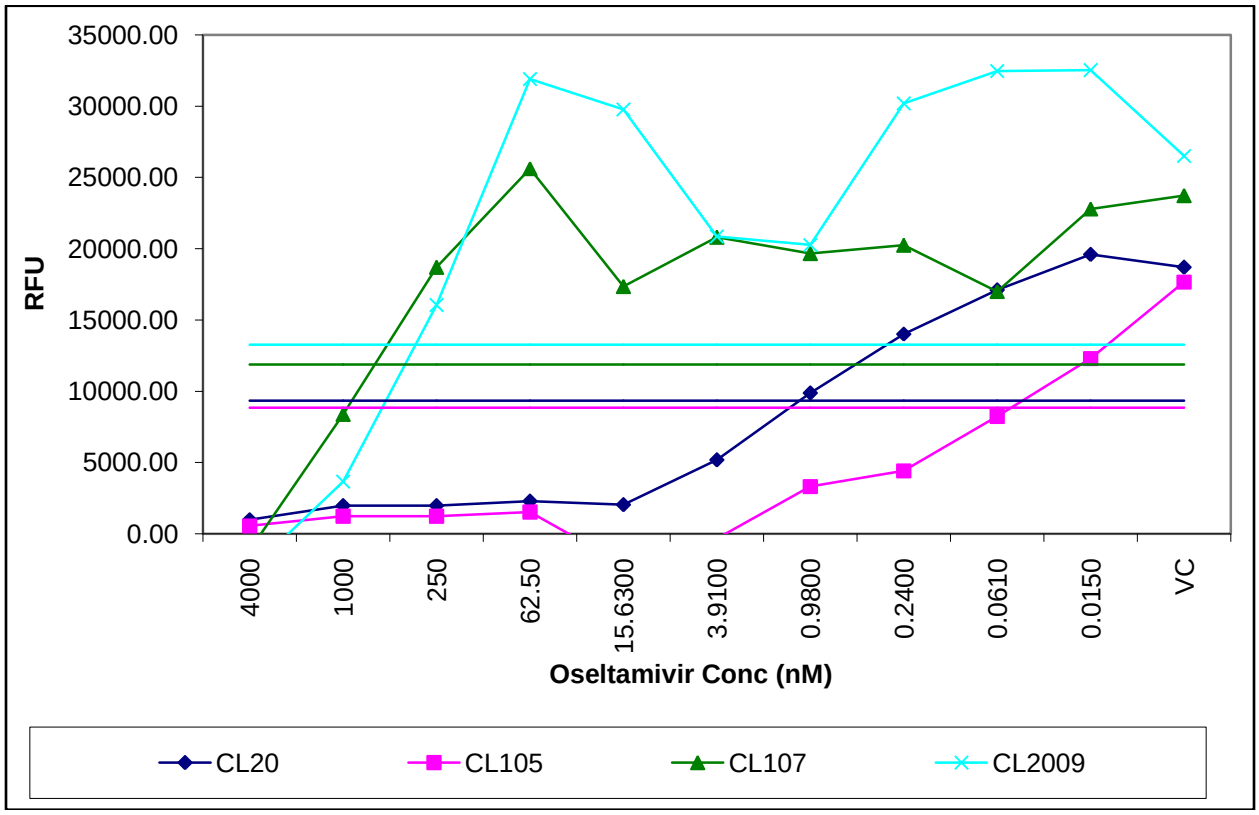


Figure 4.1 **Plotted data of NA inhibition assay**. The horizontal bars show the calculated IC_{50} for each virus (calculations from duplicates of one experiment).

RG virus	IC_{50} (nM)
CL105_w2	0.05
CL107_w2	626.02
CL2009_w2	341.07

Table 4.3 **Oseltamivir IC_{50} values of mutant viruses** (calculations from duplicates of one experiment).

2. NA kinetic properties

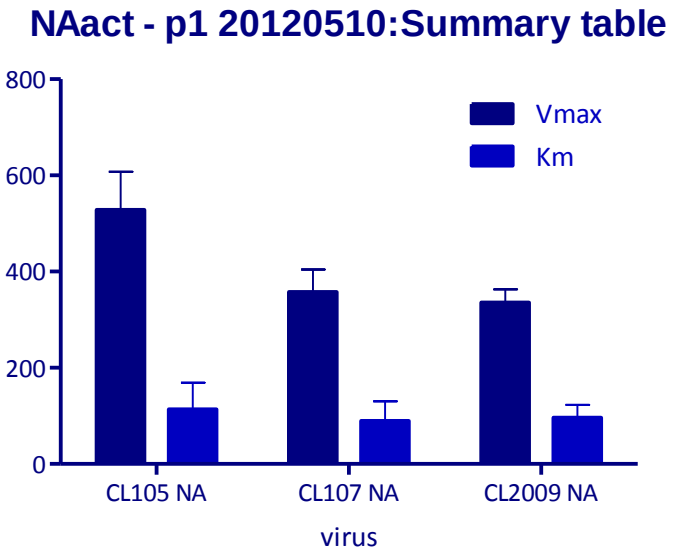


Figure 4.2 **Kinetic measurement for mutant viruses standardized on total activity**

When Oseltamivir sensitivity was determined, the NA kinetics of mutant viruses was assessed using the fluorometric substrate MUNANA to inspect the effect of Oseltamivir resistance on K_m and V_{max} values of NA enzyme.

