INVESTIGATION OF NUCLEOTIDE SEQUENCE IN NEURAMINIDASE GENE OF OSELTAMIVIR-RESISTANT INFLUENZA A (H1N1) 2009

A THESIS

BY

PAILIN KITTIKUN

Presented in Partial Fulfillment of the Requirements for the

Master of Science in Molecular Biology

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AN ABSTRACT

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Pailin Kittikun. (2014). *Investigation of nucleotide sequence in neuraminidase gene of oseltamivir-resistant influenza A (H1N1) 2009*. Master Thesis, M.S. (Molecular Biology). Bangkok: Srinakharinwirot University. Advisor Committee: Assoc. Prof. Dr. Surangrat Srisurapanon, Assoc. Prof. Dr. Teeraporn Chinchai, Assoc. Prof. Dr. Kosum Chansiri, Dr. Navin Horthongkham.

In 2009, the pandemic influenza A H1N1 (pH1N1) viruses caused occasional pandemics in North America, and epidemiologically worldwide that originated from swine. In this pandemic, neuraminidase inhibitors are an important first line of defense against pH1N1 viruses. The large-scale use of drugs for treatment of influenza infection generates resistance *de novo* and on the transmission fitness of resistant virus. There are concerns that circulating of the resistant virus might be occurred. Therefore, the aim of this study is to monitor the oseltamivir-resistant strain in the community. In this study, 85 nasal or nasopharyngeal swab samples were collected from respiratory tract infection patients whom visited at HRH Princess Mahachakri-Sirindhorn Medical Center (MSMC) during October 2010 to December 2010. By rapid test, only 85 samples were influenza A positive and were confirmed for influenza A subtype by one- step real time RT-PCR (Invitrogen, USA). The positive pH1N1 (2009) samples virus were further analysed for oseltamivir-resistance by pyrosequencing. In order to investigate for the other mutations, the nucleotide sequences of whole NA gene were performed. All the positive samples were categorized into pandemic influenza A (pH1N1) (72.94%), seasonal H1N1 (11.76%) and H3 subtype (15.29%). Only 1 sample of pH1N1 showed mutation at the position H275Y (CAT to TAT) which is the marker of oseltamivir-resistance. The resistant strain in this study was collected from an immunocompromised patient. The emergence and transmission of oseltamivir-resistant influenza A (H1N1) viruses with an H275Y mutation (H275Y in NA subtype 1 (N1) numbering) emphasize the need for close monitoring of oseltamivir resistance in hospital and community.

การตรวจหาลำดับเบสใน neuraminidase gene ของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ที่ดื้อต่อยา oseltamivir

บทคัดย่อ

ของ

ไพลิน กิตติคุณ

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาวิชาอณูชีววิทยา
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ในปี 2009 ได้เกิดการระบาดของไข้หวัดใหญ่ที่มีต้นกำเนิดมาจากหมู เป็นสายพันธุ์ใหม่ หลังจากนั้นไม่นานเชื้อไข้หวัดนี้ได้เกิดการระบาดทั่วโลก การระบาดครั้งนี้ยาในกลุ่ม neuraminidase inhibitors ถูกกำหนดเป็นยาหลักในการรักษา ทำให้มีการใช้ยาอย่างมากมาย ส่งผลให้เชื้อไวรัสไข้หวัดใหญ่เกิดการกลายพันธุ์ และอาจมีการติดต่อของเชื้อที่กลายพันธุ์ได้ ทำให้เกิดความกังวลว่าจะมีการแพร่กระจายของเชื้อไวรัสที่ดื้อยาในชุมชน ดังนั้น การศึกษานี้จึงได้เฝ้าระวังการแพร่กระจายของเชื้อไข้หวัดใหญ่ โดยทำการตรวจหาการติดเชื้อไวรัสไข้หวัดใหญ่จาก nasal/ nasopharyngeal swab ของผู้ป่วยที่มีอาการติดเชื้อของทางเดินหายใจ และมารักษาที่ ร.พ. ศูนย์การแพทย์สมเด็จพระเทพฯ ในระหว่าง เดือนตุลาคม 2010 ถึงเดือนธันวาคม 2010 ตัวอย่างตรวจโดยวิธี rapid test พบว่าติดเชื้อไข้หวัดใหญ่ ชนิด influenza A จำนวน 85 ตัวอย่างตรวจ จึงทำการตรวจยืนยันและหา subtype ของ influenza A โดย one- step real time RT-PCR (Invitrogen, USA) ในกรณีที่เป็นเชื้อไวรัสไข้หวัดใหญ่ชนิด pH1N1 (2009) ก็จะทำการตรวจเชื้อดื้อยาต่อไปโดยวิธี pyrosequencing และทดสอบหาการกลายพันธุ์ตำแหน่งอื่นๆโดยทำการตรวจหาลำดับ nucleotide ของ NA gene ทั้งหมด การศึกษานี้พบว่า มีการระบาดของเชื้อไข้หวัดใหญ่ 3 สายพันธุ์ คือ pH1N1 จำนวน 62 ตัวอย่างตรวจ (72.94%), seasonal H1N1 จำนวน 10 ตัวอย่างตรวจ (11.76%) and H3 subtype จำนวน 13 ตัวอย่างตรวจ (15.29%) จากตัวอย่างตรวจทั้งหมดของ pH1N1 พบว่ามี 1 ตัวอย่างตรวจของผู้ป่วยที่มีภูมิคุ้มกันต่ำ มีการกลายพันธุ์ที่ตำแหน่ง H275Y (CAT to TAT) ซึ่งเป็นตัวบ่งชี้ว่าเป็นเชื้อไข้หวัดใหญ่สายพันธุ์ที่ดื้อยา oseltamivir การพบเชื้อดื้อยาในโรงพยาบาล เป็นการตอกย้ำว่าควรมีการควบคุมการแพร่กระจายของเชื้อไวรัสไข้หวัดใหญ่อย่างเข้มงวด และควรมีการเฝ้าระวังการกลายพันธุ์ของเชื้อไวรัสไข้หวัดใหญ่อย่างต่อเนื่อง

The thesis titled

"Investigation of nucleotide sequence in neuraminidase gene
of oseltamivir-resistant influenza A (H1N1) 2009"

by

Pailin Kittikun

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Molecular Biology of Srinakharinwirot University.

……Dean of Graduate School
(Assoc. Prof. Dr. Somchai Santiwatanakul)
January , 2014

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	(Dr. Navin Horthongkham)

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LIST OF ABBREVIATIONS

cDNA	Complementary DNA
dsDNA	Double strand DNA
H275Y	Histidine substitution to tyrosine at position 275
HA	Hemagglutinin
IL-1	Interleukin-1
M1	Matrix protein
mRNA	Messenger RNA
NA	Neuraminidase
NAIs	neuraminidase inhibitors
NEP	nuclear export protein
NP	Nucleoprotein
NS	Nonstructural
OC	oseltamivir carboxylate
PA	Polymerase acid
PB1	Polymerase basic 1
PB2	Polymerase basic 2
R292K	Arginine substitution to lysine at position 292
RT-PCR	Reverse transcription polymerase chain reaction
TNF	Tumor necrosis factor
vRNA	viral RNA

CHAPTER I

INTRODUCTION

Influenza virus is classified in *Orthomyxoviridae* family that has negative-sense, single-stranded and 8 segmented RNA genomes. They were classified in 3 genera influenza A, B and C. All 3 genera can infect human and other species of mammals. In general, pandemic or epidemic infection caused by influenza A is infrequent[1, 2]. However, influenza A and B usually circulate as the seasonal outbreak except influenza C. In the case of influenza A virus, approximately 3-5 million people of the world population are infected influenza in each year[2] and 250,000-500,000 are dead annually[3, 4]. In 2009, the triple reassortant influenza A H1N1 viruses emerged and caused the first influenza A pandemic of the 21st century. Because of the highly contagious virus, the declaration of an influenza pandemic level 6 was announced by World Health Organization[4-6].

The prevention and intervention of influenza pandemic viruses are urgently needed. In this regard, both vaccination and anti-viral drug are considered to be the first line of prevention. Although vaccine is accepted as the method of choice in infectious disease control, in the new pandemic influenza virus, the efficacy of protection is limited by no antigen match to vaccine strains[7]. So, in early pandemic, anti-viral drug is the most suitable for both therapeutic and prophylaxis purposes[8].

There are two classes of antiviral drugs, M2 blockers (amantadine and rimantadine) and neuraminidase (NA) inhibitors (NAIs). As an early epidemic of H1N1 (2009), It was known that the pandemic influenza A H1N1 (pH1N1) had already resisted to M2 blockers. So, NAIs played an essential role for prophylaxis and treatment of influenza infection[9, 10]. The two FDA approved NAIs; oseltamivir and zanamivir are used in the clinical management of the pandemic and seasonal over 10 years ago[11]. By these reasons, oseltamivir is more popular than zanamivir. The massive used of oseltamivir was increased rapidly, since the pH1N1 viruses were highly transmissibility. Because of the large scale using of oseltamivir, the emerging of oseltamivir resistant viruses are unavoidable. Therefore, it is needed for systematic surveillance of the oseltamivir resistance[12].

Considering the emergence of oseltamivir resistant viruses, the predominant mutation conferring His275->Tyr275 (H275Y, N1 numbering) in neuraminidase (NA) gene

was reported. The other neuraminidase (NA) mutations, H275Y, N295S, and I223R, are associated to oseltamivir resistance despite the mutation positions are distance from the active site. The conformational motions of a binding site can modify binding site shape that is induced by several factors such as membrane voltage and the role of water molecules[13]. In 2011, Dr. Aeron Hurt[14] analyzed circulating pH1N1 strains and reported that resistance to oseltamivir was quite low approximately 2%. In Thailand, the mutation rates of drug-resistant strains of influenza virus vary from a report to report, probably due to the limited number of samples used for characterization. Not only the rate of oseltamivir-resistance but also the fits of virus are serious concerned. There is evidence showed that these resistant strains are capable of spreading from human-to-human and increase the risk of a public health emergency.

Hypothesis

After a period of pandemic, some resistant strains of Influenza A virus (pH1N1) might have occurred in our community.

Objectives

1. To monitor the resistant strain of Influenza A virus (pH1N1) in patients whom had experience in respiratory tract infection at HRH Princess Mahachakri-Sirindhorn Medical Center (MSMC).

2. To investigate the sequence of NA gene in the resistant strain of Influenza A virus (pH1N1).

CHAPTER II

LITERATURE REVIEW

Influenza virus is the member of family *Orthomyxoviridae*. In this family, there are three genera of influenza virus; Influenza virus A, Influenza virus B and Influenza virus C. These viruses are distinguished by differences in two major internal proteins, matrix (M) and nucleoproteins (NP). Influenza A, B and C viruses differ with respect to host range, variability of the surface glycoproteins, genome organization and morphology[15]. Only Influenza A virus is the most important pathogenic virus causing human respiratory tract infection with high morbidity and mortality rate[4]. This viruse has been recurrent epidemics every 1 to 3 years and have been pandemics every 10 to 20 years[16]. In 1981, 'Spanish' influenza was a highly contagious and caused deadly disease. It is estimated that over 40 million people were killed worldwide[16-18]. In addition, economic value was lost by pandemic's influenza. By this regard, this review focused on Influenza A virus.

Genomic of Influenza A virus

As an RNA virus, the genome of influenza A virus is formed by 8 segments of single-stranded RNA of negative polarity that vary in size from 890 to 2350 nucleotides. In total, its' genome contains 13,588 nucleotides and encodes 11 proteins[19] as shown in figure 1.

Each segment of RNA is copied into (+) strand mRNAs that serve as templates for the synthesis of proteins. The specific viral proteins are produced by each viral mRNA. Eight segments of RNA and coding proteins of Influenza A are summarized in the table 1

Table1 Genomic segments and viral proteins of Influenza A virus[20-22].

Genomic segment	Size (nt)	Coded proteins	amino acids	Main function
1	2341	PB2	759	Sub-unit of RNA-polymerase
2	2341	PB1	757	Sub-unit of RNA-polymerase
		PB1-F2		Function unknown, pro-apoptotic
3	2233	PA	716	Sub-unit of RNA-polymerase
4	1778	HA	566	Viral attachment and membrane fusion
5	1565	NP	498	Major structural component
6	1413	NA	454	Release of new virions by preventing aggregation
7	1027	M1	252	Facilitating migration of viral RNP in cell
		M2		Ion channel involved in uncoating of virus
8	890	NS1	230	Post-transcription modulation, interferon antagonist
		NS2/NEP	121	Mediates nuclear export of vRNAs

Morphology of influenza A virus

Influenza A virus is pleomorphic or spherical appearances when observed under the electron microscope. The particle has an average diameter of 100 nm and length about 300 nm[16]. For the outer surface of virion, two different glycoprotein spikes are embedded in the envelope. The virion has a lipid envelope where three viral proteins are inserted, the glycoproteins HA and NA, and small amounts of the trans-membrane protein M2. Approximately, 80 percent of the spikes are hemagglutinin (HA). The remaining 20 percent of the glycoprotein spikes consist of neuraminidase (NA). The lipid bilayer surrounded the inner protein layer formed by the matrix protein M. This protein encloses the viral genome associated with the nucleoprotein NP and with small amounts of PB1, PB2, and PA that form the RNA polymerase complex. Inside the viral particle, it contains the nuclear export protein NEP which is known as nonstructural protein NS2. Several proteins are enclosed in the capsid. The structural proteins and the segmented genomes are shown in the figure 1.

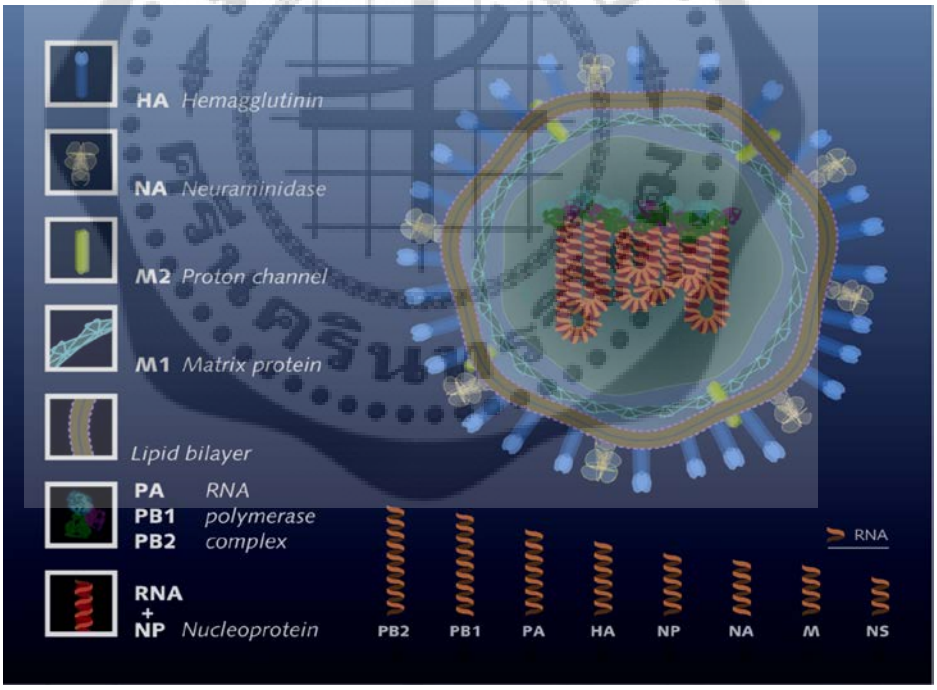

Figure 1 The segmented genomes and structural proteins of influenza A virus[23]

Subtype of influenza A virus

As describe earlier, the glycoproteins of both HA and NA on the virus envelop determine the subtypes of Influenza A virus. Based on the HA and NA gene, influenza A viruses are divided into two groups of sixteen hemagglutinin subtypes (H1-H16) as shown in figure 2. For neuraminidases, the antigen was classify into nine neuraminidase subtypes (N1-N9) as shown in figure 2[24]. All hemagglutinins and neuraminidases' subtypes can infect wild waterfowl. The various combinations of H and N results in 144 combinations and potential subtypes of influenza A viruses. The most common subtypes of human influenza virus which were identified to hemagglutinins 1, 2, and 3 and neuraminidases 1 and 2 are currently circulating. The full nomenclature for influenza virus isolates requires connotation of the influenza virus type (A or B), the host species (omitted if human in origin), the geographical site, serial number, year of isolation, and lastly, the H and N variants in brackets, for example: A/goose/Guangdong/1/96 (H5N1). Both hemagglutinin and neuraminidase are critical for virulence and responsible for the circulating influenza A virus.

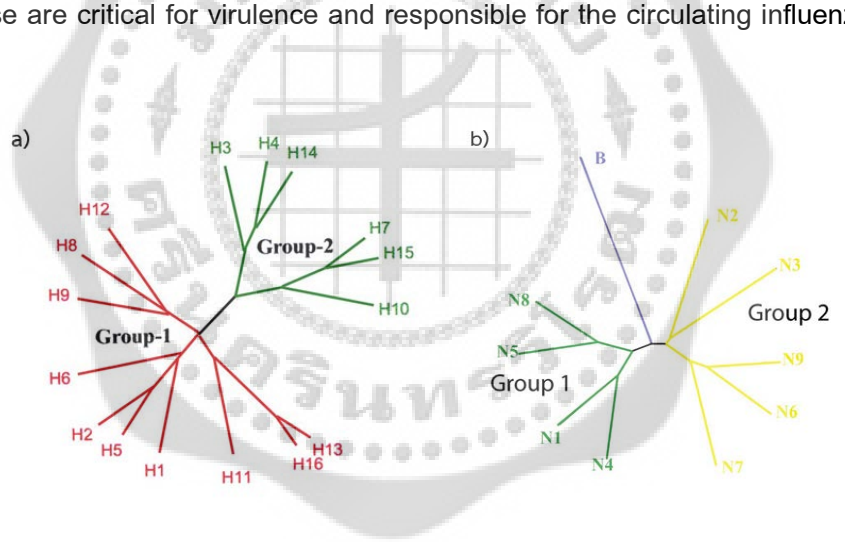

Figure 2 Subtype of hemagglutinins (HA) and neuraminidases (NA).

a) phylogenetic tree of sixteen HA subtypes of influenza A that was categorized into two groups, b) phylogenetic tree of the nine NA subtypes of together with NA from influenza B[25].

Hemagglutinin of Influenza A virus

Structure of Hemagglutinin (HA)

Hemagglutinin (HA or H) is a type 1 glycoprotein which presented at the outer surface. It composes of homotrimeric integral membrane and looks like a cylinder. The three identical monomers that constitute HA are formed into a central α-helix coil; three spherical heads contain the sialic acid binding sites. The trimer has cleaved into subunits HA_1 and HA_2, which are the disulfide-linked that contain the furin recognition motif (R-X-K/R-R). Each HA (refer as HA_0) is synthesized as precursors polypeptide of about 550 amino acid residues. Each monomer of H_0 consists of a long, helical chain anchored in the membrane by HA_2 and topped by a large HA_1 globular[15, 26, 27]. After synthesis, it was glycosylated and cleaved into a smaller polypeptide: the HA_1 and HA_2 subunits. The globular membrane-distal heads of HA_1 de-trimerize, but remain tethered to the trimeric HA stalk through residues 28–43 of HA_1. In the HA_2 subunit, the fusion peptide is extruded from its buried position in the interior of native HA trimers, and two structural rearrangements take place in the HA stalk. One is the refolding of the extended chain that links the long alpha helix with the shorter one in the native HA hairpin loop structure. These residues become helical and lead to an N-terminal extension of the coiled coil. As a result, the small helix is also recruited into the extended coiled coil and residues N-terminal to it are concurrently relocated. Therefore, the extruded fusion peptide is propelled towards the end of the newly formed coiled coil to insert into the target membrane. The other conformational change involves residues 106–112 in the membrane proximal segment of the native HA coiled coil. This unfold forms a loop structure that causes residues at the C-terminal end of each α-helix to reorient by 180º. The short helical domains pack antiparallel to the coiled coil, and residues C-terminal to this extends toward the same end in the structure of the fusion peptide[26] as shown in figure 3.