(Although there have been many studies on NA kinetics, especially V_{max} and K_m, no "standard" range can be applied for these measurements of any specific NA. This might be due to the fact that each group used different viruses for their experiments; for example wild-type H1N1 viruses (pre-2009 or 2009) or H5N1, recombinant viruses with only NA or HA/NA together (one can not exclude the effect of either HA or other segments on NA activity). Consequently, most research groups have been using

relative V_{max} and K_m, i.e. calculating the ratio between those values of "mutant" and wild-type variants, to evaluate the effects of one mutation on NA kinetic parameters.)

The viruses were first standardized for total NA activity and then the same virus input was used for kinetic measurements. Differences observed with our reversely engineered viruses were less significant compared to the results of the original H5N1 isolates; the NA activity of CL105 was only about 1.3 times as high as the ones from resistant viruses, CL107 and CL2009 (Table 4.4 and Figure 4.2).

	CL105 NA	CL107 NA	CL2009 NA
Vmax (RFU)	528.6 ± 34.98	357.6 ± 20.60	335.5 ± 12.02
Km (μM)	113.9 ± 24.38	89.54 ± 17.93	96.52 ± 11.75

Table 4.4 **V_{max} and K_m values of mutant viruses standardized on total NA activity** (duplicates of one experiment)

To ensure that we did not over-compensate for virus(es) with reduced NA activity, the assay was repeated using TCID50 for standardization. All viruses were diluted to $1.25x10^8$ infectious particles per ml.

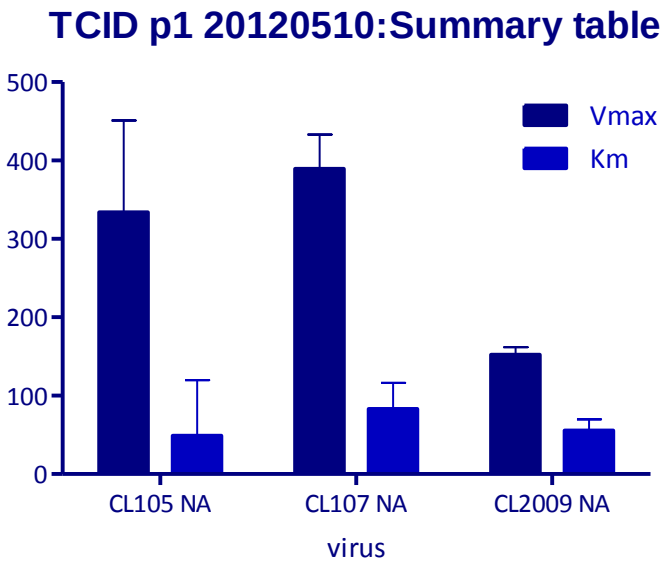


Figure 4.3 **Kinetic measurement for mutant viruses standardized on TCID**

Only when normalizing on TCID, there was a significant difference between the NA activities of sensitive and resistant viruses. Interestingly, CL107, which is a resistant virus, showed normal, or even superior, NA activity compared to the sensitive variant CL105 (Table 4.5 and Figure 4.3). Between the 2 resistant variants, CL107 had a V_{max} value twice as high as CL2009. Therefore, these results are in accordance to the previous findings (49): that in spite of the oseltamivir resistance mutation, CL107 exhibits a wild-type NA V_{max}. To explain this disparity, we performed sequence alignment of the two 2 NA genes to identify the nucleotide difference.

	CL105 NA	CL107 NA	CL2009 NA
Vmax (RFU)	334.0 ± 51.68	389.2 ± 19.37	152.3 ± 4.22
Km (µM)	49.05 ± 31.36	83.18 ± 14.71	55.69 ± 6.16

Table 4.5 **V_{max} and K_m values of mutant viruses standardized on TCID** (duplicates of one experiment)

3. Amino acid exchange

Based on the sequencing data, CL107 has 2 amino acids that are different from CL2009. One of them is near the active site of NA (residue 137), the other one is in the stalk region (residue 46). It was suspected that either one or both amino acids can be responsible for the restoration of CL107 NA activity. To investigate the impact of each mutation, we exchanged the region containing the desired mutation between the 2 viruses and examined the NA kinetic properties of the new variants.