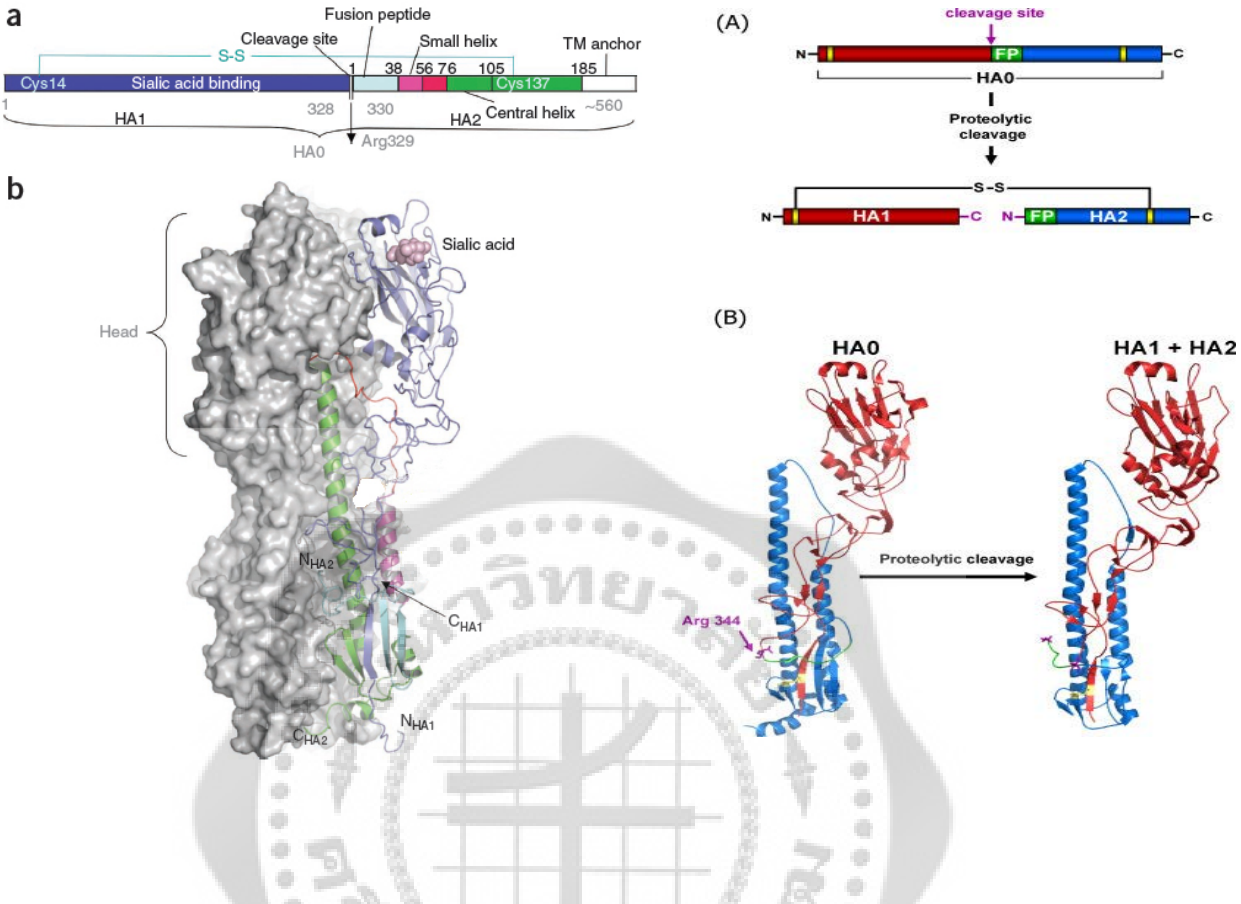

Figure 3 Linear sequence diagrams and structures of influenza A hemagglutinin.

a. linear order of sequence segments of HA_0, HA_1 and HA_2

b. trimeric structure of Influenza A hemagglutinin

A. the S-S bond between HA_1 and HA_2

B. the ribbon diagram represents a monomer of HA_0, HA_1 and HA_2 [27]

Function of hemagglutinin (HA)

HA of influenza virus plays an important role during the cell entry. It binds to the cell-surface molecule which is sialic acid as shown in figure 4[16, 28]. The HA protein is a critical determinant of virulence and pathogenicity of influenza virus. Previously, the link between cleavage site and virulence of pathogenic virus was reported[15].

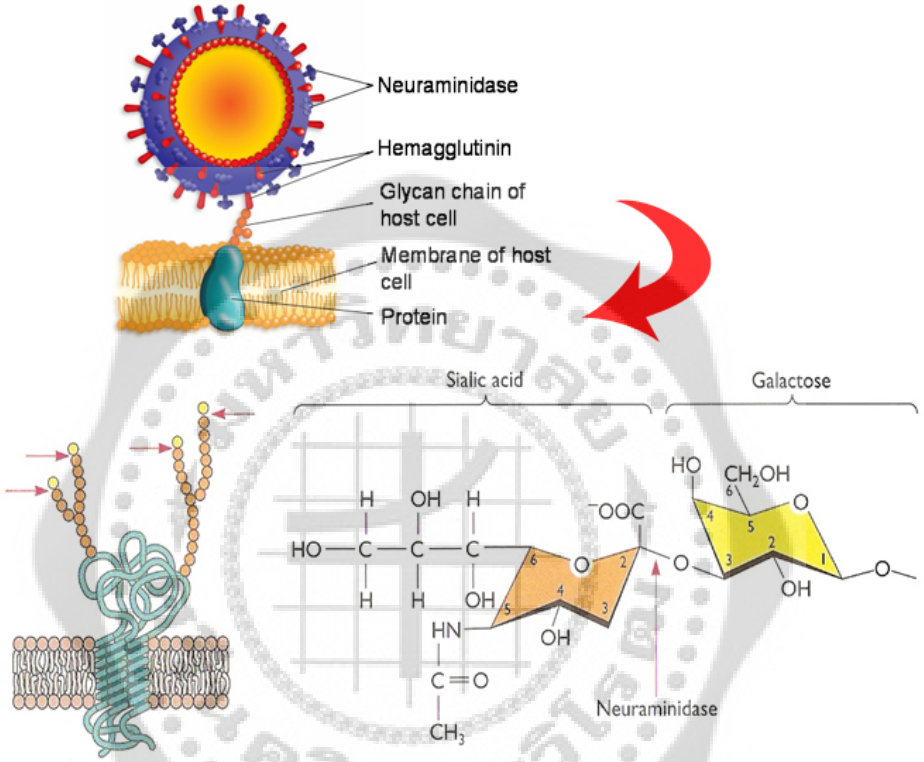

Figure 4 Binding of hemagglutinin to the cell surface and structure of sialic acid[29, 30]

The process of virus infection begins when the receptor-binding activity of HA is provided by the HA_1 subunit in the membrane-distal head domain of the molecule that is the sialic acid binding site. After binding to sialic acid residues of receptor proteins on host cells, the influenza virus is brought into the cell by endocytosis. Many of the original hemagglutinin residues are lost in this digestion, the major conformational change caused by the acidic environment in the endosome. The low pH in endosome, between pH 5 and pH 6, activates the conformational change of hemagglutinin structure. The mechanism of conformational change with HA_1 residues 323–328 and HA_2 residues 1–12 accompanies cleavage site of HA_0. A highly conserved hydrophobic region is the liberated HA_2 N-terminal domain, and due to its role in membrane fusion, it has been designated as the fusion

peptide. After cleavage, the fusion peptide relocates into an adjacent cavity and contacts several ionizable residues. The positioning of residues 1–3 of the trans-located fusion peptide also blocks access to another internal cavity in the trimer interior that contains additional ionizable residues. After cleavage shall set the trigger for subsequent conformational changes required for fusion. In the endosome, acidification of native cleaved HA is led to three prominent structural refolding events (figure 5). The "fusion-active" state of hemagglutinin triggers the fusion between the viral membrane and endosome membrane. Then the viral nucleocapsid released into the cytosol of the host cell (figure 5)[15, 26, 27] and replication cycle of virus in host cell is arisen. HA is the primary viral antigen to which is detected by the host's antibody response, and it is the only antigen inducing a virus-neutralizing response.

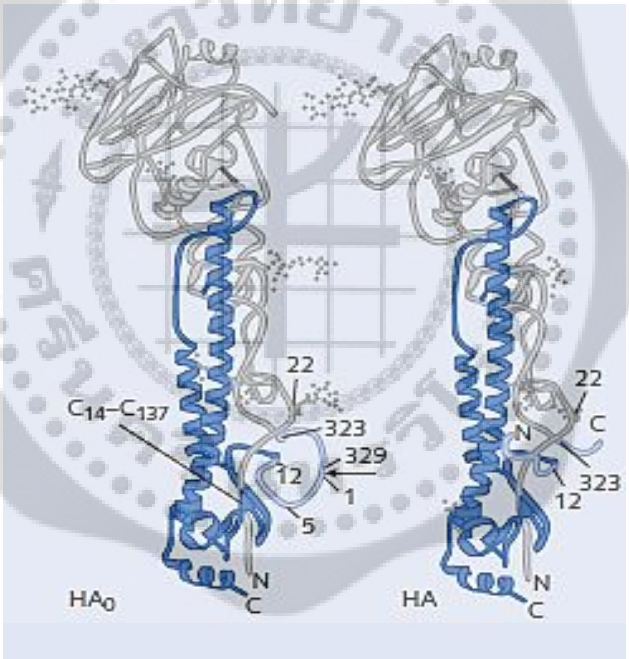

Figure 5 The ribbon diagram of the HA conformational changes with the accompany precursor cleavage.

The HA1 residues shown in grey and the HA2 residues shown in blue do not change. The residues of the HA0 loop structure (light blue, left panel) relocate following cleavage at residue 329 (arrow) such that the N-terminus of HA2 is inserted into the trimer interior of the native cleaved HA (right panel)[26].

Receptor specificity

Based on the HA, it plays a key role in the restriction of interspecies transmission. Nevertheless, the molecular basis for host range restriction is not clear. At present, it was proven that Influenza A virus infectivity is influenced by SA linked to galactose by an α2,6 linkage (Acα2,6Gal) or an α2,3 linkage (Ac α2,3Gal) on the host cell surface (Figure 6). In general, SA with 2, 6 linkages are bound by human influenza viruses (H1, H3) and 2, 3 linkages are bound by avian influenza viruses (H5, H7). In this context, both H1 and H3 viruses are the most common influenza A subtype that infect humans. New strains of influenza may spread from other animal species to humans. Alternatively, an existing human strain may pick up new genes from a virus that usually infects birds or pigs. Some of these influenza strains are species specific, which is partly due to the ability of a given hemagglutinin to bind to different sialic acid receptors on respiratory tract epithelial cells[31].

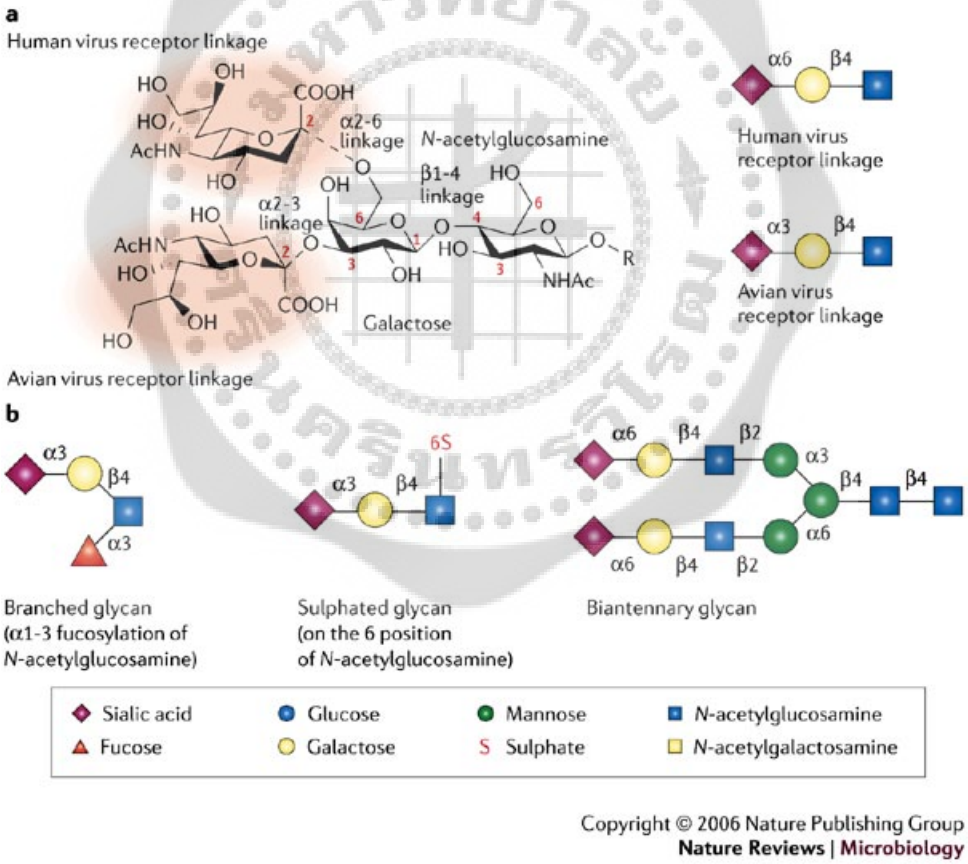

Figure 6 The difference between sialic acid receptor of human and avian [32]

Neuraminidase (NA) of Influenza A virus

Neuraminidase is an exosialidase which is coded by the 6th RNA segment. This enzyme forms mushroom-like projections from the surface of the influenza virus. Each molecule composes of homo-tetramer which assembles into head, thin stem and trans-membrane domain. This protein is glycosylated and exposed on the influenza envelop. Approximately, one viral particle has 50 tetramers. Based on the homology of amino acids in the monomer, there are nine subtypes in influenza A. Identity of viruses inside one subtype attains about 90%, whereas homology between subtypes is 50%. All subtypes of influenza A are divided into two groups. The first group consists of the neuraminidases of N1, N4, N5 and N8 subtypes, and the second one composes of N2, N3, N6 N7 and N9 subtypes[33]. By X-ray crystal structure, two groups of NA are different in their active sites despite the amino acids are conserved as shown in the figure 7. In N1 subtype, a cavity was formed in the active site. This structure might be a new target for drug design[34].

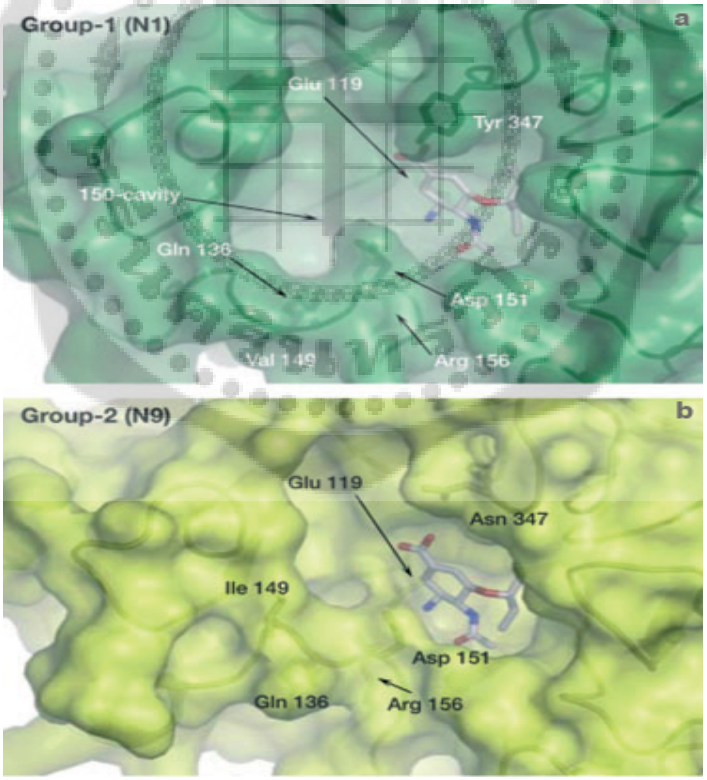

Figure 7 X-ray crystal structure of N1 and N9, the cavities in the active site of these two subtypes are different.

Structure of neuraminidase (NA)

There is about 5-10% of NA in the surface viral protein[35]. Each monomer, it comprises 470 amino acid residues. The 4 identical monomers are arranged with circular 4-fold symmetry and the enzyme active site is located centrally on each monomer. The tetramer contains 3 domains; head, stem and trans-membrane region (figure 8).

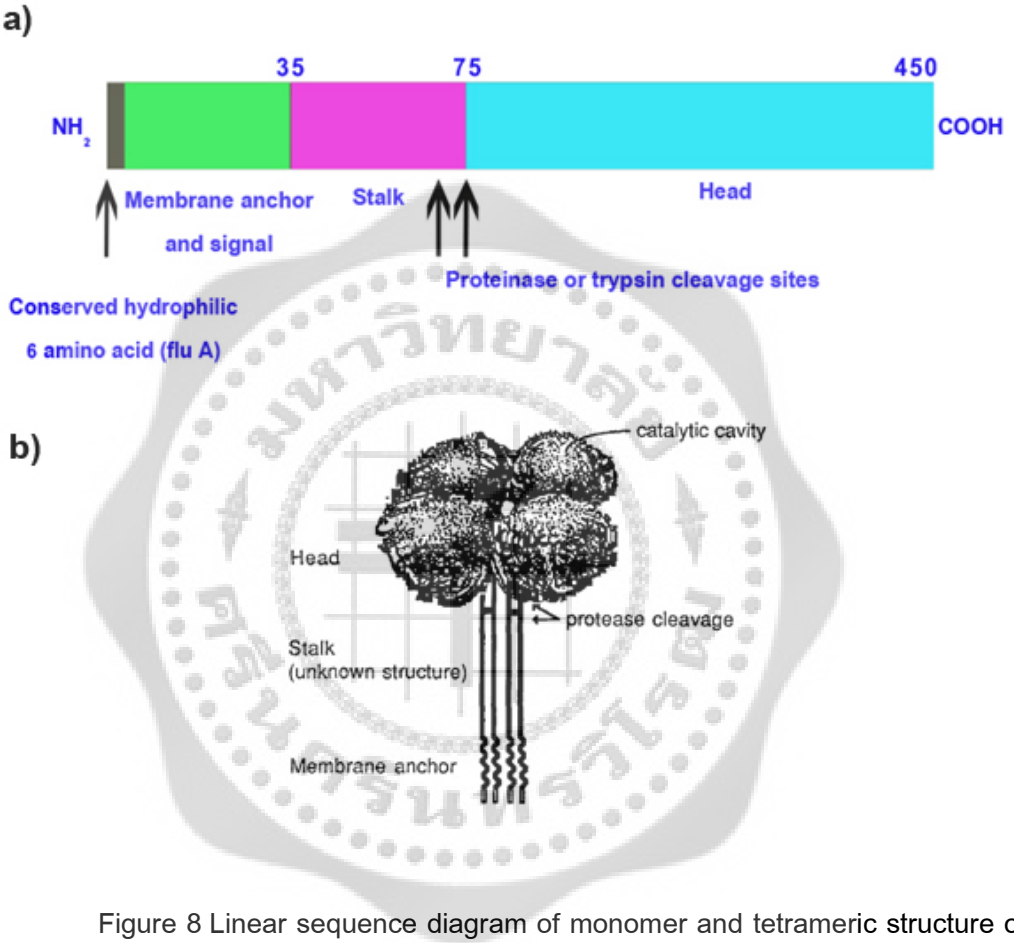

Figure 8 Linear sequence diagram of monomer and tetrameric structure of influenza neuraminidase

a. Diagram of linear amino acid sequence of neuraminidase

b. The schematically structure of tetrameric NA contains head, stem or stalk and membrane anchor or cytoplasmic membrane[35]

Head is the most important domain of neuraminidase. Both the enzyme active site and the calcium binding domain are situated in this region[33, 34]. For enzyme active site, the amino acids located within this region can be categorized by their role into 3 sets. First, amino acids which account for the catalytic function NA are Arg118, Asp151, Arg152, Arg224, Glu276, Arg292, Arg371 and Tyr406 (N2 numbering). Second, asparagine (Asn) at

the position 146 is the essential residue for the glycosylation. Third, the proline and cysteine residues provide the folding of the polypeptide chain and stabilize the 3-dimensional structure within the molecule. These amino acids are strictly conservative for all NA subtypes of Influenza A. Another enzymatic NA binding site, which was an alternative site, had been found in avian Influenza virus (N9) called HB. However, its role was not clear[33].

For calcium binding domain (figure 9), it is located inside the molecule and formed by the oxygen of the main chain residues 297, 345 and 348, as well as by the oxygen of the side chain of Asp324. Presenting of calcium in this position, it plays an important structural role in stabilizing the architecture and reactive configuration of the catalytic site[36]. Typically, the presence of the calcium ion appears to help promote enzymatic activity of neuraminidase by preventing it from denaturing.

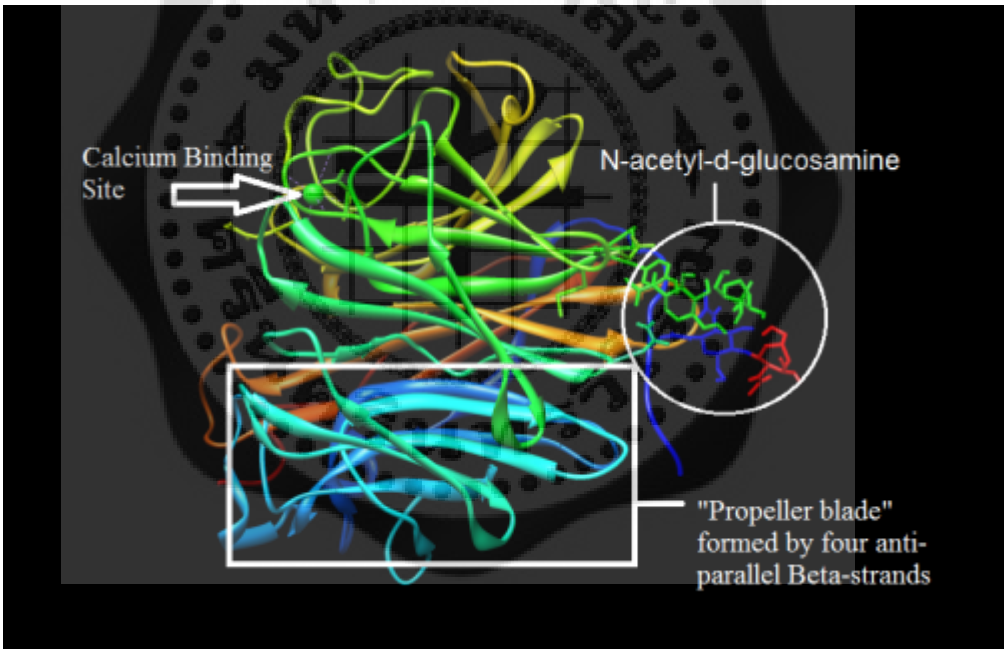

Figure 9 The ribbon diagram of NA shows a monomer which contains calcium binding site (opened arrow) and sialic acid binding site (white circle)[36]

Trans-membrane and stem domains

In recent year, evidence showed that the trans-membrane domain (TMD) of influenza neuraminidase promotes proper NA assembly. In the absence of the TMD, NA is synthesized and transported in a predominantly inactive state. NA activity was induced by progressive truncations of the stalk domain. This study also suggested that the TMD contribute to NA maturation by tethering the stalk to the membrane. The NA TMD formed a homo-tetramer and efficiently trafficked to the plasma membrane. The tetramer was formed when the TMD and enzymatic head domain drive assembly together through matching oligomeric states[37]. In concordance with the other study, the NA structure and its functions are correlated[38, 39].

Function of Influenza neuraminidase

There is data indicating that NA is relevant to different stages of infection. Firstly, it is considered that it helps the virus approach the target cells by cleavage of sialic acids from respiratory tract mucins[40]. Secondly, it may take part in the fusion of viral and cell membranes[41]. Thirdly, it facilitates budding of new virions by preventing their aggregation, caused by the interaction of the HA of the first virus with the sialylated glycans of the second one[41]. Briefly, this enzyme facilitated the mobility of the virus both to and from the site of infection. It is responsible for catalyzing the cleavage of α (2-3) or (2-6) ketosidic linkage between a SA and an adjacent sugar residue. Moreover, NA also contributes to the pathogenicity of influenza viruses[42]. There may be potentiation of the virulence of some viral strain by alteration of carbohydrate moieties from the other surface glycoprotein the HA. In addition, inducing of pro-inflammatory cytokine (IL-1, TNF) and plasminogen-binding activity of NA may promote the virulence of influenza virus. The functions of NA are not isolated. The NA activity and HA of the influenza virus act in concert and their evolution proceeds interdependently[41, 43-48].

As shown in a previous study, the influenza virus lacking NA activity can undergo multiple cycles of replication in cell culture, egg or mice[43]. It was proposed that NA activity was not required in the influenza A virus life cycle but appears to be necessary for efficient virus replication. There is functional interaction between HA and NA balancing in influenza virus infections. An optimal interplay between HA receptor-binding and NA receptor-destroying is required for efficient virus replication. Furthermore, substrate specificity of NA and the receptor specific of HA are highly correlated. Regardless of NA subtype, NA

function depends on the active site of NA, which strictly conserved. However, massive use of NAI for prophylaxis and treatment imposes strong selection for the evolution of drug resistant strain.

Life cycle of influenza virus

Infection and replication occur only in living cells with multiple steps. First, the virus uses HA glycoprotein to bind to the sialic acid receptor on host cell. Then hemagglutinin protein is cleaved by protease and virus enters the cell via endosome (figure 10-b).

Inside the cell, the hemagglutinin protein fuses the viral envelope with the vacuole's membrane, then the M2 ion channel allows protons to move through the viral envelope. The conformational change of endosome is triggered by low pH causing the core to dissemble and release the viral RNA and core protein. The viral RNA molecules, accessory proteins and RNA-dependent RNA polymerase are then released into the cytoplasm (figure 10-c).

The core proteins and viral RNA from a complex that is transported into the cell nucleus. Inner nucleus, complementary positive-sense vRNA was transcribed by using RNA-dependent RNA polymerase. The newly synthesized viral proteins are either secreted through the Golgi apparatus onto the cell surface (Figure 10-d) or transported back into the nucleus to bind viral RNA and form new viral genome particles. Other viral proteins have multiple actions in the host cell, including degrading cellular mRNA and using the released nucleotides for viral RNA synthesis and also inhibiting translation of host-cell mRNAs. Negative-sense vRNA that forms the genomes of the progeny virues, RNA-dependent RNA polymerase, and other viral proteins are assembled into a virion. Hemagglutinin and neuraminidase molecules are clustered into a bulge in the cell membrane. The vRNA and viral core proteins leave the nucleus and enter this membrane protrusion. The vRNA and viral core proteins leave the nucleus and enter this membrane. The mature virus buds off from the cell in a sphere of host phospholipid membrane coat. As virus releasing from the host cell, the viruses adhere to the cell through hemagglutinin; the mature viruses detach once their neuraminidase has cleaved sialic acid residues from the host cell. After the release of new influenza viruses, the host cell dies[27]. The new releasing viruses are ready to infect the other cells or transmit from person to person by aerosols and droplet. The

time from entry to production of new virus is on average 6 hours. Outside a human host, Influenza can survive for hours, further aiding its capacity to spread.

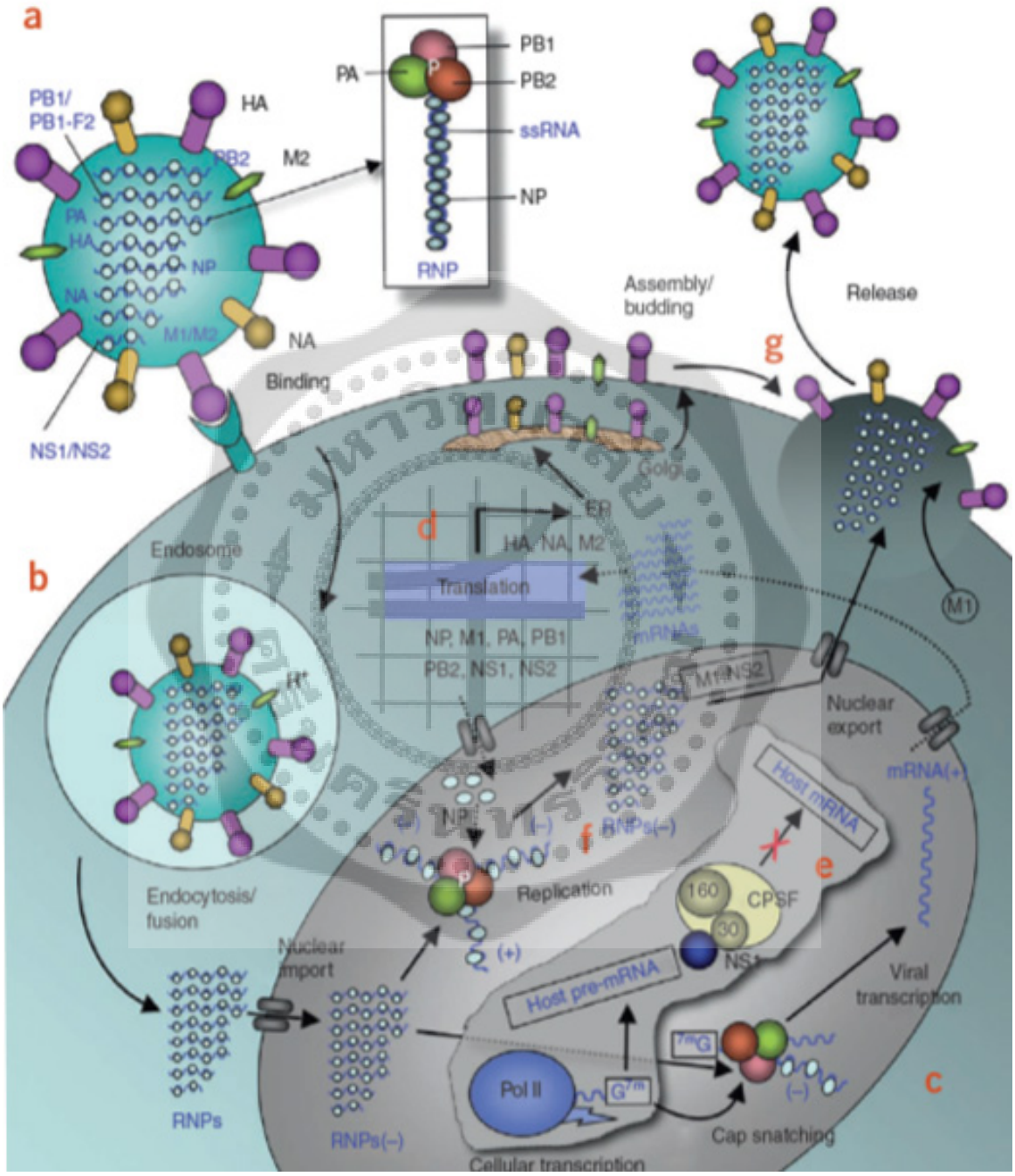

Figure 10 Life cycle of influenza A[27]

Transmission

Once an influenza virus has invaded the body and attached itself to cells lining the respiratory tract. The small particles are excreted when infected individuals cough or sneeze. An infected individual may be able to infect others prior to and during the symptomatic period. Disease spreads very quickly among the population, especially in crowded circumstances. In addition to humans, Influenza A virus can also transmit from poultry or pigs to humans and vice versa. It is appeared to occur predominantly as a result of direct contact with infected animals. Pigs and birds are believed to be particularly important reservoirs, generating pools of genetically/antigenically diverse viruses, which get transferred back to the human population via the close contact.

In a co-infected host, the segmented genome has the potential to allow re-assortment of genome segments from different strains of influenza. The re-assortment of a new strain with a mammalian strain may produce a chimeric virus that is transmissible between mammals; such mutation products may contain hemagglutinin and/or neuraminidase proteins that are unrecognizable to the immune systems of mammals. This antigenic shift consequence in a much greater population of susceptible individuals is possible and pandemic influenza might occur.

Epidemiology of Influenza A viruses

Influenza viruses have been with mankind for at least 300 years, causing epidemics every few years and pandemics every few decades. In the last century, three influenza pandemics occurred. The greatest medical disaster in history of the flu pandemic was Spanish flu in 1918–1920. The virus causing this epidemic was an H1N1 virus that was an unusually deadly influenza virus. The global mortality rate from the 1918/1919 pandemic is not known. It was estimated that one-third of the world population were infected and 3% to 6% of all encompassing populations died[49]. The second pandemic, Asian flu in 1957–1958, was the outbreak of avian influenza that originated in China in early 1956. This pandemic was relatively minor and caused by the H2N2 subtype. Ten years later, the Hong Kong Flu pandemic of 1968 and 1969 killed an estimated one million people worldwide[50]. The causative pathogenic virus in this pandemic was a strain of H3N2, which was descended from H2N2 by an antigenic shift from multiple subtype reassortment to form a new strain[51]. It was about one million people worldwide were killed in this pandemic. Since the Hong Kong flu, there were 2 relatively mild epidemics, the swine flu" scare of

1976 and the return of the Russian flu A(H1N1) in 1977. Until 1997, the starting of avian flu pandemic, H5N1 was the panzootic virus in poultry and transmits zoonotically from infected poultry to humans[42]. This virus was highly pathogenic and infected poultry across three continents, leading to huge economic losses, and transmitted to humans with lethal consequences. It is highly virulent in humans who were infected directly from chickens. Fortunately, such transmission remains inefficient in human so the number of reported H5N1 patients was relatively small [52].

Influenza A (H1N1) 2009 pandemic

The latest pandemic (March-April 2009) of Influenza A virus originated in Mexico. At first, it was called “swine flu” which was the new strain and was reported in human in the United States in April 2009. It contained many genes that were swine influenza-like in North America[53]. The virus appeared to be a new strain of H1N1, which resulted when a previous triple reassortment of bird, swine and human flu viruses further combined with a Eurasian pig flu virus. As a ‘triple reassortant’ virus from the swine ancestor, the HA, NP and NS genes of the virus were obtained from the classical swine that closely related to swine flu 1981. The M and NA genes were originated from an avian virus, which was found in the swine outbreak in 1979 and circulated since then in Eurasia. The PA, PB1 and PB2 genes were traced to triple reassortant swine viruses. This virus was a mixture of influenza viruses that had circulated in avian, human and swine for many years ago[54]. The gene lineages of Influenza A (H1N1) 2009 pandemic were shown in figure 11. This virus spread from human-to-human worldwide. Soon after, the confirmed cases of pandemic influenza were reported from more than 214 countries on 1st August 2010, and the death toll was reached to 18,449 cases[4-6]. Although the virus was highly contagious, its severity was slightly moderate. At the beginning of pandemic, it was known that the influenza A (H1N1) 2009 have the resistance marker (S31N in M2) to the amantadine and are sensitive to oseltamivir and zanamivir[55, 56].

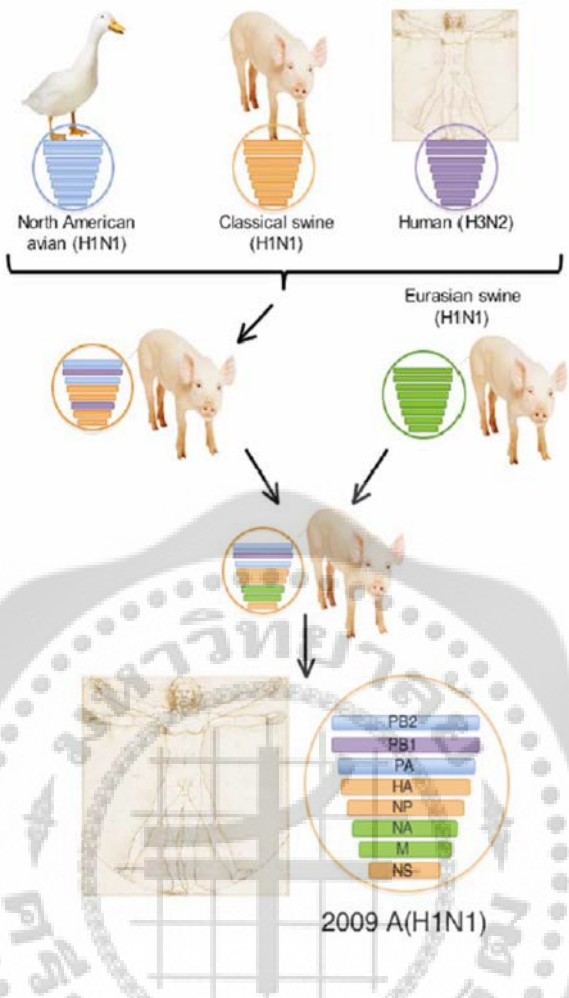

Figure 11 The lineage of the pandemic influenza A (H1N1) 2009[54]

Influenza epidemic in Thailand

In Thailand, as an endemic area of influenza, the epidemic of these viruses was recorded as the following:

In 1918, as a consequence of Spanish flu, Thai people were infected and died from pneumonia. In Bangkok, more than 500,000 people were infected and 3.5% of infected people died with pneumonia[57].

In 1957, during the pandemic of Asian flu, Influenza A/Singapore/1/57 (H2N2) was the etiologic agent. However, no death toll was recorded[58].

In 1968-1975, the epidemic of influenza A (H3N2) was occurred and influenza strain A/Hong Kong/1/68 (H3N2) was isolated in that year. More than 150,000 were infected[59]. This subtype continued epidemics of influenza nearly annually, although the rates and severity of influenza illness vary substantially from year to year.

In 1978, during Russian flu pandemic, the outbreak of H1N1 influenza in Thailand was peaked in February – March 1978. Most of the infected people were young adults[60].

During 1978-2004, both subtypes of Influenza A virus, H3N2 and H1N1 were still circulated in seasonal flu. During this period, these annual epidemics are generally due to genetically drifting strains of influenza that differ slightly from previous strains.

From 2004-2008, the annual adjusted incidence of hospitalized influenza pneumonia across all age groups was 83, 22, 42 and 66 per 100,000 persons in 2005, 2006, 2007 and 2008, respectively.

As influenza A pandemic in 2009, the first two cases of these novel strains in Thailand were identified on 12 May. After a two-week lapse, more cases were reported, building up into an exponential growth phase in early June. One month later, A(H1N1) pdm09 virus transmission was detected in all 76 Thai provinces, and 65 deaths were confirmed to be associated with the infection. Data from the Ministry of Public Health (MOPH) reported that 2 pandemic waves occurred during the initial 12-month outbreak period. The first wave began in May 2009, peaked in July, and subsided in December; the second wave began in January 2010, peaked in early February, and subsided in April. A third pandemic wave occurred during the latter part of 2010. During 2009–2010, a total of 234,050 influenza cases were reported in Thailand. Of these, 47,433 were laboratory-confirmed to be pH1N1 virus infections; 347 deaths were associated with the confirmed cases. Intensive responses to the pandemic, including pharmaceutical interventions and the other control measurements, were apparently effective in reducing pandemic influenza transmission, especially during the first wave. The reverse tendency reflected a decrease in transmission rates after the comprehensive interventions. However, the lack of sustainable interventions in the population as well as the inability to prevent further transmission of the widespread virus resulted in the pandemic's second and third wave. The last wave occurred in July 2010 and continued through the end of the pandemic period in October 2010. During 18 months of pH1N1, it was estimated that about one-half of the Thai population might have been infected with[61].

Diagnosis of Influenza virus infection

In infected patients, the symptoms alone can be diagnosed as influenza infection. Clinical findings can identify patients with influenza-like illness (ILI) but are not effective for confirming or excluding the diagnosis of influenza. The influenza-like illness (ILI) cann't identify because there are several different pathogens can produce illness with similar clinical symptoms. During recognized influenza epidemic periods, clinical diagnoses of influenza have correlated highly (>70%) with laboratory diagnosis of influenza infection. Testing for influenza may help to improve influenza diagnosis and guide antiviral treatment. A number of laboratory methods are available in the diagnosis of influenza as following:

1. Rapid Tests

The diagnostic time is rapid. It takes about 10-30 minutes. This test detects influenza A or B virus antigen directly from clinical specimens: throat swab, nasal swab, or nasal wash. The principles of these test are immunochromatography or enzyme immunoassays assay for detection[62]. On average, rapid tests generally have more than 70% sensitivity and 90% specificity for viral antigen detection[63, 64]. Most rapid tests are commercial available[44].

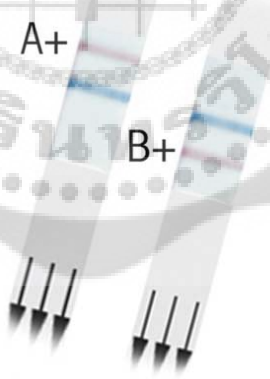

Figure12 Commercial test stripe for testing Influenza A and B antigens [65]

2. Reverse transcription polymerase chain reaction (RT-PCR)

By this method, influenza RNA is reverse transcribed into complement DNA (cDNA). A reverse transcriptase-PCR (RT-PCR) technique has three major steps. The first step is reverse transcription (RT), in which RNA is reverse transcribed to cDNA using reverse transcriptase. This step is very important in order to perform PCR since DNA polymerase can act only on DNA templates[66]. The next step involves the denaturation of the dsDNA at 95°C, so that the two strands separate and the primers can bind again at lower temperatures and begin a new chain reaction. Then, the temperature is decreased until it reaches the annealing temperature which can vary depending on the set of primers used, their concentration, the probe and its concentration (if used), and the cations concentration. The main consideration, of course, when choosing the optimal annealing temperature is the melting temperature (Tm) of the primers and probes (if used). The annealing temperature chosen for a PCR depends directly on length and composition of the primers. The final step of PCR amplification is DNA extension from the primers. This is done with thermo stable Taq DNA polymerase, usually at 72°C, the temperature at which the enzyme works optimally. The length of the incubation at each temperature, the temperature alterations, and the number of cycles are controlled by a programmable thermal cycler. The analysis of the PCR products depends on the type of PCR applied. If a conventional PCR is used, the PCR product is detected using agarose gel electrophoresis and ethidium bromide (or other nucleic acid staining)[67].