One of the 2 amino acid differences between CL107 and CL2009 is at position 137. Wild-type CL107 had an A at this position while CL2009 has a T. To generate CL107 A137T and CL2009 T137A variants, we switched the flanking regions of these 2 amino acids. Sequencing data confirmed that new variants were identical to original ones apart from the mutation of interest. These new variants were aimed to be used for 2 different investigations (including NA kinetic assay and NA protein expression) to identify the mechanism by which they affect NA enzyme.

4. NA kinetic assays

In the first experiment, CL2009 T137A displayed higher V_{max} when compared to CL107 A137T (but still lower to wild-type CL2009). This result supported the hypothesis that residue 137 is responsible for restoration of NA V_{max} value (Figure 4.4). To confirm this observation, NA kinetic measurement was repeated for all viruses using different stocks. However, the result was not reproducible in subsequent assays. In fact, we obtained opposite findings with different virus stocks (Figure 4.5).

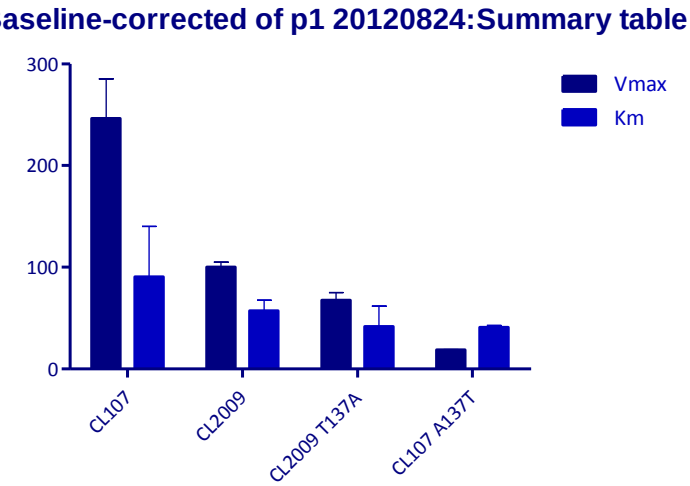


Figure 4.4 **Kinetic measurements for mutant viruses containing amino acid exchange at position 137** in accordance to the hypothesis that residue 137 might be responsible for the changes in V_{max} of NA enzyme (calculated values in Table 4.6 below)

	CL107	CL2009	CL2009 T137A	CL107 A137T
Vmax (RFU)	246.4 ± 17.19	100.0 ± 2.1	67.59 ± 3.34	18.72 ± 0.08
Km (µM)	90.67 ± 21.91	57.03 ± 4.73	41.64 ± 8.88	40.89 ± 0.73

Table 4.6 **V_{max} and K_m values of CL107 and CL2009 and their counterparts in the first experiment** (duplicates of one experiment)

	CL107	CL2009	CL2009 T137A	CL107 A137T
Vmax (RFU)	240.7 ± 10.19	667.5 ± 41.12	98.81 ± 3.73	691.9 ± 52.71
Km (µM)	99.78 ± 14.21	131.8 ± 25.11	71.57 ± 10.04	120.9 ± 29.25

Table 4.7 **V_{max} and K_m values of CL107 and CL2009 and their counterparts in the second experiment** using another virus stock (duplicates of one experiment)

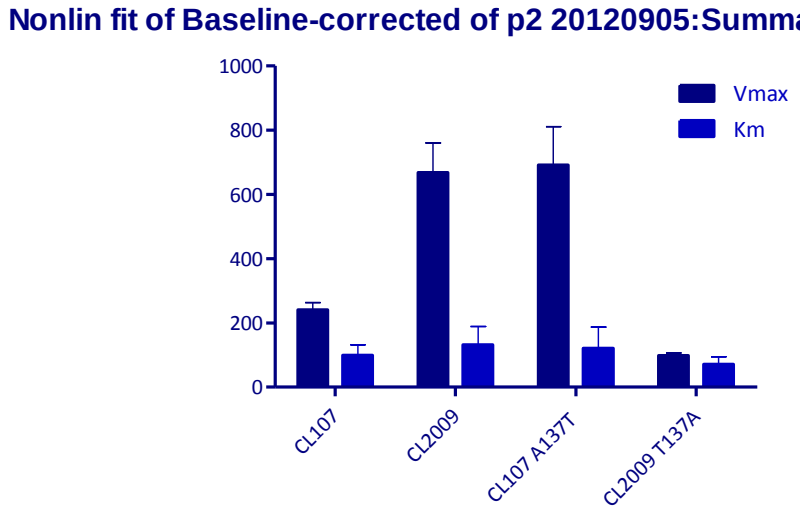


Figure 4.5 **Kinetic measurements for mutant viruses containing amino acid exchange at position 137** shown opposite phenotypes for different virus stocks (calculated values in Table 4.7 above)

As shown in Tables 4.6 and 4.7, while CL107 displayed similar V_{max} and K_m, both CL2009 and the conversion variants demonstrated opposite phenotypes in the two experiments using two different virus stocks. Although there were changes in either V_{max} or K_m when introducing mutation at position 137, these changes were not consistent and could not be statistically compared between different stocks (each experiment had different linear range for calculation). Sequence analysis confirmed that all viruses retained the correct amino acid sequence, therefore further investigation are required to conclude the effect of residue 137 on NA enzymatic properties. Consequently, due to inconclusive results from kinetic experiments, we focused to study NA expression of the variants of interest.