3. Real time RT-PCR

Real time RT-PCR is the amplifying method of short RNA sequence to cDNA and showed amplicon of reaction in real time. Principle of this technique, the first step is similar to RT-PCR technique. Next step, probe or fluorescence dye binds DNA sequence and then releases fluorescent light for thermal cycle detection [68].

4. Hemagglutination inhibition (HI)

Hemagglutination inhibition test is simple to perform and requires inexpensive equipment and reagents. The principle of this test is described briefly. A hemagglutinin (e.g., influenza virus) is mixed with its homologous antibody in suitable amounts and erythrocytes are then added, hemagglutination does not occur. Using this property, one can test the potency of an antiserum to a virus hemagglutinin by measuring the minimum amount of antiserum (e.g., highest dilution of antiserum) necessary to inhibit

hemagglutination completely. The procedure is as follows: serial dilutions of patient's sera are allowed to react with a fixed dose of viral hemagglutinin, followed by the addition of agglutinable erythrocytes[69]. The advantages of HI tests are relatively easy and inexpensive to perform. The disadvantage of HI is not as sensitive as enzyme immunoassay (EIA) or radioimmunoassay (RIA)[70].

Test	Sensitivity	Turnaround time	Advantages	Disadvantages
Conventional cell culture	About 100% (less than RT-PCR)	At least 4–5 days	• Highly sensitive and specific • Isolate for characterisation • Recovers novel and divergent strains • Recovers other respiratory viruses	• Dependent on specimen quality and transport • Slow turnaround time • Labour-intensive, requires technical skill • Specialised equipment (PC3 laboratory for pandemic influenza)
Rapid cell culture (shell vial with IF)	56%–100% (generally 70%–90%)	1–4 days	• Quicker turnaround time than conventional cell culture • Relatively inexpensive	• Dependent on specimen quality and transport • Less sensitive than conventional cell culture • May miss divergent strains
IF for rapid antigen detection	60%–100% (generally 70%–90%)	2–4 hours	• Rapid turnaround time • Provides assessment of specimen quality	• Labour-intensive • Interpretive skill required (subjective) • Fluorescent microscopy required • No isolate for antigenic characterisation
Nucleic acid testing (RT-PCR)	About 100% (greater than cell culture)	< 1 day	• Highly sensitive and specific • Less dependent on specimen quality and transport • Typing and subtyping possible • Molecular analysis by genome sequencing • Detects other respiratory viruses (in multiplex assays) • More rapid turnaround time with real-time PCR assays	• Expensive • Labour-intensive (depending on assay) • Technical skill and specialised equipment required • Potential for cross-contamination (false positives) • No isolate for antigenic characterisation • May miss divergent strains
Rapid antigen (point-of-care) tests	59%–93% (generally about 70%)	15–30 minutes	• Rapid turnaround time • Less technical skill required • Specimen transportation not required	• Expensive • Lower sensitivity (false negatives) • False positives (interpreting faint bands) • No isolate for antigenic characterisation
Serology (CF, HAI, IF, neutralisation, EIA)	Up to 100%	1–3 weeks	• Useful where specimens for virus detection not obtained or collected too late, or laboratory facilities limited	• Delayed diagnosis • Requires paired serum specimens • Variable sensitivity and specificity • Labour-intensive, requires technical skill

RT-PCR = reverse transcription polymerase chain reaction. PC3 = Physical Containment level 3. IF = immunofluorescence. CF = complement fixation. HAI = haemagglutination inhibition assay. EIA = enzyme immunoassay.

Figure 13 Comparison of diagnostic techniques for human influenza virus infection[65]

Antiviral agents

Vaccines and antiviral drugs are clearly efficacious in preventing infection or treating illness. In general, an immune response is stimulated by vaccines that can protect against influenza. Therefore, the vaccine containing the antigens resemble those of the circulating viruses is urgently needed. Unfortunately, in the early of the novel influenza virus pandemic, no antigen matched vaccine is available. In contrast, the antiviral drug is independent of the antigenic make-up of the circulating viruses. The drugs are of importance in the influenza management, including treatment and prophylaxis. In prophylaxis, they must be administered daily throughout the period of risk of exposure. They are necessary for pandemics when supplies of vaccines, especially in the early months, are likely to be severely limited.

Antiviral drug is considered to be a best choice in control measurement during the early months of a pandemic. The effectiveness of drugs is 70–90% as prophylaxis and period of treatment is only 1.5 days. Currently, antiviral drugs have two classes for the treatment of influenza viruses infection; blockers of M2 protein or adamantane (amantadine and rimantadine) and neuraminidase inhibitors (zanamivir and oseltamivir)[10].

M2 inhibitor

M2 inhibitor (amantadine and rimantadine) is an antiviral drug for inhibiting of M2 protein in influenza virus. It is a proton channel that functions during virus entry to acidify the interior of virus particles when they are in the endosome[26]. Amantadine (1-adamantamine hydrochloride; Symmetrel) and its analogue, rimantadine (-methyl-1-adamantanemethylamine; Flumadine), are the first class of antiviral drugs licensed for influenza infections. This drug can inhibit an early step in the influenza A virus replication cycle. It interferes with the function of the viral M2 protein (Figure 14). Both amantadine and rimantadine have been effective at low dose defense to all subtypes of influenza A viruses. However, it has no defense effect on influenza B viruses. Furthermore, amantadine and rimantadine have never been considered for therapy in a pandemic. As a consequence of M2 inhibitor treatment, drug resistant viruses may appear in about one-third of infected patients. Moreover, the resistant mutants are fully pathogenic and transmissible[71]. Comparison to the NAI resistance, when it occurred, the mutant is far to a lesser frequent than with the adamantanes, and most of the mutants might be less infectious and transmissible than wild type[72-74].

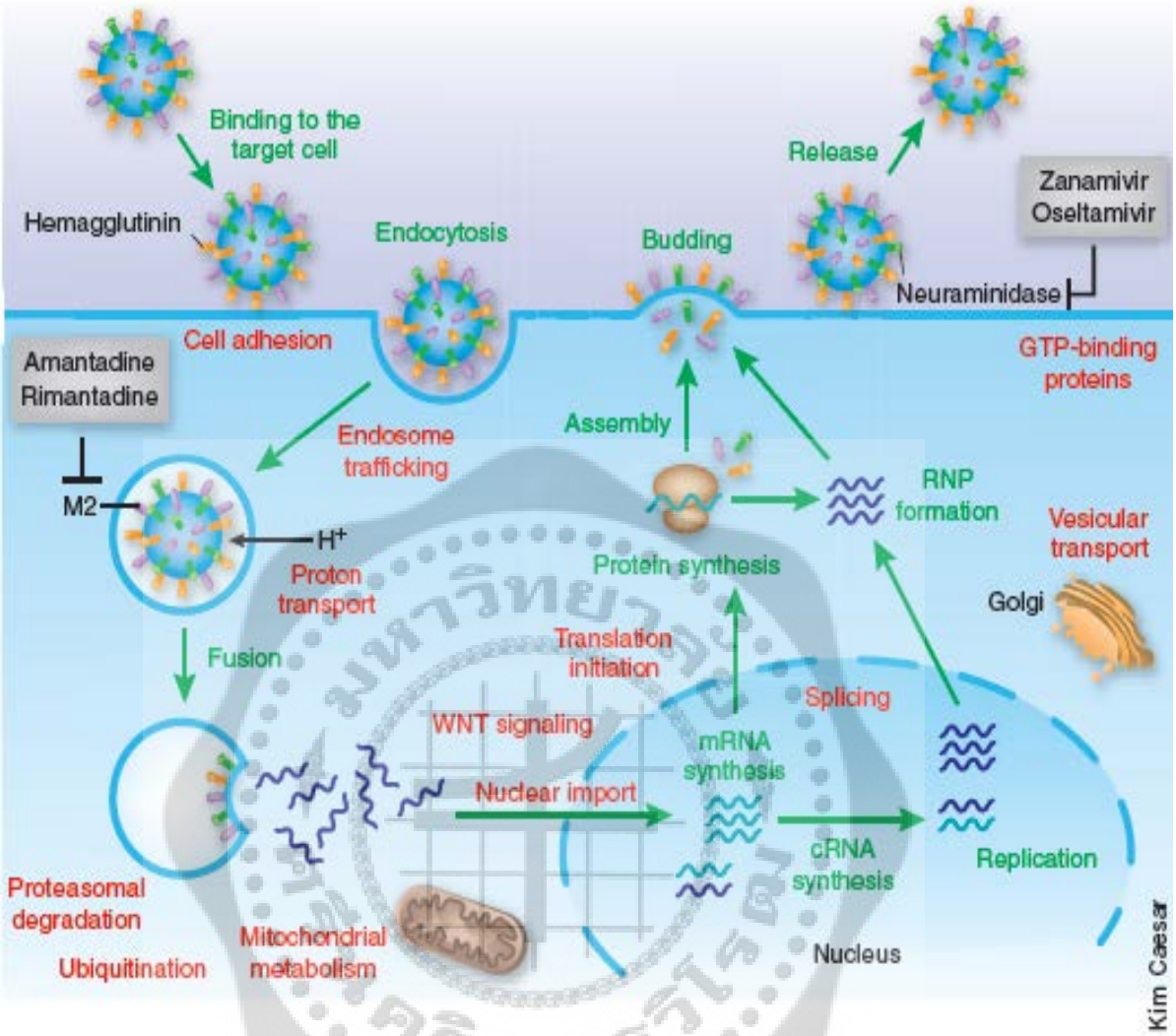

Figure 14 Mechanisms of antiviral drugs.

The replication cycle of influenza virus (attachment, internalization, replication and exit from the host respiratory cell) is illustrated. Amantadine blocks viral internalization and uncoating. Neuraminidase inhibitors prevent the neuraminidase function from releasing budding viruses and dispersing virions (adapted from Ji-Young Min and Kanta (2010) Subbarao with permission[31].

The neuraminidase inhibitor

The other class of anti-influenza drug is the neuraminidase inhibitors (NAIs). These drugs are rationally designed using the 3-dimensional structure of the influenza neuraminidase. They are sensitive to both influenza A and influenza B. Recently; only two approved NAIs were available, oseltamivir and zanamivir. Both are different in their bioavailability and structures as shown in figure 15. Base on this sense, zanamivir shows poor absorption when taking in; therefore, it was made as an inhaled product. For oseltamivir, it has been sensible to administer orally. In comparison, oseltamivir is well-known and more effective in drug administration for influenza treatment and prophylaxis.

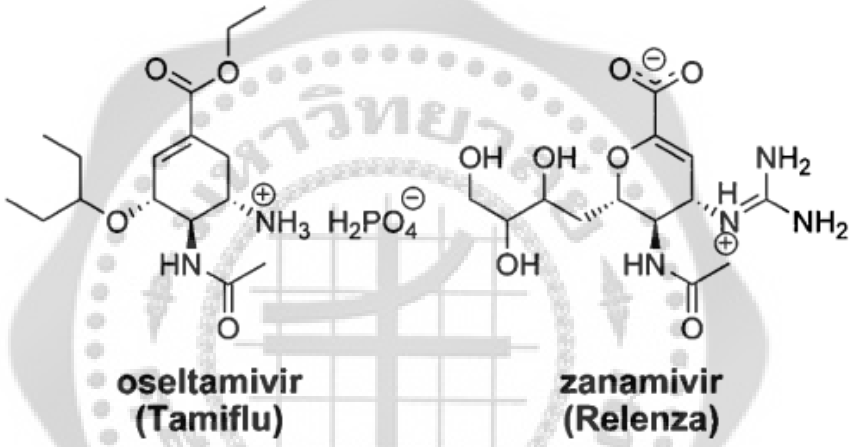

Figure 15 Neuraminidase inhibitor of influenza A virus: oseltamivir, zanamivir[75]

Oseltamivir drug

Oseltamivir is an orally administered antiviral drug for the treatment and prophylaxis of influenza A and B infections. In uncomplicated acute influenza infection, it is generally well tolerated in adults and children aged over 1 year. Oseltamivir is an ethyl ester prodrug requiring ester hydrolysis in liver where it converses to its active form, oseltamivir carboxylate (OC). The half-life of OC is about 6—10 h[76] and is not dose-dependent kinetics[77]. Currently, oseltamivir is an important class of antiviral drugs available for the treatment of seasonal and pandemic influenza. In Thailand, oseltamivir treatment using in the national hospital is free of charge; therefore, it is massive used in each influenza epidemic[61].

Neuraminidase and Neuraminidase inhibitor interaction

The active site of neuraminidase enzyme is the highly conserved region which the tetramer contains several invariant residues[78]. Two parts of NA are concerned with this view. The first part is an enzyme active site which interacted with the substrate. It consists of the amino acids in the position; R118, D151, R152, R224, E276, R292, R371 and Y406 as shown in Figure 16. The second part is framework residues, which is essential for the structure and stabilization of the active site. This part comprises of E119, R156, W178, S179, D198, I222, E227, H274, E277, N294 [79-81]. These are conserved residues, including three arginines at positions 118, 292 and 371 which interact with the carboxylate of the sugar substrate. NAIs binding to this active site can interrupt the virus replication cycle[82]. Although these positions are critical and highly conserved, the mutation viruses in the active site or framework region of NA were recently isolated after neuraminidase inhibitors' treatment. Mutations in these regions confer various levels of neuraminidase inhibitors' resistance[83].

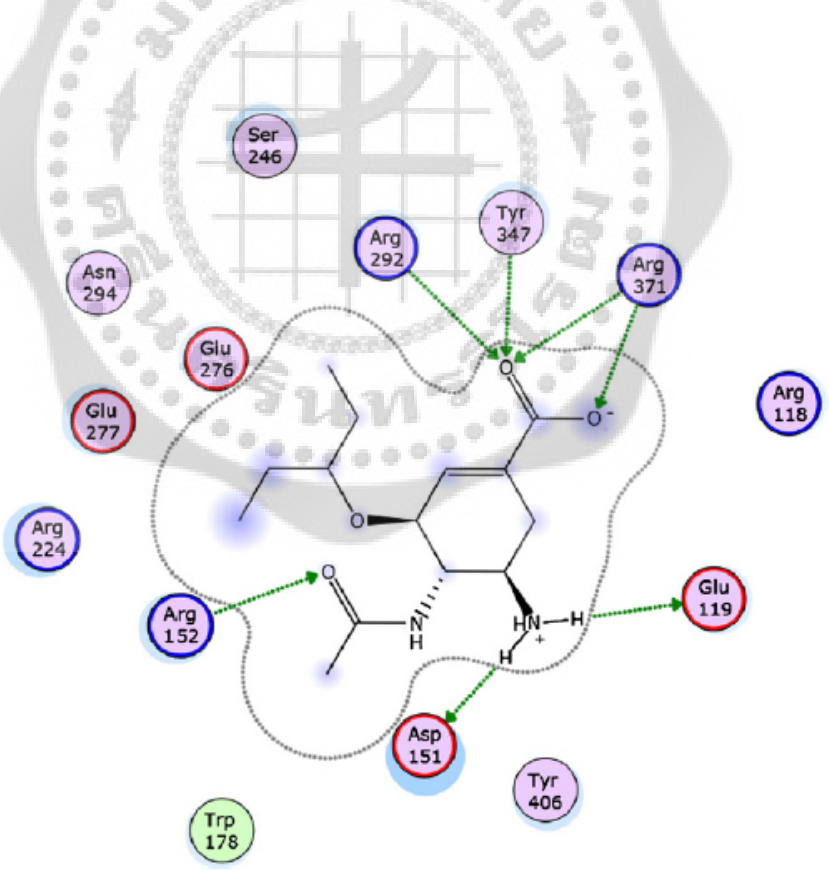

Figure 16 The active site of N1 neuraminidase interact with oseltamivir[75]

Neuraminidase inhibitor resistance

Although administration of NA inhibitors may significantly reduce influenza virus transmission, but the risk in the emergence of drug-resistant variants is unavoidable. While on therapy, the development of oseltamivir resistant virus might occur in infected patients, including immunocompromised individuals, depending on age, treatment regimens, and other differences[84]. These resistant variants characterized to date have contained precise mutations in the NA of specific subtypes. Despite the highly conserved of amino acids in the active site of neuraminidase enzyme, the mutations have been identified at both catalytic and framework residues. The location of the NA mutation is reportedly associated with the degree of NAI resistance [85].

The mutations at definite conserved residues of NA may lead to different degrees of NA functional loss. Mutation of the catalytic residue (R292K) resulted in greater loss of NA activity and thermo-stability than mutation of the framework residue (E119V). The changes in NA functional activity caused by the R292K and E119V mutations should both disrupt the HA-NA balance. Although both mutations decreased the NA activity, one observation reported that only the R292K mutation significantly impaired the HA-NA balance[86]. The amino acid changing at position R292K may result in complete resistance. The mutation of R to K is linked to a single nucleotide exchange of AGA to AAA in the NA gene. Position 292 is highly significant because mutation may induce resistance not only against the substance oseltamivir, but also against zanamivir. As previously reported, by NA inhibition assay, the virus with the R292K mutation was resistant to zanamivir (13-fold) and oseltamivir carboxylate (>30,000-fold)[87]. However, it is still not clear whether the R292K-NA mutation changed the enzymatic activity and/or substrate specificity of NA. The other possibility is that the NA expression level varies with different mutations. Furthermore, influenza virus carrying an R292K mutation in the neuraminidase gene is not transmitted in ferrets[73]. In general, mutations within the enzymatic site that confer resistance to NA inhibitors would also reduce NA enzyme activity and thereby compromise viral fitness[88]. On the other hand, the mutations at framework residues (H274Y, E119G/A/D) appear to confer resistance to only zanamivir or oseltamivir carboxylate[85]. In addition, the other in framework residues E119V or E227D mutation also confers resistance to oseltamivir carboxylate, but the mechanism of resistance is not clear.

Oseltamivir resistant virus

The common mutations which are arising from oseltamivir selection pressure are subtype specific[89]. For N1 subtype, mutation at the position H275Y (using N1 numbering) is predominate whereas the R292K is the most frequent detection in influenza virus of the N2 subtype[90, 91]. Regarding to the transmission of resistant virus, the transmissibility of the H275Y variant was comparable to that of the circulating oseltamivir-sensitive strains[92, 93]. Focus on the NA activity, it was indicated that the H275Y mutation conferring oseltamivir resistance can lead to NA functional loss in pH1N1 and seasonal H1N1 viruses. Despite decreasing in NA activity, viruses carrying the H275Y mutation could transmit to direct contact and respiratory droplet contact ferrets[94]. The other paper described that H275Y mutation not only reduces in the enzymatic activity of NA, but also forces a parallel mutation in the receptor binding domain of the HA to retain a balance of activity[95]. Besides these substitution mutations, massive deletions of 270 amino acids, including most residues that constitute the active center of the NA enzyme, have been identified in NAI-resistant influenza A/H1N1 variants. Most of these mutants were obtained after in vitro passages under NAI pressure and in a volunteer treated with peramivir. A number of published data summarized that these NAI resistant mutants exhibited compromised viral fitness and virulence[73, 83, 96-98].

At present, oseltamivir-resistant A/H1N1 viruses are detected worldwide. So far, these emerging viruses are relative efficiency. However, the biologic fitness and transmissibility are modified in the different host species. Moreover, the neuraminidase (NA) mutation responsible for the NAI-resistance phenotype was found to vary according to the drug and the NA subtype. With the great concern in resistant virus epidemic, it is emphasized that the surveillance of novel resistant strain is needed in each geographic region.

CHAPTER III

Materials and methods

Materials and Instruments

Materials

1. QIAmp® Viral RNA Kit (Qiagen, Valencia, USA)
2. SuperScript. III Platinum® One-Step Quantitative RT-PCR (Invitrogen, California, USA)
3. Subtype H1 of Influenza A real time RT-PCR Kit (Shanghai ZJ Bio-Tech, Shanhai,China)
4. Subtype H3 of Influenza A real time RT-PCR Kit (Shanghai ZJ Bio-Tech, Shanhai, China)
5. One step RT-PCR SensiFAST™ One-Step RT-PCR Kit (Bioline, USA)
6. SuperScript III Synthesis System for RT-PCR (Invitrogen,California, USA)
7. Taq DNA Polymerase, recombinant (Invitrogen, California, USA)
8. Gel/PCR fragments extraction kit (real-biotage, USA)
9. BigDye® Terminator v1.1 Cycle Sequencing Kit (ABI, USA)

Instruments

1. LightCycler® 2.0 (Roche, USA)
2. Thermal cycler (eppendoff, Germany)
3. Swift Maxpro™ Thermal cycler (Ehco, USA)
4. PyroMark ID (Biotage headquarters, Sweden)
5. Maestro Nano® spectrophotometry (Maestrogen, USA)
6. ABI PRISM®3130 DNA sequencer (ABI, USA)
7. Thermo® Multi RF(Thermo, USA)

Methods

1. Clinical specimens, positive and negative controls

Clinical specimens

During July 2010 – October 2010, nasal or nasopharyngeal swabs were collected in sterile normal saline from 85 patients who had influenza like illness (ILI) at HRH Princess Mahachakri-Sirindhorn Medical Center (MSMC). Samples were screened for influenza antigen by immune-chromatography test kit. All positive samples were further confirmed by real time RT-PCR.

Positive control viruses

Clinical isolate which was confirmed by National Institutes of Health (NIH) Thailand for pandemic influenza A H1N1 (2009) was used as a positive control for real time RT-PCR assay. The positive controls were kindly gifts from Prof. Dr. Ruengpung Sutthent, Department Microbiology, Faculty of Medicine Siriraj Hospital.

2. Pandemic influenza H1N1 detection

2.1 Primer and probe designation

Primers and probes using in real time RT-PCR were followed CDC protocol of real time RT-PCR for influenza A (H1N1)[99]. Primers for amplification and sequencing of pandemic influenza H1N1 (pH1N1) by pyrosequencing assay were followed CDC protocol. Primers for the direct sequencing whole NA genome was used according to Graham et al[100]. All primers and probes using in this study were shown in table 2.

Table 2 Oligonucleotide probes, primers, complementary sequence of probes and primer used in this study.

Virus	Gene target	primer/probe name	Sequence (5'-3')	Position	Amplicon length	Reference
Primer for Real-time RT-PCR						
Influenza A	H1	SW H1 Forward	GTG CTA TAA ACA CCA GCC TYC CA	915-937[a]	115	(99)
Influenza A	H1	SW H1 Reverse	CGG GAT ATT CCT TAA TCC TGT RGC	1007-1030[a]		(99)
Primer of one step RT-PCR assay for pyrosequencing						
Influenza A	N1	Uni-sw-B-F780	GGGGAA GAT TGT YAA ATC AGT YGA	780-804[b]	516	(99)
Influenza A	N1	Uni-sw-B-R1273	CWA CCCA GAA RCA AGG YCT TAT G	1273-1296[b]		(99)
Primer of amplification of HA and NA for direct sequencing						
Influenza A	H1	swHAf1	ATGAAGGCAATACTAGTAGT	14-33[a]	1700	(100)
Influenza A	H1	swHAr1	TTAAATACATATTCTACACT	1695-1714[a]		(100)
Influenza A	N1	swNAf1	ATGAATCCAAACCAAAAGAT	3-22[b]	1409	(100)
Influenza A	N1	swNAr1	TTACTTGTCAATGGTAAATG	1393-1412[b]		(100)
Probe for Real-time RT-PCR						
Influenza A	H1	SW H1 Probe	CA GAA TAT ACA "T"CC RGT CAC AAT TGGARA A[c]	941-970[a]		(99)
Primer for Swine-H1N1 NA-H274 detailed pyrosequencing						
Influenza A	N1	Uni-sw-B-F804	GYT GAA TGC MCC TAA TT	804-821[b]		(99)
Primer for direct sequencing (ABI 3130)						
Influenza A	N1	swNAf1	ATGAATCCAAACCAAAAGAT	3-22[b]		(100)
Influenza A	N1	swNAr2	TATCCAAACACCATTGCCGT	1060-1079[b]		(100)
Influenza A	N1	swNAr1	TTACTTGTCAATGGTAAATG	1393-1412[b]		(100)

[a]From HA gene; A/Bangkok/INS427/2010(H1N1) GenBank accession number CY071351

[b]From NA gene; A/Bangkok/INS427/2010(H1N1) GenBank accession number CY071353

[c]Taqman® probe are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and quenched internally at a modified residue with BHQ1 with a modified 3'-end to prevent probe extension by Taq polymerase

2.2 RNA preparation

The supernatant from Influenza A positive sample was extracted by QIAmp® Viral RNA Kit (Qiagen, Valencia, USA) which followed the manufacturer's instructions. Briefly, 140 µl of the supernatant was added to 560 µl of AVL buffer, which contained 5.6 µl of carrier RNA solution, and mixed thoroughly by pulse-vortex for 15 seconds. The suspension was incubated at room temperature for 10 minutes. Then 560 µl of 95% ethanol to the solution was added and vortex for 15 seconds. The 630 µl of the solution was transferred to QIAamp Mini spin column and centrifuged at 8,000 rpm for 1 minute.

The supernatant from Influenza A virus positive sample was extracted by QIAmp® Viral RNA Kit (Qiagen, Valencia, USA) which followed the manufacturer's instructions. Briefly, 140 µl of the supernatant was added to 560 µl of AVL buffer, which contained 5.6 µl of carrier RNA solution, was mixed thoroughly by pulse-vortex for 15 seconds and incubated at room temperature for 10 minutes. The solution of 95% ethanol 560 µl was added and vortex for 15 seconds. All 630 µl of the solution was transferred to QIAamp Mini spin column and centrifuged at 8,000 rpm for 1 minute. The supernatant in a collection tube was discarded. The spin column was washed with 500 µl buffer AW1 and centrifuged at 8,000 rpm for 1 minute. The supernatant in a collection tube was discarded. The spin column was washed with 500 µl buffer AW2 and centrifuged at 8,000 rpm for 1 minute. The supernatant in a collection tube and centrifuged at 13,000 rpm for 3 min was discarded. A new 2 ml micro-centrifuge tube was placed into the column and centrifugation at 13,000 rpm for 1 minute. The 1.5 ml micro-centrifuge tube was placed to QIAamp Mini spin column. RNA was eluted by adding 60 µl of AVE buffer and equilibrated at room temperature for 1 minute, and then centrifuged at 8,000 rpm for 1 minute. The extracted RNA was used for real-time PCR detection immediately; otherwise they were kept at -80°C for long storage.

2.3 One-step real-time RT-PCR assay

Real-time RT-PCR was performed by using a Taqman® probe system. Reaction mixtures were prepared by using SuperScript. III Platinum® One-Step Quantitative RT-PCR (Invitrogen, California, USA) according to the manufacturer's instructions. Real-time PCR master mix contained 12.5 µl of 2X One-step real-time, 0.5 µl of 10 µM SWH1 Taqman® probe, 0.5 µl of 40 µM SWH1-F primer, 0.5 µl of 40 µM SWH1-R primer, 0.5 µl of 5 U/µl SuperScript. III Platinum® One-Step Quantitative RT-PCR (Invitrogen, California, USA), 5.5 µl of Nuclease free water, and 5 µl of RNA template. The real-time PCR cycles were

performed as following 30 minutes at 50°C, 2 minutes at 95°C. The cycles were consisted of 45 cycles of 15 seconds at 95°C and 30 seconds at 55°C by using LightCycler® 2.0 (Roche, USA). The fluorescent signals of LightCycler® were collected during annealing and extension steps (55°C) and amplification data were analyzed by the LightCycler® 4.5 software according to the manufacturer's instructions.

For the influenza H3N2 and H1N1 seasonal flu subtype were performed by using Influenza H3 real time RT-PCR® One-Step Quantitative Kit (Shanghai ZJ Bio-Tech, Shanhai, China) Reaction mixes were prepared by adding 18 µl of real-time PCR master mix, 1 µl of enzyme mix,1 µl of Nuclease free water and 5 µl of RNA template. The real-time PCR cycles were performed as following 10 minutes at 45°C, 15 minutes at 95°C. The cycles were consisted of 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C by using LightCycler® 2.0 (Roche, USA). The results were analyzed by using Ct value in channel FAM. The positive result would show Ct value ≤ 38.

3. Oseltamivir-resistant identification

3.1 One step RT-PCR assay for pyrosequencing

Neuraminidase gene of influenza was amplified by using one step RT-PCR SensiFAST™ One-Step RT-PCR Kit (Bioline, USA). Briefly, amplification reaction mixtures were prepared by adding 25 µl of 2x SensiFAST One-Step Mix, 2 µl of 10 µM Uni-sw-B-F780, 2 µl of 10 µM Uni-sw-B-R1273, 0.5 µl of Reverse transcriptase (5 U/µl), 1 µl of RiboSafe RNase Inhibitor, 17.5 µl of Nuclease free water and 2 µl of sample RNA to 0.2 ml thin-walled tube. PCR cycle was performed as following; 20 minutes at 45°C, 1 minutes at 95°C for one cycle and followed by 45 cycles of 15 seconds at 95°C, 45 seconds at 55°C, 45 seconds at 72°C with a final extension 5 minutes at 72°C by using Swift Maxpro™ Thermal cycler (Ehco, USA). After one-step RT-PCR reaction, products were checked by gel electrophoresis. Expected band was 516 base-pairs under UV light.

3.2 Pyrosequencing

Pyrosequencing was performed on PyroMark Q96 ID system (Biotage headquarters, Sweden) with WHO-published sequencing primer and Golden Reagent kit (Qiagen, Valencia, USA) with 10 nucleotide-dispensing cycles. Pyrosequencing reaction mix1 was prepared by adding 1.6 µl of 10 µM Uni-sw-B-F804 sequencing primer, 38.4 µl of 1X annealing buffer, mixed thoroughly and aliquot 40 µl of reaction mix 2 to each well of 96

well PSQ plate. Pyrosequencing reaction mix 2 was prepared by adding 20 µl of PCR product, 3 µl of streptavidin sepharose HP bead, 40 µl of Binding buffer, 17 µl of nuclease free water. Reaction mix 2 was mixed by the shaker at 1,400 rpm for at least 10 minutes. After that, reaction mix 2 was washed with 70% ethanol for 5 seconds by using vacuum prep tool and followed by denature buffer for 5 seconds and shifting 3 times, placed in wash buffer for 10 seconds. The vacuum pressure was turned off and carefully shaking Vacuum Prep Tool on 96 well PSQ plate in order to release beads. PSQ plate was placed on hot plate at 80°C for 2 minutes and room temperature for 10 minutes.

The PyroMark Gold Q96 Reagents (Biotage headquarters, Sweden) were placed at room temperature before processing. The enzyme and substrate in PyroMark Gold Q96 Reagents were dissolved by 620 µl of Milli-Q 18.2 MΩ water. After that, PyroMark Q96 Instrument and computer were turned on. The automatic Pyromark® ID program would calculate volume of enzyme, substrate, dATP, dGTP, dCTP, dTTP (PyroMark Gold Q96 Reagents) for each run. All dNTP, enzyme and substrate were added according to template. The PyroMark Q96 Cartridge and PSQ plate was placed in Pyromark ID. The machine cover was closed and pressed start button at pyrosequencer software. Data analysis was followed manufacturer's instructions of the PyroMark Q96 analysis software user guide.

4. Analysis of the relationship between the NA and HA gene for Oseltamivir drug resistance cases

4.1 cDNA synthesis for Sanger sequencing

cDNA was synthesized by using SuperScript III Synthesis System for RT-PCR (Invitrogen, California, USA). The reaction mixtures 1 (1 reaction) were prepared by adding 1 µl of 10 µM uni12, 1 µl of 10 mM dNTP Mix, 5 µl of RNA and was adjusted to 13 µl by distilled water. The reaction mixtures were incubated at 65°C for 5 minutes and incubated on ice for at least 1 minute. Reaction mixtures 2 were prepared by adding 4 µl of 5X First-Strand Buffer, 1 µl of 0.1 M DTT, 1 µl RNaseOUT™ Recombinant RNase Inhibitor, 1 µl of SuperScript™ III RT (Invitrogen, California, USA). Added 9 µl of reaction mixture 2 to mixtures 1. Then, mixed by pipetting gently up and down and incubated tube followed; at 50°C for 50 minutes, and inactivated the reaction by heating at 70°C for 15 minutes, hold at 4°C. In some cases, amplification of PCR targets (those >1 kb) may require the removal of RNA complementary to the cDNA. In this study, removing of RNA complementary was

performed by using one µl (2 units) of *E. coli* RNase H added in the mixture and subsequently incubated at 37°C for 20 minutes.

4.2 Amplification of NA gene for direct sequencing

The PCR reaction (for 1 reaction) was prepared by adding 5 µl of 10X PCR Buffer, 1.5 µl of 50 mM $MgCl_2$ 1µl of 10 mM dNTP Mix, 1µl of swHAf1/swNAf1 primer (10 µM), 0.5 µl of *Taq* DNA polymerase (5 U/µl), 2 µl of cDNA and was adjusted to 50 µl by distilled water. The cycle PCR reaction was performed as following:3 minutes at 94°C followed by 35 cycles of 45 seconds at 94°C, 1 minutes at 45°C, 2 minutes at 72°C, final extension 7 minutes at 72°C and hold at 4°C. by using Swift Maxpro™ Thermal cycler (Ehco, USA). Products were checked by gel electrophoresis. Gel was stained for 15 minutes with Gel red™ (Biotium, USA). Expected band was 1,409 bp for NA gene under UV light.

4.3 Direct sequencing

PCR products were purified by Real Genomic Inc. HiYield™ Gel/PCR fragments extraction kit (real-biotage, USA) that used for direct nucleotide sequencing. Briefly, added 5 volume of DF buffer for 1 volume of PCR product and then applied the PCR product to DF column. Centrifuged at 13,000 rpm for 30 seconds and discarded supernatant. Added 600 µl of wash buffer (ethanol add) in column, centrifuged at 13,000 rpm for 30 seconds, discarded supernatant and washed 600 µl of 70% ethanol, centrifuged at 13,000 rpm for 30 seconds. The DF column was centrifuged at 13,000 rpm for 2 minutes and 20-50 µl of Elution buffer was applied to the center of column, incubated for 2 minutes at room temperature. Column was centrifuged at 13,000 rpm for 2 minutes. The purified PCR product was measured by spectrophotometer (Maestrogen, USA). The concentration of purified PCR product should be >20 ng/µl and A260/A280 ratio should be 1.7 to 1.9. The sequencing reactions were performed by using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA). The mixture contains 4 µl of sequencing reaction mix, 2 µl of BigDye sequencing buffer, 4 µl of 0.8 pmol sequencing primer, 6.8 µl of 25 ng/µl PCR product and added water to 20 µl. The cycle sequencing reaction was performed as following:1 minutes at 96°C for one cycle and followed by 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C with final cycle 4 minutes at 60°C and then soaks at 4°C by using Swift Maxpro™ Thermal cycler (Ehco, USA). The sequencing product was precipitated with 75% Isopropanol, Briefly, 60 µl Isopropanol and 20 µl of DNase-RNase free water were mixed and added to 20 µl of sequencing product, vortex and kept in dark

for 15 minutes. After incubation, centrifuge (Thermo® Multi RF, USA) at 2000 RCF at least 45 minutes. The plate was turned over tissue paper and centrifuged at 700 RCF for 1 minute to get rid a left over solution, the plate was kept at -20 °C until used. The dried pellet's product was resuspended in 10 µl of Hidiformamide and transferred to Genetic Analyzer.

5. Data analysis

The NA sequences from Genbank (influenza sequence database) and clinical sample No. 14, in this study were edited and assembled by DNA Baser V. 4 software (Heracle BioSoft S.R.L., Romania). The 16 sequences of NA genes were selected and aligned by using clustralW function in MEGA program version 5.2.2 software. All reference data of sequences belong to N1 subtype which were obtained from the influenza sequence database (Influenza Virus Resource: http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU. html at the same time as shown in the following;

1. CY138642.1A/American black duck/New Brunswick/00321/2010(H1N1),
2. HM138502.1 (A/California/07/2009(H1N1)),
3. CY071353.1(A/Bangkok/INS427/2010(H1N1)),
4. CY096292.1(A/Bangkok/INS477/2010(H1N1)),
5. CY096364.1(A/Bangkok/INS491/2010(H1N1)),
6. CY096428.1(A/Bangkok/INS500/2010(H1N1)),
7. CY058837.1(A/Guangdong/2361/2009(H1N1)),
8. HM145748.1(A/Haishu/SWL110/2010(H1N1)),
9. CY096124.1(A/KhonKaen/INS451/2010(H1N1)),
10. CY055213.1(A/Malaysia/5112/2009(H1N1)),
11. CY057074.1(A/Mexico/InDRE797/2010(H1N1)),
12. CY039988.1(A/Nonthaburi/102/2009(H1N1)),
13. CY082966.2 (A/Thailand/ CUH2358/ 2010(H1N1)),
14. NA resistance (clinical sample No. 14)

The phylogenetic tree analysis of nucleotide substitution was constructed Neighbor-Joining method with Kimura's two-parameter model and 1000 bootstrap replicates, Transitions + Transversions, Uniform rates by using MEGA program version 5.2.2. The

percent identity and diversity distance of nucleotide was analyzed by DNAStar program (DNASTAR, USA).

The amino acid of NA clinical sample No. 14 were translated by using ExPASy-Translate tool website (http://web.expasy.org/translate/). The alignment of amino acid in 15 sequences of NA genes and clinical sample No. 14 were aligned by using ClustalW function in MEGA program version 5.2.2 software.

Chapter IV

Results

4.1 pH1N1 positive cases by rapid test

In this study, a total of 85 clinical samples which were collected during the peak of epidemic from July 2010 to October 2010, showed positive for Influenza A antigen by immuno-chromatography (rapid test). As shown in table 3, the range of median age was between 1-10 years and the incidence of influenza infection was raised to peak in September 2010. The demographic data of these positive cases were grouped by sex, age and month.

Table 3 The demographic data of Influenza A positive cases were grouped by sex, age and month

Date	Sex			Age							
	Male	Female	Total	<1	1-10	11-20	21-30	31-40	41-50	>51	Unknown data
July	0	1	1		1						
August	2	1	3		2		1				
September	21	33	54	2	24	7	6	8	3	4	
October	16	11	27	0	16	3	1	0	5	0	2

4.2 Confirmed pH1N1 influenza positive cases

Since the sensitivity and specificity of rapid tests are limited, all positive samples were further confirmed in the existing of Influenza A antigen by real-time RT-PCR. By this technique, the presence of influenza A had been confirmed and the subtype of influenza A virus had been identified simultaneously as shown in figure 17-19.