5. NA expression and Western Blot

To investigate whether the NA mutations resulted in lower NA activities because of reduced protein expression, we performed Western Blot on NA of mutant viruses using

the same TCID value as for NA activity assay. Co-staining with NP was used as a control for the amount of virus in each sample. Cell pellet of an NA expression experiment was used as positive control. Other samples included NA protein harvested from a co-transfection experiment using NA of all 3 viruses and NS of WSN33 in 293T cells (1:1 ratio, to increase protein yield (70)).

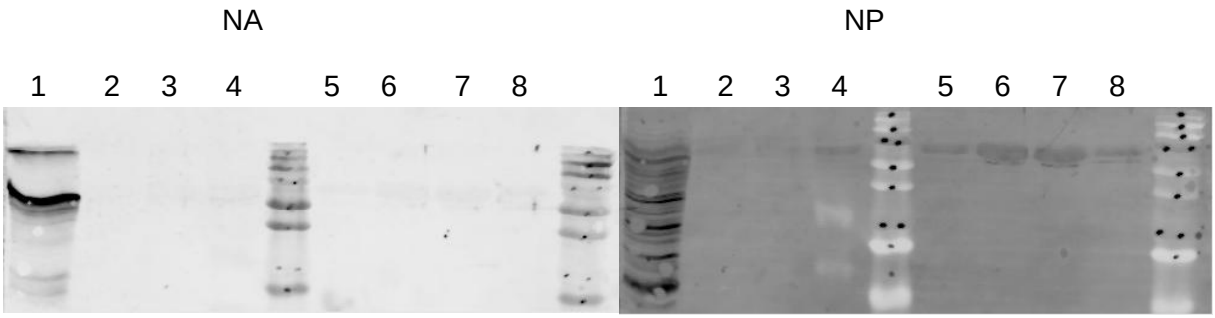


Figure 4.6 **Western Blot for NA protein expression and virus samples**
1, positive control from NA expression experiment.
2 – 4, NA protein (of CL105, CL107 and CL2009, respectively) from co-transfection experiment with NS.
5 – 8, mutant virus samples of CL20 (a control virus with normal NA activity), CL105, CL107 and CL2009, respectively).

No visible NA bands were detected except for positive control. NA proteins from co-transfection experiment also gave no band although they gave very strong fluorescent signal in activity assay (data not shown). When blotted with NP antibody, only thin bands were observed in all virus samples with similar intensity corresponding to similar TCID of all samples. The ratio of NP/NA per virus particle is between 5 and 10, hence one explanation, based on the fact the NP only reached detectable level, was that the amount of NA protein used for Western Blot was not sufficient to produce detectable fluorescent signal.

To improve visibility of Western Blot bands, virus samples were concentrated before loading. 30ml of virus infection of MDCK cell supernatant was used for

ultracentrifugation. The pellet was then resuspended in 100µl of PBS (about 300 times concentration). In parallel, cell pellets from 1ml of the same MDCK infections (resuspended 50ul of PBS) were also included in Western Blot to examine the level of virus particles remained inside the cells. 5ul of all samples was used for blotting. Figures 4.7 and 4.8 demonstrated 2 different blots, against NP and NA segments respectively, of the same set of sample.

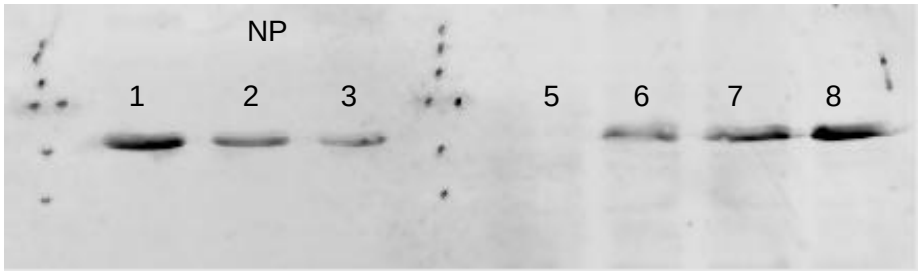


Figure 4.7 **Western Blot for NA proteins with concentrated samples, NP blotting.**
1 – 3, concentrated supernatant from CL105, CL107 and CL2009 infection.
5, positive control from NA expression experiment.
6 – 8, cell pellet of CL105, CL107 and CL2009 infection.