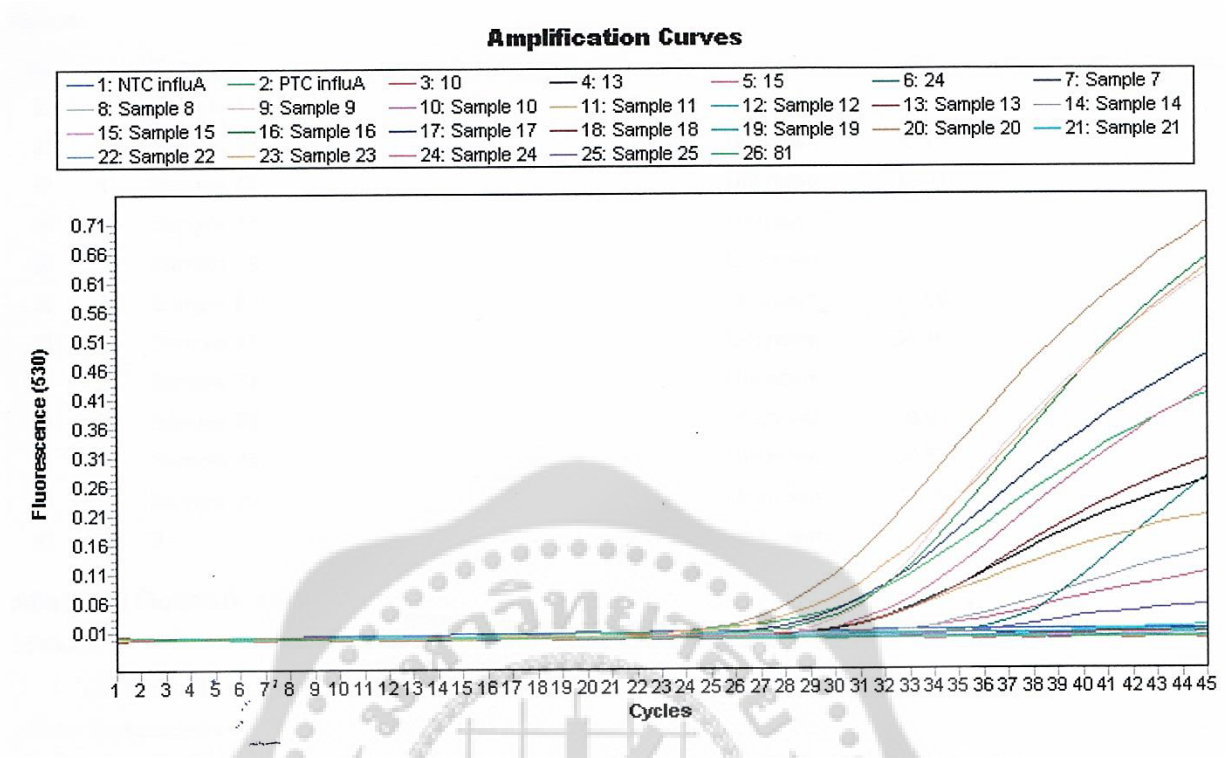

Figure 17 Amplification curves of pandemic influenza A (pHIN1) by real-time RT-PCR assay

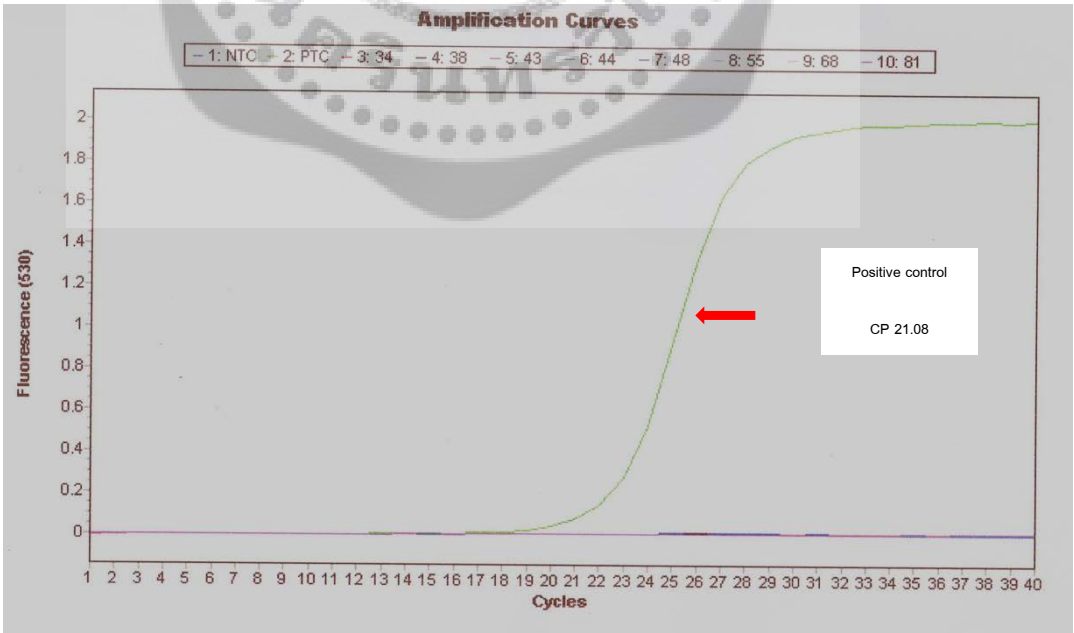

Figure 18 Amplification curves of pandemic H1 subtype by real-time RT-PCR assay

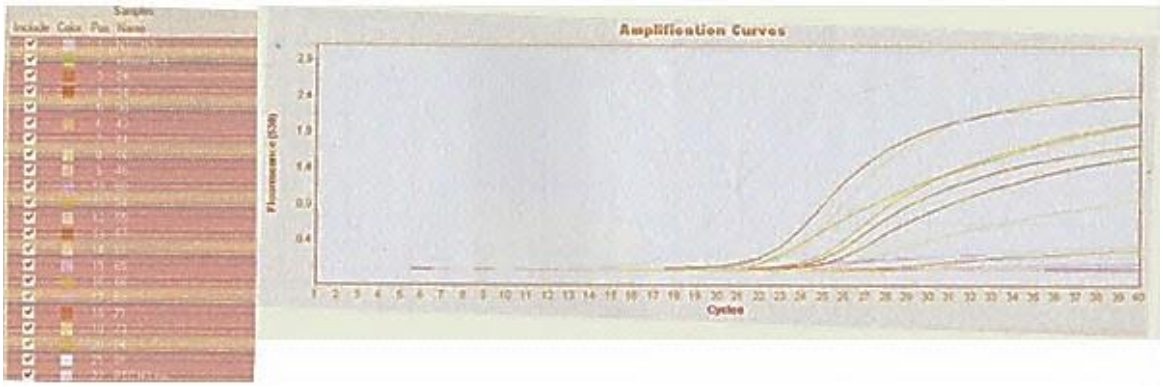

Figure 19 Amplification curves of seasonal H1 subtype by real-time RT-PCR assay

In concordance with rapid test, all of the positive samples showed positive by real-time RT-PCR. For influenza A virus subtyping, the percentage of pH1N1, seasonal H1N1, and H3 were 72.94% 11.76% and 15.29%, respectively as categorized in table 4.

Table 4 The numbers of Influenza A positive samples in various subtypes during the pandemic of pH1N1

One-step real-time RT-PCR (pH1N1)		
Positive (pH1N1)	Positive H3 subtype	Positive seasonal H1 subtype
62/85	13/85	10/85
(72.94%)	(15.29%)	(11.76%)

According to the data shown in table 4, the majority of influenza A viruses were pH1N1. In order to investigate the pattern of epidemiology, the prevalence of Influenza virus subtypes was summarized in each month during the outbreak. As shown in figure 20, the prevalence of pH1N1 was peaked in September 2010. However, the seasonal Influenza viruses H3N2 and H1N1 were co-circulated in the community at the same time.

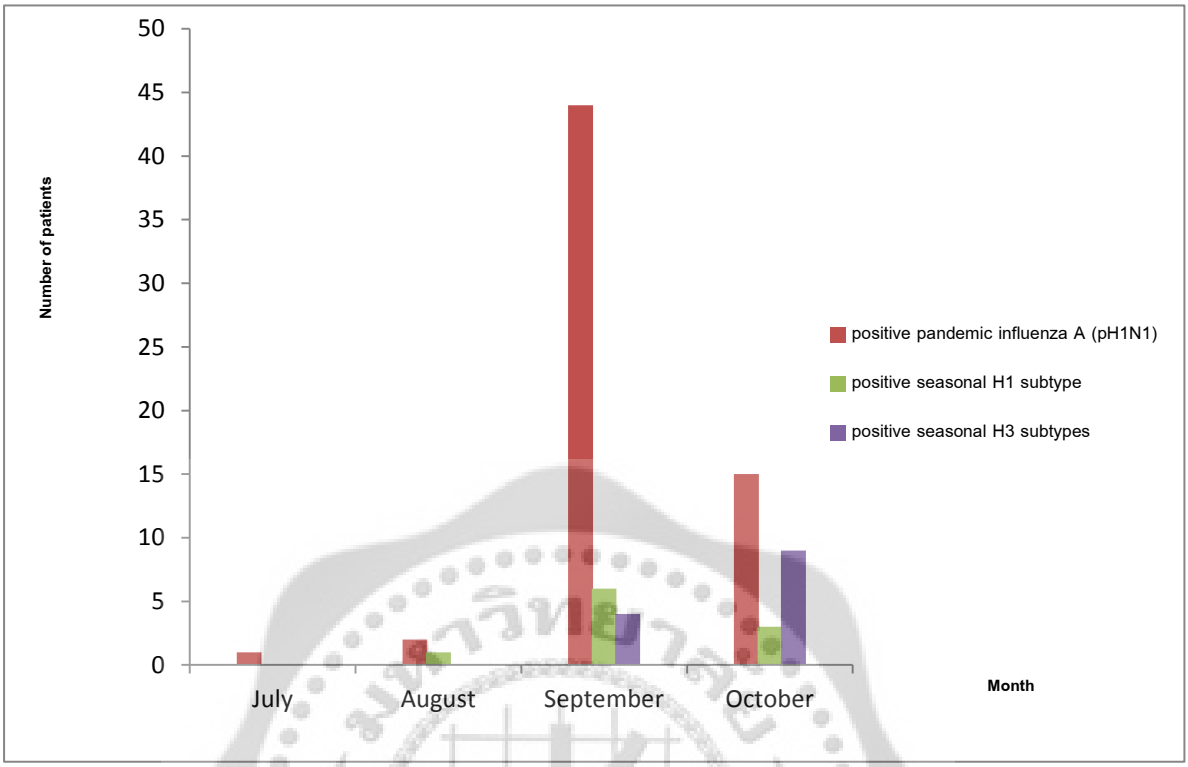

Figure 20 Influenza A subtype pH1N1 is predominated during the outbreak and reach the peak in September 2010.

4.3 Identification of oseltamivir-resistant virus (mutation H275Y)

4.3.1 One step RT-PCR assay for pyrosequencing

During the pandemic, the large-scale used of oseltamivir was essential. Therefore, the consequence of oseltamivir-resistance is inevitable. In this regards, the total 62 pH1N1 positive by real time RT-PCR and positive control viruses were investigated for the H275Y neuraminidase mutation. Positive control viruses and RNA samples from 62 pH1N1 positive samples were examined by One step RT-PCR SensiFAST™ One-Step RT-PCR kit (Bioline, USA). This assay was amplified by using 1 pair of primer, Uni-sw-B-F780 and Uni-sw-B-R1273. The amplification product of 516 bp DNA in 1% agarose gel electrophoresis was proven as seen in figure 21. The total of 62 clinical samples could be amplified and used for H275Y mutation detection by pyrosequencing assay.

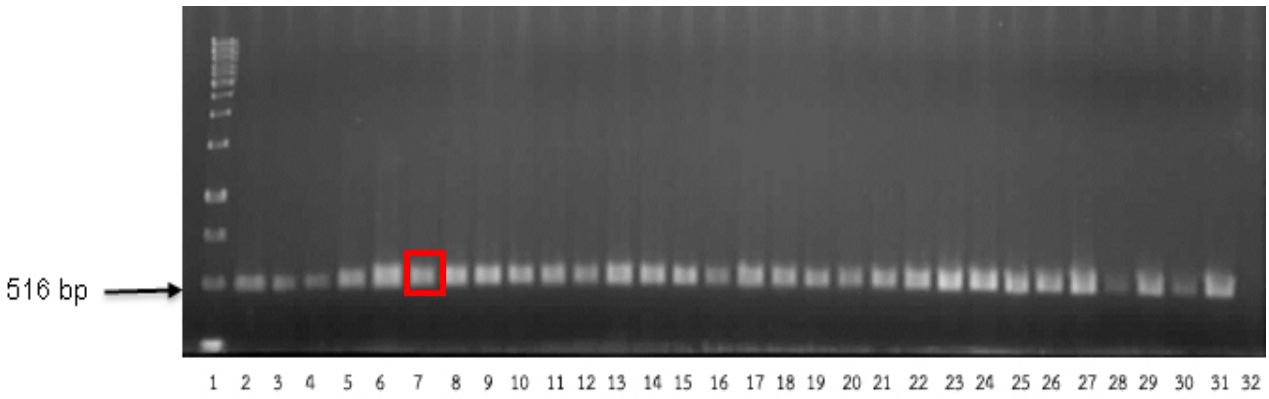

Figure 21 Agarose gel electrophoresis of one-step RT-PCR

Lane 1 1 kb DNA ladder (fermentus™)
Lane 2 RNA number 4
Lane 3 RNA number 5
Lane 4 RNA number 8
Lane 5 RNA number 12
Lane 6 RNA number 29
Lane 7 RNA number 14 (oseltamivir resistant sample)
Lane 8 RNA number 16
Lane 9 RNA number 17
Lane 10 RNA number 18
Lane 11 RNA number 20
Lane 12 RNA number 25
Lane 13 RNA number 27
Lane 14 RNA number 31
Lane 15 RNA number 32
Lane 16 RNA number 33
Lane 17 RNA number 36
Lane 18 RNA number 37
Lane 19 RNA number 39
Lane 20 RNA number 41
Lane 21 RNA number 45
Lane 22 RNA number 49
Lane 23 RNA number 52
Lane 24 RNA number 64
Lane 25 RNA number 72
Lane 26 RNA number 78
Lane 27 RNA number 79
Lane 28 RNA number 82
Lane 29 RNA number 83
Lane 30 RNA number 86
Lane 31 RNA number 87
Lane 32 RNA number 16 (Repeated sample)

4.3.2 Identification for oseltamivir-resistant strain at the position H275Y by pyrosequencing assay

The PCR products, as earlier described, were sequenced to identify the NA mutations at position H275Y, which confers resistance to oseltamivir. By using pyrosequencing assay, the 516 base-pairs of NA genes were sequenced. The nucleotide sequence at position 825 was mutated from C to T. Therefore, at the position 275 of NA gene amino acid sequence was substituted of tyrosine (encoded by TAC) for histidine (encoded by CAC). This mutation which is the marker of oseltamivir resistance was found in only one sample of 62 pH1N1 positive samples (1.61%) as shown in figure 22. In this study, the oseltamivir-resistant sample belonged to an immuno-compromised patient who was diagnosed to be myelocytic leukemia. The mutation of this specific nucleotide was similar to that of the positive control shown in figure 23.

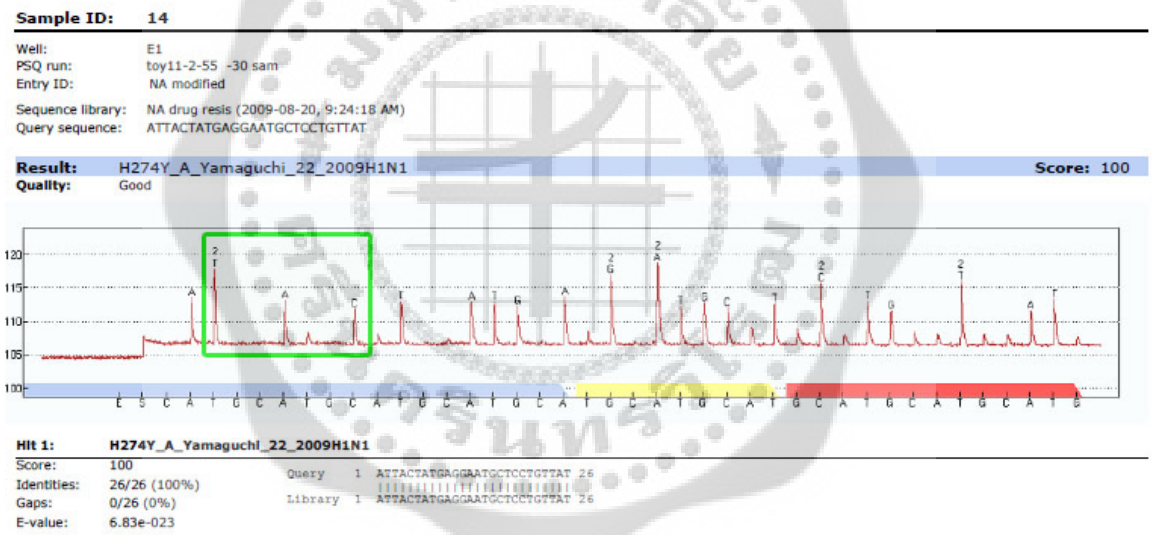

Figure 22 Detection of oseltamivir-resistance sample at the position 825 of nucleotide sequence

The green box showed mutation at the position 825 (CAC to TAC). This sequence was derived from the clinical sample collection from the leukemic patient.

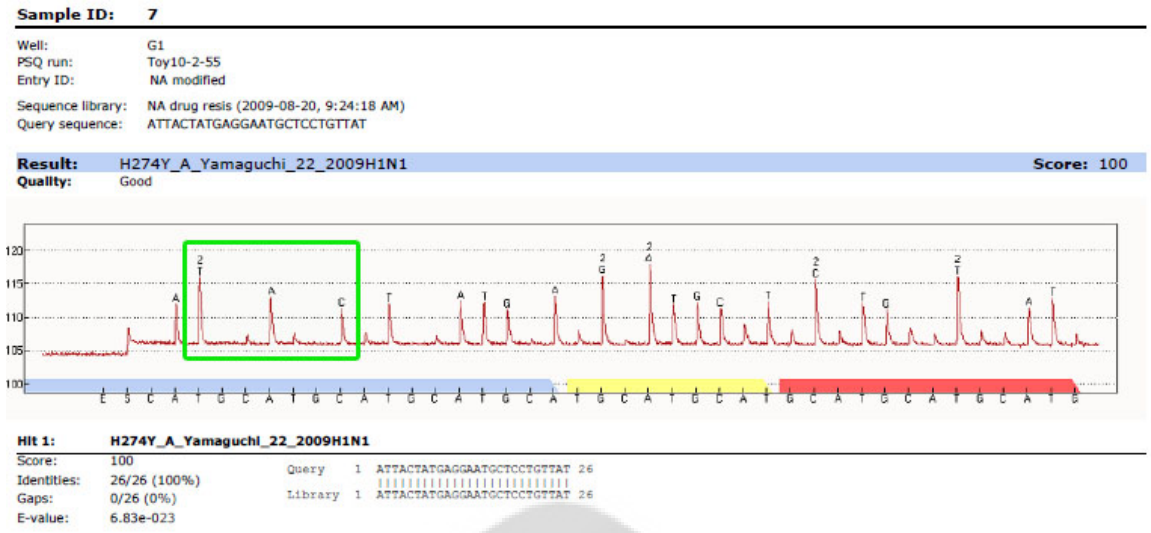

Figure 23 The pandemic influenza A H1N1 (2009) mutant which is oseltamivir-resistant virus was performed as a positive control.

The position of CAC to TAC in NA gene was shown in the green box.

4.2.3 Direct sequencing

4.2.3.1 Sequence analysis of neuraminidase gene

In order to investigate the other nucleotide mutations, the partial sequence of NA gene was performed. The amplifying product of 1,265 bp was sequenced by ABI 3130 DNA sequencer (Perkin-Elmer Cetus, USA). The sequence of nucleotide was edited and analyzed by DNA baser program (Heracle BioSoft S.R.L., Romania). The nucleotide sequence of NA gene was shown in appendix B.

The electropherogram printout from the automated sequencer of partial NA gene and it's deduce amino acid sequence in this study are attached in the appendix B

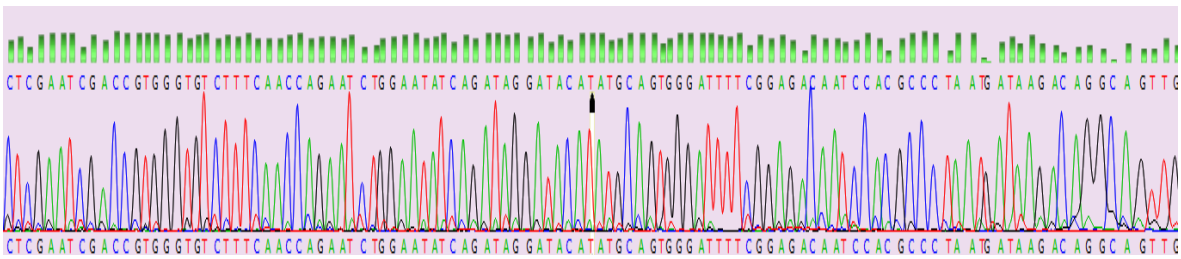

Figure 24 Chromatogram of nucleotide sequences at position 825-828 showed the mutation and resulting in amino acid substitution at H275Y.

4.2.3.2 Phylogenetic tree of nucleotide sequences in NA gene

The partially sequences of clinical samples No. 14 was compared to 13 NA sequences from GenBank database and phylogenetic tree analysis was performed. From phylogenetic analysis, it was found that clinical sample was closely related to A/Thailand/CU-H2358/2010 strain as shown in figure 25. Percent identities of nucleotides from both strains are 99.8%.