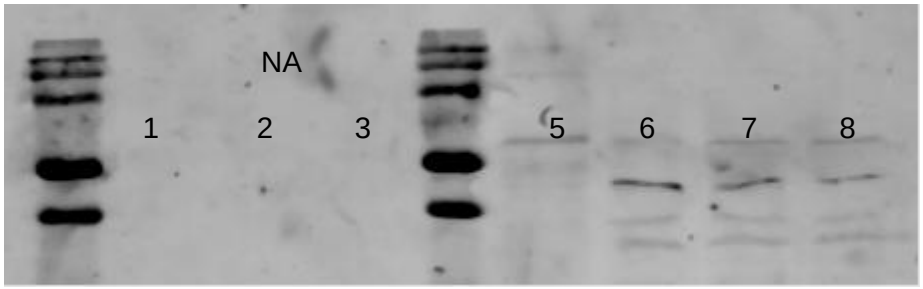


Figure 4.8 **Western Blot for NA proteins with concentrated samples, NA blotting.**
1 – 3, concentrated supernatant from CL105, CL107 and CL2009 infection.
5, positive control from NA expression experiment.
6 – 8, cell pellet of CL105, CL107 and CL2009 infection.

Again, no NA band was detected in supernatant samples although they were concentrated 300 times. In cell pellet samples, NA bands were visible but incomparable. On the other hand, NP bands for all samples (except NA positive control) were clearly visualized. According to the brightness of those bands, we suspect that there were more

viral particles retained inside or attached to MDCK cells in case of resistant virus. Between the 2 resistant viruses, CL2009 had a lower level of NA expression on non-secreted viral particles.

V Discussion

NAIs play an important role in influenza pandemic preparedness owing to their broad spectrum and potency as well as their prophylactic and therapeutic effectiveness (52). Among the NA mutations conferring NAI resistance, especially oseltamivir, H274Y is the most studied one. This is not only because it is the most common one but also because of its various effects on different N1 backgrounds. The situation is more pronounced for H5N1 viruses because of its complicated pathogenesis and diverse genetic variations. Moreover, based on epidemiological data and experimental results, the occurrence of H274Y could be a consequence of or could lead to adaptive changes during influenza virus evolution (30,31). To gain more insight into the impacts of H274Y mutation on the NA protein and to identify other H274Y-supportive-mutation(s), we characterized NA genes of different H5N1 viruses in a seasonal H1N1 background. The characterization involved both enzymatic assays and protein expression experiments.

IC_{50} values of mutant viruses were first confirmed and all the variants displayed IC_{50}s in accordance to their wild-type counterparts (tables 4.1 and 4.3). Although IC_{50} values have been used as standards to evaluate drug susceptibility profile of a virus toward a specific drug, there is no clear definition on the IC_{50} range of sensitive and resistant viruses. The Global Neuraminidase Inhibitor Susceptibility Network (now known as the

ISIRV (International Society of Influenza and other Respiratory Viruses) Antiviral Group)) has criteria to define NAI resistance: to be classified as resistant, one isolate, with known and characterized drug resistance conferring mutation(s), should have IC_{50} values 100–10,000 times higher than the normal range for that specific type/subtype and should be clearly recognizable based on their NAI sensitivity. Moreover, IC_{50} values differ among influenza virus type/subtype and affected by NAI assay type as well as drug tested (71). Resistance to NAIs is not an absolute measurement, but the IC_{50} value should be at least 10-fold higher than the one of similar virus at the same season. The ISIRV Antiviral Group also released a reference panel of influenza viruses (including influenza B, seasonal H1N1, H3N2 and H1N1pdm2009 viruses) with well-established IC_{50} values (72). However, due to the lack of precise consensus for comparison, many research groups have defined resistance as “reduced susceptibility” (about 10-fold increase in IC_{50}), resistant (between 10 and 1000-fold increase in IC_{50}) and “highly resistant” (more than 1000-fold increase in IC_{50}) (73,74). Similarly, throughout this work, we use the term “sensitive” to refer to viruses with IC_{50} values less than 10nM and “resistant” to refer to viruses with IC_{50} values >100-fold higher than the sensitive ones.

Enzymatic properties of mutant viruses were then examined in NA kinetic assays. We first tested all viruses with standardized total NA activity, as in the NA inhibition assay to calculate IC_{50} values. Interestingly, there was no significant difference among sensitive CL105 and resistant variants as well as between 2 resistant variants, CL107 and CL2009C, as seen in preliminary experiments with wild-type H5N1 viruses. We suspected that when using similar NA activity as input we already compensated for the reduction, if any, of resistant virus. Hence we used TCID value as a new input. Other