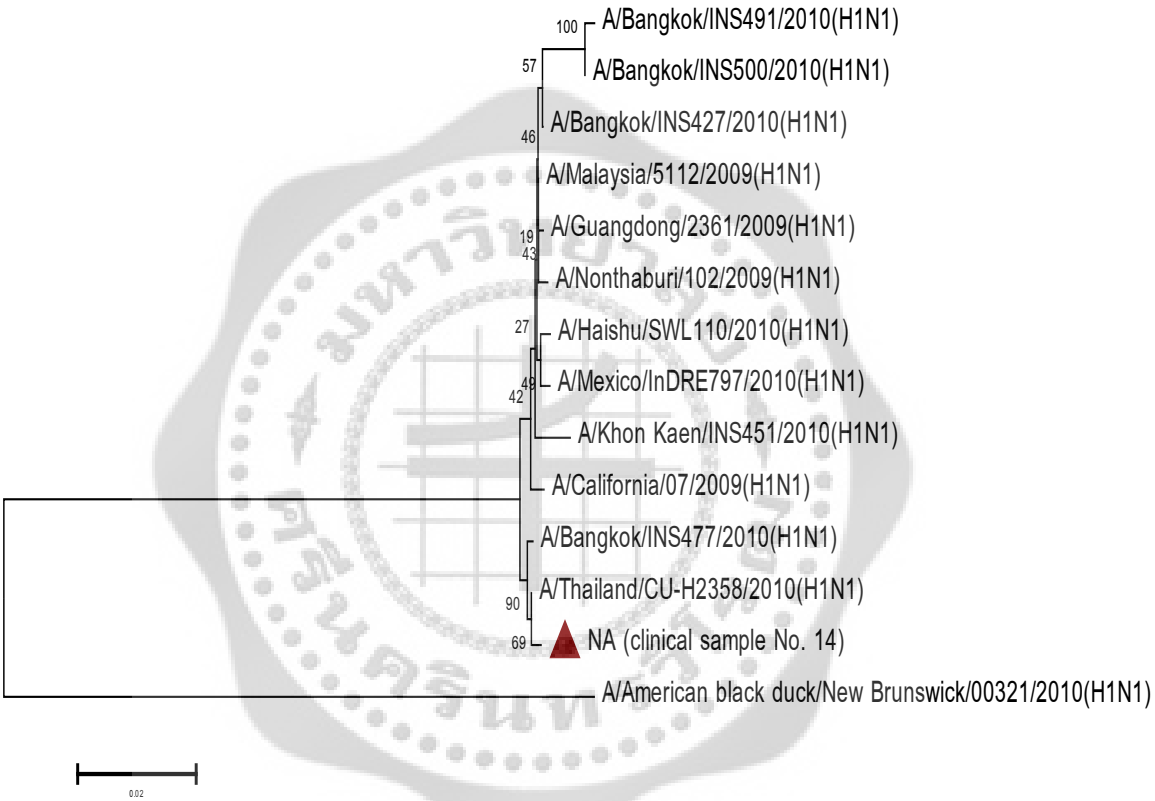

Figure 25 Phylogenetic tree of partially NA gene from clinical sample and NA sequences from GenBank database.

4.3.3.3 Distances of nucleotide sequences in NA gene were computed by DNAStar program

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1		81.7	81.4	81.8	80.9	81.1	81.3	81.4	81.3	81.5	81.6	81.3	81.7	81.5	1
2	18.9		99.6	99.2	98.7	98.9	99.6	99.4	99.1	99.7	99.4	99.5	99.2	99.1	2
3	19.2	0.4		99.4	99.1	99.3	99.8	99.7	99.3	99.9	99.7	99.8	99.4	99.3	3
4	18.9	0.8	0.6		98.6	98.7	99.4	99.3	98.9	99.5	99.3	99.4	99.8	99.7	4
5	19.8	1.3	0.9	1.4		99.8	99.0	98.8	98.4	99.1	98.8	98.9	98.6	98.4	5
6	19.6	1.1	0.7	1.3	0.2		99.1	99.0	98.6	99.2	99.0	99.1	98.7	98.6	6
7	19.2	0.4	0.2	0.6	1.0	0.9		99.7	99.3	99.9	99.7	99.8	99.4	99.3	7
8	19.2	0.6	0.3	0.7	1.2	1.0	0.3		99.1	99.8	99.7	99.6	99.4	99.3	8
9	19.3	1.0	0.7	1.1	1.6	1.4	0.7	0.9		99.4	99.1	99.2	98.9	98.7	9
10	19.1	0.3	0.1	0.5	1.0	0.8	0.1	0.2	0.6		99.8	99.8	99.5	99.4	10
11	19.0	0.6	0.3	0.7	1.2	1.0	0.3	0.3	0.9	0.2		99.6	99.4	99.3	11
12	19.3	0.5	0.2	0.6	1.1	1.0	0.2	0.4	0.8	0.2	0.4		99.4	99.2	12
13	18.9	0.8	0.6	0.2	1.4	1.3	0.6	0.6	1.1	0.5	0.6	0.6		99.8	13
14	19.1	1.0	0.7	0.3	1.6	1.4	0.7	0.7	1.3	0.6	0.7	0.8	0.2		14
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	

1 CY138642
2 HM138502
3 CY071353
4 CY096292
5 CY096364
6 CY096428
7 CY058837
8 HM145748
9 CY096124
10 CY055213
11 CY057074
12 CY039988
13 CY082966
14 NA_(clinical_sample_No

Figure 26 Percent identity and divergence of nucleotides from NA genes.

GenBank accession number

1 CY138642.1A/American black duck/New Brunswick/00321/2010(H1N1),

2 HM138502.1 (A/California/07/2009(H1N1)),

3 CY071353.1(A/Bangkok/INS427/2010(H1N1)),

4 CY096292.1(A/Bangkok/INS477/2010(H1N1)),

5 CY096364.1(A/Bangkok/INS491/2010(H1N1)),

6 CY096428.1(A/Bangkok/INS500/2010(H1N1)),

7 CY058837.1(A/Guangdong/2361/2009(H1N1)),

8 HM145748.1(A/Haishu/SWL110/2010(H1N1)),

9 CY096124.1(A/KhonKaen/INS451/2010(H1N1)),

10 CY055213.1(A/Malaysia/5112/2009(H1N1)),

11 CY057074.1(A/Mexico/InDRE797/2010(H1N1)),

12 CY039988.1(A/Nonthaburi/102/2009(H1N1)),

13 CY082966.2 (A/Thailand/ CUH2358/ 2010(H1N1)),

14 NA resistance (clinical sample No. 14)

The amino acid of NA gene was translated by using ExPASy-Translate tool website (http://web.expasy.org/translate/), the nucleotide sequence was translated in the amino acid sequence as followed:

Amino acid sequence of NA with reference strain (A/Thailand/CUH2358/2010(H1N1)H274Y)

```
A/Thailand/CU-H2358/2010_H1N1_      MNPNQKIITIGSVCMTIGMANLILQIGNIISIWISHSIQLGNQSQIETCN 50
NA                                  ----------------------QIGNIISIWISHSIQLGNQSQIETCN 26
                                                           *************************

A/Thailand/CU-H2358/2010_H1N1_      QSVITYENNTWVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIY 100
NA                                  QSVITYENNTWVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIY 76
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      SKDNSIRIGSKGDVFVIREPFISCSPLECRTFFLTQGALLNDKHSNGTIK 150
NA                                  SKDNSIRIGSKGDVFVIREPFISCSPLECRTFFLTQGALLNDKHSNGTIK 126
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      DRSPYRTLMSCPIGEVPSPYNSRFESVAWSASACHDGINWLTIGISGPDN 200
NA                                  DRSPYRTLMSCPIGEVPSPYNSRFESVAWSASACHDGINWLTIGISGPDN 176
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      GAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTIMTDGPSDGQ 250
NA                                  GAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTIMTDGPSDGQ 226
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      ASYKIFRIEKGKIVKSVEMNAPNYYYEECSCYPDSSEITCVCRDNWHGSN 300
NA                                  ASYKIFRIEKGKIVKSVEMNAPNYYYEECSCYPDSSEITCVCRDNWHGSN 276
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      RPWVSFNQNLEYQIGYICSGIFGDNPRPNDKTGSCGPVSSNGANGVKGFS 350
NA                                  RPWVSFNQNLEYQIGYICSGIFGDNPRPNDKTGSCGPVSSNGANGIKGFS 326
                                    *******************************************.****

A/Thailand/CU-H2358/2010_H1N1_      FKYGNGVWIGRTKSISSRKGFEMIWDPNGWTGTDNNFSIKQDIVGINEWS 400
NA                                  FKYGNGVWIGRTKSISSRKGFEMIWDPNGWTGTDNNFSIKQDIVGINEWS 376
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      GYSGSFVQHPELTGLDCIRPCFWVELIRGRPKENTIWTSGSSISFCGVNS 450
NA                                  GYSGSFVQHPELTGLDCIRPCFWVELIRGRPKENTIWTSGSSISF----- 421
                                    *****************************************

A/Thailand/CU-H2358/2010_H1N1_      DTVGWSWPDGAELPFTIDK 469
NA                                  -------------------
```

The amino acid coding sequence of partial of NA gene from oseltamivir-resistant sample was compared to the amino acid sequence of CY071353.1, CY096292.1, CY096364.1, CY096428.1, AF250356.2, CY058837.1, HM145748.1, CY096124.1, CY055213.1, CY057074.1, CY039988.1, GU271960.1, GU183801.1, GU183817.1 and CY082966.2. All of these sequences are in N1 subtype as showed in appendix B.

4.2.3.4 Amino acid sequence alignment

The amino acid sequence of oseltamivir-resistant sample and the other sequences were aligned. The pH1N1 sequences that were selected are the following; CY071353.1, CY096292.1, CY096364.1, CY096428.1, AF250356.2, CY058837.1, HM145748.1, CY096124.1, CY055213.1, CY057074.1, CY039988.1, GU271960.1, GU183801.1, GU183817.1 and CY082966. A particular position, 274-276, the amino acid in each sequence diverged from one another as shown in figure 28. The substitution of amino acid at this point confers the neuraminidase inhibitor resistance.

Figure 27 The alignment of the amino acid of the NA gene segments from oseltamivir resistant sample (NA red letter) and the other resistant strains.

The amino acids substitution at position 274-276 in the roll bar is the marker of oseltamivir resistance.

Chapter V

Discussion and conclusion

All 85 positive samples in this study were examined by rapid influenza diagnosis test (RIDT). These clinical specimens were performed in parallel with the positive and negative controls. Regarding to the current reference standard, the total of 85 specimens were further analyzed by rRT-PCR. Each paired result was concordant. In general, the sensitivity of rapid tests has varied considerably from each other. The strong correlation might be due to the high prevalence of Influenza infection rate, and the selected test-kit was at a height of sensitivity. However, it is accepted that rRT-PCR offers several advantages over RIDT, including the ability to identify influenza subtype and increasing the sensitivity of viral detection[101]. Despite a number of advantages of rRT-PCR technique, the rapid influenza diagnostic tests are still useful. It is sensible in describe the seasonality of influenza, estimate the cost of illness, increase the sensitivity of surveillance and conduct outbreak response. It is also effective tools for influenza research, surveillance, and outbreak investigations[102].

The first case of influenza A pH1N1 infection in Thailand was reported in May 2009. During the third wave of pandemic, this study was conducted since July to October, 2010. As categorized by rRT-PCR, the prevalence of influenza A subtype pHIN1, seasonal H1N1, and H3 infection were 72.94%, 11.76% and 15.29%, respectively. Comparison to the other two published datas in Thailand, the first one reported that the incidence of influenza A subtype pH1N1 were 54%, H3N2 were 35%, H1N1 were 7.6% and 4.1% were not subtyped[103]. The second study reported that 76.3% were influenza A subtype pH1N1 influenza viruses while 23.7% were seasonal influenza A (H3N2/H1N1) influenza viruses[104]. The discrepancy in percentage in the first report might be due to the different wave of the pandemic. However, the pattern of Influenza subtype in these studies was quite similar. Regarding to seasonal flu, all the study conducted in that period showed that Influenza A subtype H3 and H1N1 were co-circulated. Among three subtypes, the prevalence of pH1N1 infection was the highest and peaked in September 2009. The epidemiological character of circulating influenza virus, it was reported that the predominant circulating influenza was pH1N1 subtype and co-circulation of seasonal influenza viruses continued[105].

Oseltamivir resistance pH1N1 viruses

In Thailand, oseltamivir was introduced after the outbreak of avian influenza in 2004. Nevertheless, it was not commonly used at that time and was not used widely. Until the influenza A (H1N1) pandemic in 2009, it became the first-line drugs in both prophylaxis and treatment and was used in the national hospital with free of charge. The widely use of oseltamivir potentially promoted the evolution of drug-resistant strains[106, 107]. However, at the time of specimen collection, the incidence rate of oseltamivir-resistant was quite low. The first case of oseltamivir resistant (pH1N1) was reported in 2010[108]. In this study, one of the 64 clinical samples (1.6%) showed the evidence of oseltamivir-resistant viruses by pyrosequencing. During the same period, several oseltamivir-resistant studies were performed. Chittaganpitch et al[109] reported that the prevalence of oseltamivir-resistant pandemic A(H1N1) virus was still low as 1.31%. The other one by Payungporn et al[108] demonstrated that no oseltamivir resistant strain (0%) was found during the first wave of the outbreak (April-December, 2009) in Thailand. Subsequently, the numbers of oseltamivir resistant strains were 0.24% and 0.61%, respectively during the second wave (January-April, 2010) and third wave (May-October, 2010). Totally, 4 of the 1,288 specimens (0.31%) were positive for oseltamivir resistant strains as detected by real-time RT-PCR. In concordance with WHO reports, the incidence of viruses resistant to antivirals remains very low.

For the mechanism of drug resistance, two mechanisms were proposed. The first one involves in the volume of amino acid at the position H275Y itself. The cytosine was substituted by thymine at position 825 in the NA gene. The deduced amino acids had been changed (H275Y) which was also demonstrated throughout this study. Some scientists use 'N1 numbering' (H275Y) and some use the actual 'N2 numbering' (H274Y).The detected mutation associated with single amino acid substitution of the histidine (H) to tyrosine (Y) at position 275 confers high-level resistant to oseltamivir. By 3D structure, the H275Y substitution is located in the active site of the enzyme NA[110]. Based on the NA structure, the large side chain of amino acid interferes with NA-NAI binding. Previous study suggested that the binding activity depend on the volume occupied by the amino acid side chain. The replacement of His274 with larger side chain residues (Tyr or Phe) reduced the NA sensitivity to oseltamivir. Loss of binding was demonstrated in the His274Tyr and His274Phe mutant NA but not in His274Asn, His274Gly, His274Ser, or His274Gln. The replacement of His274 with smaller side chain residues (Gly, Asn, Ser, and Gln) resulted in

enhanced or unchanged sensitivity to oseltamivir carboxylate. Furthermore, the volume occupied by the amino acid side chain at position 274 can influence the sensitivities of influenza to both oseltamivir carboxylate and zanamivir[90]. This mutation is the most common of Influenza A subtype N1. Moreover, several studies stated that H275Y was NA subtype and oseltamivir specific. For susceptibility evaluation of this mutant, it was indicated that this variant demonstrated on average a 1,466-fold reduction in oseltamivir susceptibility compared to wild-type A (H1N1) viruses[104]. In this study, the other mutation was invested by whole NA genome sequenced. No other amino acid mutation in the other position was detected. However, the susceptibility of this mutant was not analyzed.

The second mechanism is the influence of the other amino acids which form the hydrophobic pocket. The mutant gives rise to oseltamivir-resistance is due to the binding affinity. The changing of amino acid H275Y has an impact on the rotation of E276 in the active site. E276 and R224 form a salt link which is necessary to form the hydrophobic pocket. Alteration of this formation interferes with the accommodation the ethyl propoxy side chains of NAIs. Substitution of the amino acid residues with different side chain (volumes, charge, and polarity) at position 274 can shift the equilibrium between the two orientation states of the side chain of Glu276. A bulky side chain residue at position 274 was going to block the reorientation of Glu276 required for the high-affinity binding of oseltamivir carboxylate to NA. The sensitivity to oseltamivir carboxylate is reduced. Conversely, introducing a smaller side chain residue at position 274 was going to create the conformation of NA favoring the reorientation of Glu276. This would result in an unfavorable binding pocket for substrate due to the hydrophobicity of the pocket [90, 111]. In considering to the other NA mutation, E119V, R292K, N294S, H274Y, and R152K were associated with resistance to oseltamivir[110, 112]. Some mutations, i.e., the R292K and H274Y mutation, lead to a functionally defective enzyme with compromised viral fitness. It suggested that viruses carrying these mutations are unlikely to be of significant clinical consequence in man[74, 97, 113, 114]. In contrast, another report described that the currently circulating pandemic 2009 H1N1 virus had a high potential to acquire drug resistance without losing fitness[115].

Phylogenic analysis of neuraminidase gene

Influenza strain A/American black duck/New Brunswick/0032/2010(H1N1) used for comparing as an out-group. In this study, the A/Thailand/CU-H2358/2010(H1N1) strain and clinical specimen were located in the same node. Additional supported information was found that both strains confer H275Y which is the significant marker for oseltamivir-resistance. The distance of phylogenetic analysis of nucleotide in NA sequence from clinical sample No.14 and A/Thailand/CU-2358/2010(H1N1) strain was 0.00054 as shown in figure 25. The result showed both NA sequence strains were closely genetic related. The analysis of percent identity of both strains is 99.8% that show supported evidence.

Neuraminidase variation

Most of the resistant viruses' pH1N1, which were found in Thailand, carried an H275Y substitution in their NA gene. As reported by Makkoch et al[116], genetic sequence of both strains (A/Thailand/CU-H2358/2010(H1N1) and clinical sample No. 14) contained the similar NA substitutions 44S, 241I, 276Y. These mutation positions in NA gene of both strains showed that they had identical nucleotide sequences for partial segment of NA.

Immunocompromised host

As specify earlier, the oseltamivir-resistant virus in this study was found in a 28-year-old man who was diagnosed as acute promyelocytic leukemia. He was admitted because of febrile neutropenia, coughs and injected pharynx. Thus, the suspicion of bacterial infection was resulted treatment with antibiotic and specimen collection at that time. This nasopharyngeal sample was collected from the affected patients during the chemotherapy treatment. From the medical records, whether this infected patient was exposed to oseltamivir is still a question. Since he was referred from the other hospital, no history of oseltamivir exposure was available. His report indicated that this hematologic malignant patient was infected by influenza virus. In this case, two possible hypotheses were proposed. First, it might be community-acquired. The oseltamivir-resistant viruses and the other influenza A subtypes were co-circulated during the end of third wave. On the other hand, the H275Y mutant was drug-induced after receiving neuraminidase inhibitors. In immunocompromised individuals, prolonged illness and viral shedding of drug-resistant virus have been demonstrated [117, 118].

A number of reports focusing on immunocompromised patients have shown a high mortality rate. According to World Health Organization (WHO) reports, approximately 30% of oseltamivir-resistant cases occurred in severely immunocompromised patients and 70% in non- immunocompromised patients. Within the latter, 69% of the cases were associated with drug use, including cases occurring during or after oseltamivir treatment and/or peramivir treatment. The emergence of drug-resistant virus might occur in long-term treatment of oseltamivir. Interestingly, 31% occurred in patients who had not used antiviral drugs prior to occurrence of the resistant virus. Fortunately, at this time, the occurring mutant is incompetent by the lack of apparent detriment to viral fitness and transmissibility. In this regards, it is necessary to prevent the immune-compromised individuals from influenza infection. In case of unavoidable, careful consideration of the rapid development of drug resistance during antiviral treatment is needed.

Conclusion

During the third wave of pH1N1 pandemic, the strong correlation between rapid influenza test and rRT-PCR might be due to the high prevalence of Influenza infection rate, and a high sensitivity of the selected test kit. For epidemiologic character, it was reported that pH1N1 subtype was predominant circulating influenza, and the other seasonal influenza viruses were co-circulation. Regarding to seasonal flu, all the study conducted in that periods showed that Influenza A subtype H3 and H1N1 were co-circulated. In order to investigate the oseltamivir-resistant strain, one clinical sample showed the evidence of oseltamivir–resistant viruses by pyrosequencing. The mutation in NA gene at position H275Y was demonstrated. This mutation with single amino acid substitution of the histidine (H) to tyrosine (Y) at position 275 confers high-level resistant to oseltamivir. For Influenza A subtype N1, the mutation at this position is the most common. However, the result of phylogenetic tree showed that influenza strain in this study was similar to the mutant virus strain in Thailand. This strain has mutation in 44S, 241I and 276Y position. This oseltamivir-resistant specimen belongs to a 28-year-old man who was diagnosed as acute promyelocytic leukemia. From the medical records, whether this infected patient was exposed to oseltamivir is still a question. Therefore, two possible hypotheses were proposed. First, it might be community-acquired. Second, the H275Y mutant was drug-induced after receiving neuraminidase inhibitors. This finding indicated that oseltamivir-resistant pH1N1 viruses circulated in Nakon-nayok and the nearby area. Continued

surveillance studies are necessary to monitor whether these viruses will persist. At present, the occurring mutant is incompetent by the lack of apparent detriment to viral fitness and transmissibility.