groups also utilized a similar approach for NA kinetic assay albeit they based on plaque forming unit calculations (31,51,75). When using comparable TCID inputs for all mutant viruses, we observed substantial differences in enzymatic parameters of oseltamivir sensitive and resistant variants. CL2009C, a resistant one, exhibited a reduced V_{max} and an increase K_m when compared to sensitive variant CL105. There was about 2-fold difference in V_{max} of CL105 (relative V_{max} 334.0 U/min) and CL2009C (152.3 U/min), similar to what detected by other groups with the same H274Y mutation in H1N1 or H5N1 backgrounds (31,51). The main observation was that, although bearing H274Y oseltamivir resistance mutation, CL107 possessed high enzymatic activity, even slightly higher than the susceptible CL105 (389.2 and 334.0 U/min respectively). This was also in compliance with our preliminary results (V_{max} 1673 and 1677 RFU for CL107 and CL105 respectively). However, the discrepancy in K_m was not as pronounced as for wild-type H5N1 viruses. Wild-type CL105 had a very low K_m, about 5-fold lower than K_m of CL107 and even 50-fold lower than K_m of CL2009C; whereas in seasonal H1N1 background NAs of CL105 and CL2009C displayed comparable K_m values and about 2-fold lower compared to that of CL107 (table 4.8). Since mutant viruses only contained the NA segment of their H5N1 counterparts, it was suspected other genes might play a role in low K_m phenotype. Further investigations are required to identify the involving factors.

Recombinants	CL105	CL107	CL2009
Relative V_{max}	334.0±34.98	389.2±20.60	152.3±12.02
K_m (μM)	49.05±24.38	83.18±17.93	55.69±11.75

Table 4.8 **NA enzymatic parameters of mutant viruses** (Values were means of duplicates of one experiment).

High V_{max}, high enzymatic activity, could be an intrinsic properties of NA or could result from enhanced NA expression. To identify the actual cause of increased V_{max} of CL107, we performed Western blot to quantify the level of NA expression. In parallel with NA antibody, we used NP antibody to control the virus inputs (which were diluted to the same TCID as in NA kinetic assay). Samples from protein expression experiment were also included. Those were NA plasmids (of CL105, CL107 and CL2009C) co-transfected with NS plasmids of WSN33 strain (1:1 ratio) in 293T cells (70). Transfectants were harvested and the same amount was used in Western blot. This experiment gave no NA specific band (Figure 4.6) although the NA from co-transfection experiment displayed very high activity according to standardization step (data not showed). For the virus samples, albeit there was signal for NP protein, it was difficult to clearly visualize the band. The ration of NP/NA in 1 influenza virus particle is between 5 and 10 (Rapid reference to Influenza, © 2006 Elsevier Ltd, All rights reserved). Hence one possible explanation was that when NP protein only reached detectable level, the amount of NA used in Western blot needed to be increased. Consequently, we re-generated recombinant variants in a larger volume to be able to concentrate the samples. In this experiment, MDCK infection supernatants were concentrated 300 times then used in Western blot together with cell pellet samples from the same infection. NP specific staining presented bands with different intensity in inverse proportion. CL105

had the most intense band among the concentrated supernatant samples whereas its cell pellet sample gave the weakest band of 3 viruses. In contrast, the intensity of CL2009C concentrated sample was the lowest one though its cell pellet was the strongest band of all samples. Therefore, we hypothesized that H274Y mutation had reduced the amount of viruses secreted from infected MDCK cells (CL2009C concentrated sample shown band with lowest intensity), resulting in increased level of viruses retained inside (or attached to) the cells (CL2009C cell pellet sample with strongest signal). One possible explanation was that H274Y mutation might interfere with protein transport pathway, causing lesser amount of NA presented on cell surface even when it was normally expressed. Although CL107 contained H274Y, its NA also had other amino acid changes when compared to CL2009C NA sequence. So we had another hypothesis that these mutations somehow diminished the effect of H274Y and restored the level of secreted viruses (represented in increased intensity of CL107 concentrated supernatant band when compared to the one of CL2009C). Detailed studies on individual mutation are necessary to identify any responsible mutation as well as to understand the restoration mechanism.

Sequence alignment suggested that 2 amino acid differences between CL107 and CL2009C NA might be responsible for restoration of NA activity. Those mutations were at positions 46 and 137; one in stalk region and one near the active site respectively. To understand the mechanisms by which the mutations caused effect, we exchanged a short sequence of 2 mutant NAs containing residue 137, generating CL107 A137T and CL2009C T137A variants. These two reversion variants were then examined in NA kinetic assay together with their original recombinant counterparts. Results from the first

experiment confirmed our observation in that reversion variants demonstrated opposing phenotype, CL2009C T137A had increased V_{max} relative to CL107 A137T. More interestingly, sequencing of clinical specimens taken on the first day of admission revealed that CL107 already contained A137, even before starting oseltamivir treatment (49), this implied that A137 might be a permissive mutation, supporting the emergence of oseltamivir resistance mutation H274Y by increase NA enzymatic activity. Nevertheless, results from reversion experiments were not reproducible when we tested different virus stocks. In fact, new virus stocks showed conflicting phenotype for both CL107 and CL2009.

All of these works, generating recombinant viruses, enzymatic characterization and protein expression quantification, were performed at the AMC, Amsterdam during 1 year training on human epithelial cell culture and reverse genetics. The effect of mutation at position 46 was not investigated because there was no sufficient time to finish the experiments in Amsterdam and it would require more time and effort to perform those in our laboratory in Vietnam.

VI Summary results

We successfully cloned NA of H5N1 isolates into the WNS33 backbone for transfection and confirmed drug susceptibility phenotype of reversely engineering viruses. We also demonstrated differences in NA activities of sensitive and resistant viruses. Based on sequencing results, we suspected that 2 amino acids on NA gene, at positions 137 and 46, of CL107 could play a role in restoration of its NA activity. We first investigated the

effect of residue 137 on NA enzymatic properties; although preliminary results supported the hypothesis that A137 might play a role but those were not reproducible. Hence, it is still unclear which one is the responsive mutation and, moreover, how it caused the changes, i.e. effects on level of expression and/or intrinsic enzymatic properties.

Chapter 5

Summary and future plans

Initially, the results and training described here were meant to be the foundation for a larger (PhD) project on H5N1 adaption in human (and porcine and avian) airway epithelial cell cultures. The first stage was the training on reverse genetics of influenza viruses (at MRC National Institute for Medical Research, Mill Hill, London [UK] and at the Department of Medical Microbiology at the Academic Medical Centre, Amsterdam [NL]) and cell culture techniques (Academic Medical Centre), followed by setting up those techniques in Vietnam in the second stage and ultimately implementing these techniques using human lung materials and H5N1 isolates from Vietnam (final stage). The first stage was accomplished successfully as described in chapter 3. Two different approaches of reverse genetic techniques for influenza virus were transferred to Vietnam. The first one, achieved in London, involved restriction cloning resulting in an H5N1 HA gene without the polybasic cleavage site (the determinant for high pathogenicity). The second approach involved in one-step cloning method to produce WSN33 (an attenuated influenza laboratory strain) recombinant virus. Besides mastering the techniques, I also obtained basic results supporting subsequent experiments. Characterization of recombinant viruses revealed amino acid variations between 2 oseltamivir resistant H5N1 isolates with different NA enzymatic properties. These differences could contribute to restore the activity of NA enzyme, individually or in combination, either by improving its intrinsic properties or by increasing the level of protein expression. Unfortunately, no conclusive results regarding the precise mutations and the underlying mechanism were achieved due to time limitation. All the characterization experiments were performed in Amsterdam, where the necessary

assays are well implemented. We wished to continue the work in Vietnam but it was unfeasible because it would exceed the time allowance.

Unlike the first stage, we encountered huge challenges in the second stage. Obtaining fresh human lung tissue is a necessity to establish the airway epithelial cell culture system. We tried to set up a collaboration with the cardiopulmonary surgery and pathology departments of Cho Ray hospital (one of largest hospitals in Ho Chi Minh city, Vietnam) but have not succeeded to obtain the type of materials we needed after several attempts, due to hospital regulations and restrictions. Consequently, we intended to shift our focus to porcine airway cell system, which - although dissimilar to the human model- plays an important role in influenza adaptation and reassortment. Again, it was impractical since we do not have an animal facility in or near our laboratory that allows performing sterile removal and dissection of porcine lung. Most of all, experiments on H5N1, either wild-type or genetically modified, viruses would require working in biosafety level 3 environment which was infeasible at the time writing this thesis and also in the near future due to personal circumstances.

H5N1 viruses are rapidly evolving and have become endemic in poultry in many countries. Although there are only very few incidences of proven or suspected human-to-human transmission, H5N1 viruses continue to be a pandemic threat due to their ongoing circulation and high pathogenicity and case fatality rate in sporadic human infections (76,77). Since the start of this project, more studies on H5N1 and oseltamivir–the drug of choice for H5N1 treatment (76) – susceptibility have been published. In two recent articles statistical analysis on the number of avian H5N1 NAI resistant viruses isolated in Indonesia (76) and Vietnam (77) is described. Both identified additional

mutations (I222T/M/V, R430W, H252Y), apart from the well-known H274Y mutation, that may contribute to the reduced susceptibility to oseltamivir of those isolates. Both studies also emphasized the importance of continual monitoring of H5N1 drug susceptibility as well as establishing a consensus for the definition of resistance that would lead to clinical failure. Since H5N1 viruses display a very wide range of IC_{50} values and different clades have different baselines, it requires a clear cut-off to classify the susceptibility profile of one specific virus. Many laboratories use a 10-fold change in IC_{50} (compared to wild-type counterpart), yet clade 1 and clade 2 H5N1 viruses are already a few fold different in oseltamivir IC_{50}. For example, in one study, Gubareva *et al* demonstrated that IC_{50} values of avian H5N1 viruses isolated in Vietnam in 2009-2011 could range from 0.04nM to more than 500nM depending on HA clades (77). This would make it more challenging to draw conclusions on the clinical significance of one specific mutation (75).

On the topic of airway epithelial cells, numerous groups have been working with differentiated primary epithelial cells to study influenza infections. Tumpey *et al* (78) have successfully isolated and differentiated ferret tracheal primary epithelial cells, and then utilized the system to investigate influenza tropism. Ferrets have been widely used to assess influenza pathogenicity and transmissibility owing to their similarities in clinical signs, illness and also physiological conditions to humans. In this study, the Tumpey group demonstrated that 95% α2,6-linked SA receptors were present on ciliated cells and α2,3-linked SA receptors were exclusively present on non-ciliated cells. This is in accordance with findings in human culture systems (79). Moreover, they also showed that human influenza viruses predominantly infected ciliated cells whereas avian viruses

solely infected non-ciliated cells. Infection by avian viruses resulted in substantial necrosis and induced high level of pro-inflammatory cytokines and chemokines. These findings are consistent with H5N1 infection in humans, and therefore ferret tracheal epithelial cell cultures may represent a model to study influenza viral tropism and host interaction during infection. Ferret cell culture systems have the advantage over human models of being easier to set up and maintain. Moreover, comparison among different models and species might provide valuable information and shed more light in influenza evolution and pathogenesis. Another group, Olsen and colleagues (80), used a similar approach to establish swine airway epithelial cell cultures for investigation of influenza A virus infection and replication. Previously, they used a submerged monolayer of porcine respiratory epithelia cells to examine swine H3N2 viral infection. However, that system did not fully reproduce the cellular conditions of the porcine respiratory tract. Hence they developed a differentiated epithelial cell culture (grown at air-liquid interface) by modulating the conditions applied for human respiratory epithelial cell cultures. This resulted in a culture model suitable to study influenza as well as other respiratory pathogens of pigs. Using this system, they successfully demonstrated productive infection and replication of influenza viruses and illustrated that the differences in viral infection characteristics here are also seen *in vivo*. Furthermore, they also described optimal concentrations of retinoic acid and epidermal growth factor for the growth of those cells. Differentiated porcine epithelial cells were not only similar to the porcine lower respiratory tract in sialic acid expression but also promoted influenza infection without the need of exogenous trypsin, in concordance to differentiated human respiratory cells. Taken all of these achievements together, porcine airway epithelial cell

cultures may provide a useful model to study influenza infection and adaptation/reassortment and other respiratory diseases of pigs.

Although the precise mechanisms facilitating H5N1 viruses to cross the species barrier is not fully understood, a prerequisite for human-to-human transmission is a change in receptor binding preference of the viral HA protein, fromα2,3-linked to α2,6-linked sialic acid receptors. Numerous studies have identified and characterized HA mutations which are responsible for changes in the receptor binding profile of H5N1 viruses. In one of our recently published papers, Crusat *et al* (81), we detected substitutions in the HA gene that could play a role during the adaptation process of H5N1 viruses to the human host. Interestingly, those mutations (A134V, I151F and E186D) were present in different proportions in the original clinical specimens and subsequently cultured samples. The E186D mutation was not even detected in the original clinical sample. These observations indicate that changes in H5N1 receptor preference can surface rapidly after during human infection and can be selected in culture, which may disguise the real adaptation process. It is also worth noting that HA mutations can also affect H5N1 drug susceptibility through mutual interaction of HA and NA, even though these have displayed no clinical impacts until now (19).

Based on the findings mentioned above and by adapting and utilizing various airway epithelial cell cultures, we can study different aspects of influenza infection and drug susceptibility. We intend to continue this project and to investigate the adaptation of H5N1 viruses after serial passage either with or without selective pressure. Based on the preliminary results achieved and the techniques that have been transferred, we plan to serially passage H5N1 viruses, with different oseltamivir susceptibility and NA kinetic

properties, in well-differentiated airway epithelial cell system(s) (depending on the feasibility of the technique, either a human and/or porcine culture system would be employed) and subsequently characterize progeny viruses to investigate the influenza adaptation process. Passaging experiments could be performed in the presence and absence of oseltamivir, with and without mutations of the HA (and/or NA) gene that confer a receptor specificity switch, in a natural or reversely engineered background, to examine the impacts of drug pressure as well as mutual effects of HA and NA segments.

H5N1 viruses to be studied should be selected from different clades circulating in poultry and associated with human disease in different regions of Vietnam between 2001 and now. By (investigating and) comparing the adaptive process of these clades, we may be able to partly explain why one clade is dominant over another. Taken these observations together, we can gain insight into the evolution of influenza viruses, in particular H5N1 viruses and their HA/NA functional balance. This, in turn, would inform influenza pandemic preparedness and control programmes of avian influenza.

Related publications

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