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APPENDIX

APPENDIX A

REAGENT

1. Gel Red™

1 M NaCl (w/v)	10	ml
Gel Red™ (1,000X)	50	µl
Distilled water	89.50	ml
Adjusts to 100 ml		

2. 1 M NaCl

NaCl (1×58.44)	58	g
Distilled water	1000	ml
Total	1000	ml

3. 0.5X TBE buffer

10X TBE buffer	15	ml
Distilled water	285	ml
Total	300	ml

4. 1% (w/v) agarose gel

Agarose	1	g
0.5X TBE buffer	100	ml
Total	100	ml

Mel until gel dissolve and place at 55°C in water baht

APPENDIX B

Alignment of NA gene

Alignment Nucleotide of NA gene influenza viruses by clustal W

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CLUSTAL 2.1 multiple sequence alignment

A/Guangdong/2361/2009_H1N1_CY0      CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Malaysia/5112/2009_H1N1_CY05     CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Nonthaburi/102/2009_H1N1_CY0     CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Khon_Kaen/INS451/2010_H1N1_C     CAAATTGGGAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Bangkok/INS491/2010_H1N1_        CAAATTGGAAACATAATCTTAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Bangkok/INS500/2010_H1N1_CY0     CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Bangkok/INS427/2010_H1N1_CY0     CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Haishu/SWL110/2010_H1N1_HM14     CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Mexico/InDRE797/2010_H1N1_CY     CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/California/07/2009_H1N1_HM13     CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Thailand/CU-H2358/2010_H1N1_     CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
NA__clinical_sample_No.14_         CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/Bangkok/INS477/2010_H1N1_CY0     CAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGG 50
A/American                         CAAATTGGAAATATAATCTCAATATGGGTTAGTCATTCAATCCAAACTGG 50
                                   ******** ** ******* ******* **** ** ***** ***  ***

A/Guangdong/2361/2009_H1N1_CY0     GAATCAAAATCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/Malaysia/5112/2009_H1N1_CY05     GAATCAAAATCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/Nonthaburi/102/2009_H1N1_CY0     GAATCAAAATCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/Khon_Kaen/INS451/2010_H1N1_C     GAATAAAAATCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/Bangkok/INS491/2010_H1N1_        GAATCAAAATCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/Bangkok/INS500/2010_H1N1_CY0     GAATCAAAATCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/Bangkok/INS427/2010_H1N1_CY0     GAATCAAAATCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/Haishu/SWL110/2010_H1N1_HM14     GAATCAAAATCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/Mexico/InDRE797/2010_H1N1_CY     GAATCAAAATCAGATTGAAACATGCAATCAAAGTGTCATTACTTATGAAA 100
A/California/07/2009_H1N1_HM13     GAATCAAAATCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/Thailand/CU-H2358/2010_H1N1_     GAATCAAAGTCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
NA__clinical_sample_No.14_         GAATCAAAGTCAGATTGAAACATGCAACCAAAGCGTCATTACTTATGAAA 100
A/Bangkok/INS477/2010_H1N1_CY0     GAATCAAAGTCAGATTGAAACATGCAATCAAAGCGTCATTACTTATGAAA 100
A/American                         AAGCAAGAGCCACCCTGAAACATGCAATCAAAGTATCATTACCTACGAAA 100
                                    *  * *  **  ************ *****  ******* ** ****

A/Guangdong/2361/2009_H1N1_CY0     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/Malaysia/5112/2009_H1N1_CY05     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/Nonthaburi/102/2009_H1N1_CY0     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/Khon_Kaen/INS451/2010_H1N1_C     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/Bangkok/INS491/2010_H1N1_        ACAACACTTGGGTAAATCAGACATACGTTAACATCAGCAACACCAACTTT 150
A/Bangkok/INS500/2010_H1N1_CY0     ACAACACTTGGGTAAATCAGACATACGTTAACATCAGCAACACCAACTTT 150
A/Bangkok/INS427/2010_H1N1_CY0     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/Haishu/SWL110/2010_H1N1_HM14     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/Mexico/InDRE797/2010_H1N1_CY     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/California/07/2009_H1N1_HM13     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/Thailand/CU-H2358/2010_H1N1_     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
NA__clinical_sample_No.14_         ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/Bangkok/INS477/2010_H1N1_CY0     ACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACTTT 150
A/American                         ACAATACTTGGGTGAATCAGACATACCTTAACATAAGTAATACCAATTTA 150
                                   **** ******** ***********  ******* ** ** ***** **

A/Guangdong/2361/2009_H1N1_CY0     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/Malaysia/5112/2009_H1N1_CY05     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/Nonthaburi/102/2009_H1N1_CY0     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/Khon_Kaen/INS451/2010_H1N1_C     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/Bangkok/INS491/2010_H1N1_        GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/Bangkok/INS500/2010_H1N1_CY0     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/Bangkok/INS427/2010_H1N1_CY0     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/Haishu/SWL110/2010_H1N1_HM14     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/Mexico/InDRE797/2010_H1N1_CY     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/California/07/2009_H1N1_HM13     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCAGGCAATTCCTCTCT 200
A/Thailand/CU-H2358/2010_H1N1_     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
NA__clinical_sample_No.14_         GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/Bangkok/INS477/2010_H1N1_CY0     GCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCT 200
A/American                         ATTGCAGAACAGACTGTGGCTCCAATAACATTAGCAGGTAATTCCTCTCT 200
                                     *** * **** * **** * *  * * ****** ** ***********
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A/Guangdong/2361/2009_H1N1_CY0      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/Malaysia/5112/2009_H1N1_CY05      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/Nonthaburi/102/2009_H1N1_CY0      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/Khon_Kaen/INS451/2010_H1N1_C      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/Bangkok/INS491/2010_H1N1_         CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/Bangkok/INS500/2010_H1N1_CY0      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/Bangkok/INS427/2010_H1N1_CY0      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/Haishu/SWL110/2010_H1N1_HM14      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/Mexico/InDRE797/2010_H1N1_CY      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/California/07/2009_H1N1_HM13      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTGTAAGAA 250
A/Thailand/CU-H2358/2010_H1N1_      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
NA__clinical_sample_No.14_          CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/Bangkok/INS477/2010_H1N1_CY0      CTGCCCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAA 250
A/American                          CTGTCCCATCAGTGGATGGGCTATATACAGCAAGGATAATGGTATAAGGA 250
                                    *** **  * ********************* ** ** **  ** **** *

A/Guangdong/2361/2009_H1N1_CY0      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/Malaysia/5112/2009_H1N1_CY05      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/Nonthaburi/102/2009_H1N1_CY0      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/Khon_Kaen/INS451/2010_H1N1_C      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/Bangkok/INS491/2010_H1N1_         TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGT 300
A/Bangkok/INS500/2010_H1N1_CY0      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGT 300
A/Bangkok/INS427/2010_H1N1_CY0      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/Haishu/SWL110/2010_H1N1_HM14      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/Mexico/InDRE797/2010_H1N1_CY      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/California/07/2009_H1N1_HM13      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/Thailand/CU-H2358/2010_H1N1_      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
NA__clinical_sample_No.14_          TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/Bangkok/INS477/2010_H1N1_CY0      TCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGC 300
A/American                          TAGGTTCCAAAGGAGATGTATTTGTCATCAGAGAGCCTTTTATTTCATGC 300
                                    * ******** ** ***** ******** ** ** ** ** ** *****

A/Guangdong/2361/2009_H1N1_CY0      TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/Malaysia/5112/2009_H1N1_CY05      TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/Nonthaburi/102/2009_H1N1_CY0      TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCCTGCTAAA 350
A/Khon_Kaen/INS451/2010_H1N1_C      TCCCCCCTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/Bangkok/INS491/2010_H1N1_         TCCCCCTTGGAATGCAGGACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/Bangkok/INS500/2010_H1N1_CY0      TCCCCCTTGGAATGCAGGACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/Bangkok/INS427/2010_H1N1_CY0      TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/Haishu/SWL110/2010_H1N1_HM14      TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/Mexico/InDRE797/2010_H1N1_CY      TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/California/07/2009_H1N1_HM13      TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/Thailand/CU-H2358/2010_H1N1_      TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
NA__clinical_sample_No.14_          TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/Bangkok/INS477/2010_H1N1_CY0      TCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAA 350
A/American                          TCGCACATGGAATGCAGAACTTTCTTTCTAACTCAAGGGGCCTTGTTGAA 350
                                    ** * * ********** ** *****  * ************ ** * **

A/Guangdong/2361/2009_H1N1_CY0      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/Malaysia/5112/2009_H1N1_CY05      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/Nonthaburi/102/2009_H1N1_CY0      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/Khon_Kaen/INS451/2010_H1N1_C      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/Bangkok/INS491/2010_H1N1_         TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACTC 400
A/Bangkok/INS500/2010_H1N1_CY0      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACTC 400
A/Bangkok/INS427/2010_H1N1_CY0      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/Haishu/SWL110/2010_H1N1_HM14      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/Mexico/InDRE797/2010_H1N1_CY      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/California/07/2009_H1N1_HM13      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/Thailand/CU-H2358/2010_H1N1_      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
NA__clinical_sample_No.14_          TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/Bangkok/INS477/2010_H1N1_CY0      TGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCC 400
A/American                          TGACAAGCATTCCAATGGAACCGTTAAAGACAGAAGCCCTTATAGAACCC 400
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A/Guangdong/2361/2009_H1N1_CY0      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Malaysia/5112/2009_H1N1_CY05      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Nonthaburi/102/2009_H1N1_CY0      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Khon_Kaen/INS451/2010_H1N1_C      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Bangkok/INS491/2010_H1N1_         TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Bangkok/INS500/2010_H1N1_CY0      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Bangkok/INS427/2010_H1N1_CY0      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Haishu/SWL110/2010_H1N1_HM14      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Mexico/InDRE797/2010_H1N1_CY      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/California/07/2009_H1N1_HM13      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Thailand/CU-H2358/2010_H1N1_      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
NA__clinical_sample_No.14_          TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/Bangkok/INS477/2010_H1N1_CY0      TAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTT 450
A/American                          TAATGAGCTGTCCTGTTGGTGAAGCTCCCTCTCCATACAATTCAAGGTTT 450
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A/Guangdong/2361/2009_H1N1_CY0      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Malaysia/5112/2009_H1N1_CY05      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Nonthaburi/102/2009_H1N1_CY0      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Khon_Kaen/INS451/2010_H1N1_C      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Bangkok/INS491/2010_H1N1_         GAATCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Bangkok/INS500/2010_H1N1_CY0      GAATCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Bangkok/INS427/2010_H1N1_CY0      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Haishu/SWL110/2010_H1N1_HM14      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Mexico/InDRE797/2010_H1N1_CY      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/California/07/2009_H1N1_HM13      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Thailand/CU-H2358/2010_H1N1_      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
NA__clinical_sample_No.14_          GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/Bangkok/INS477/2010_H1N1_CY0      GAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATTGGCT 500
A/American                          GAGTCTGTTGCTTGGTCGGCAAGTGCTTGCCACGATGGCATTAGCTGGTT 500
                                    ** ** ** ******** *********** ** ******** *  *** *

A/Guangdong/2361/2009_H1N1_CY0      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Malaysia/5112/2009_H1N1_CY05      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Nonthaburi/102/2009_H1N1_CY0      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Khon_Kaen/INS451/2010_H1N1_C      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Bangkok/INS491/2010_H1N1_         AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Bangkok/INS500/2010_H1N1_CY0      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Bangkok/INS427/2010_H1N1_CY0      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Haishu/SWL110/2010_H1N1_HM14      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Mexico/InDRE797/2010_H1N1_CY      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/California/07/2009_H1N1_HM13      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Thailand/CU-H2358/2010_H1N1_      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
NA__clinical_sample_No.14_          AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/Bangkok/INS477/2010_H1N1_CY0      AACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGT 550
A/American                          GACAATTGGTATTTCCGGCCCAGACAATGGGGCAGTGGCTGTATTGAAGT 550
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A/Guangdong/2361/2009_H1N1_CY0      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Malaysia/5112/2009_H1N1_CY05      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Nonthaburi/102/2009_H1N1_CY0      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Khon_Kaen/INS451/2010_H1N1_C      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Bangkok/INS491/2010_H1N1_         ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Bangkok/INS500/2010_H1N1_CY0      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Bangkok/INS427/2010_H1N1_CY0      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Haishu/SWL110/2010_H1N1_HM14      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Mexico/InDRE797/2010_H1N1_CY      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/California/07/2009_H1N1_HM13      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Thailand/CU-H2358/2010_H1N1_      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
NA__clinical_sample_No.14_          ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/Bangkok/INS477/2010_H1N1_CY0      ACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTG 600
A/American                          ACAATAGCATAATAACAGATACTATCAAGAGTTGGAGAAACAACATATTG 600
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A/Guangdong/2361/2009_H1N1_CY0      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/Malaysia/5112/2009_H1N1_CY05      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/Nonthaburi/102/2009_H1N1_CY0      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/Khon_Kaen/INS451/2010_H1N1_C      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/Bangkok/INS491/2010_H1N1_         AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/Bangkok/INS500/2010_H1N1_CY0      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/Bangkok/INS427/2010_H1N1_CY0      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/Haishu/SWL110/2010_H1N1_HM14      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/Mexico/InDRE797/2010_H1N1_CY      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/California/07/2009_H1N1_HM13      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGT 650
A/Thailand/CU-H2358/2010_H1N1_      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACCAT 650
NA__clinical_sample_No.14_          AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACCAT 650
A/Bangkok/INS477/2010_H1N1_CY0      AGAACACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACCAT 650
A/American                          AGGACACAAGAATCTGAATGTGCCTGCATCAATGGTTCTTGCTTTACCAT 650
                                    ** ******** *********** **  * ****************  *

A/Guangdong/2361/2009_H1N1_CY0      AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/Malaysia/5112/2009_H1N1_CY05      AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/Nonthaburi/102/2009_H1N1_CY0      AATGACCGATGGACCAAGTGATGAACAGGCCTCATACAAGATCTTCAGAA 700
A/Khon_Kaen/INS451/2010_H1N1_C      AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGGA 700
A/Bangkok/INS491/2010_H1N1_         AATGACCGATGGACCAAATGATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/Bangkok/INS500/2010_H1N1_CY0      AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/Bangkok/INS427/2010_H1N1_CY0      AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/Haishu/SWL110/2010_H1N1_HM14      AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/Mexico/InDRE797/2010_H1N1_CY      AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/California/07/2009_H1N1_HM13      AATGACCGATGGACCAAGTAATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/Thailand/CU-H2358/2010_H1N1_      AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGAA 700
NA__clinical_sample_No.14_          AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/Bangkok/INS477/2010_H1N1_CY0      AATGACCGATGGACCAAGTGATGGACAGGCCTCATACAAGATCTTCAGAA 700
A/American                          AATGACTGATGGACCAAGTAATGGTCAGGCTTCATACAAGATTTTCAAAA 700
                                    ****** ********** * ***  ***** *********** ****  *

A/Guangdong/2361/2009_H1N1_CY0      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Malaysia/5112/2009_H1N1_CY05      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Nonthaburi/102/2009_H1N1_CY0      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Khon_Kaen/INS451/2010_H1N1_C      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Bangkok/INS491/2010_H1N1_         TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Bangkok/INS500/2010_H1N1_CY0      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Bangkok/INS427/2010_H1N1_CY0      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Haishu/SWL110/2010_H1N1_HM14      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Mexico/InDRE797/2010_H1N1_CY      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/California/07/2009_H1N1_HM13      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Thailand/CU-H2358/2010_H1N1_      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
NA__clinical_sample_No.14_          TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/Bangkok/INS477/2010_H1N1_CY0      TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTAT 750
A/American                          TAGAAAAGGGAAAGGTAGTAAAATCAGTTGAGTTGAATGCCCCTAATTAC 750
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A/Guangdong/2361/2009_H1N1_CY0      CACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Malaysia/5112/2009_H1N1_CY05      CACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Nonthaburi/102/2009_H1N1_CY0      CACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Khon_Kaen/INS451/2010_H1N1_C      CACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Bangkok/INS491/2010_H1N1_         CACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Bangkok/INS500/2010_H1N1_CY0      CACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Bangkok/INS427/2010_H1N1_CY0      CACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Haishu/SWL110/2010_H1N1_HM14      TACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Mexico/InDRE797/2010_H1N1_CY      TACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/California/07/2009_H1N1_HM13      CACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Thailand/CU-H2358/2010_H1N1_      TACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
NA__clinical_sample_No.14_          TACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/Bangkok/INS477/2010_H1N1_CY0      CACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGT 800
A/American                          CACTATGAAGAGTGTTCCTGTTATCCTGATGCTAGTGAGGTGATGTGTGT 800
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A/Guangdong/2361/2009_H1N1_CY0      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/Malaysia/5112/2009_H1N1_CY05      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/Nonthaburi/102/2009_H1N1_CY0      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/Khon_Kaen/INS451/2010_H1N1_C      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/Bangkok/INS491/2010_H1N1_         GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/Bangkok/INS500/2010_H1N1_CY0      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/Bangkok/INS427/2010_H1N1_CY0      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/Haishu/SWL110/2010_H1N1_HM14      GTGCAGGGATAACTGGCATGGCTCAAATCGACCGTGGGTGTCTTTCAACC 850
A/Mexico/InDRE797/2010_H1N1_CY      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/California/07/2009_H1N1_HM13      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/Thailand/CU-H2358/2010_H1N1_      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
NA__clinical_sample_No.14_          GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/Bangkok/INS477/2010_H1N1_CY0      GTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACC 850
A/American                          ATGCAGGGACAATTGGCATGGTTCAAATCGGCCATGGGTGTCCTTCAATC 850
                                     ******** ** ******** ** ***** ** ******** ***** *

A/Guangdong/2361/2009_H1N1_CY0      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Malaysia/5112/2009_H1N1_CY05      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Nonthaburi/102/2009_H1N1_CY0      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Khon_Kaen/INS451/2010_H1N1_C      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Bangkok/INS491/2010_H1N1_         AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Bangkok/INS500/2010_H1N1_CY0      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Bangkok/INS427/2010_H1N1_CY0      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Haishu/SWL110/2010_H1N1_HM14      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Mexico/InDRE797/2010_H1N1_CY      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/California/07/2009_H1N1_HM13      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Thailand/CU-H2358/2010_H1N1_      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
NA__clinical_sample_No.14_          AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/Bangkok/INS477/2010_H1N1_CY0      AGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGAC 900
A/American                          AGAATCTAGAGTATCAAATAGGATATATATGCAGCGGAGTTTTTGGAGAC 900
                                    ******* ** ***** ******** ******** **  **** ******

A/Guangdong/2361/2009_H1N1_CY0      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Malaysia/5112/2009_H1N1_CY05      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Nonthaburi/102/2009_H1N1_CY0      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Khon_Kaen/INS451/2010_H1N1_C      AATCCACGCCCTAATGATAAGAAAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Bangkok/INS491/2010_H1N1_         AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Bangkok/INS500/2010_H1N1_CY0      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Bangkok/INS427/2010_H1N1_CY0      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Haishu/SWL110/2010_H1N1_HM14      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Mexico/InDRE797/2010_H1N1_CY      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/California/07/2009_H1N1_HM13      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Thailand/CU-H2358/2010_H1N1_      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
NA__clinical_sample_No.14_          AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/Bangkok/INS477/2010_H1N1_CY0      AATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAA 950
A/American                          AATCCACGCCCCAATGACGGGACAGGCAGTTGTGGTCCAGTATCTTCTAA 950
                                    ********** *****   ** ********************* *****

A/Guangdong/2361/2009_H1N1_CY0      TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Malaysia/5112/2009_H1N1_CY05      TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Nonthaburi/102/2009_H1N1_CY0      TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Khon_Kaen/INS451/2010_H1N1_C      TGGAGCAAACGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Bangkok/INS491/2010_H1N1_         TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Bangkok/INS500/2010_H1N1_CY0      TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Bangkok/INS427/2010_H1N1_CY0      TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Haishu/SWL110/2010_H1N1_HM14      TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Mexico/InDRE797/2010_H1N1_CY      TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/California/07/2009_H1N1_HM13      TGGAGCAAATGGAGTAAAAGGGTTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Thailand/CU-H2358/2010_H1N1_      TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
NA__clinical_sample_No.14_          TGGAGCAAATGGAATAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/Bangkok/INS477/2010_H1N1_CY0      TGGAGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTT 1000
A/American                          TGGGGCATATGGGGTGAAAGGGTTTTCGTTTAGATACGGTAACGGTGTTT 1000
                                    *** *** * **  * ***** ***** ** * ****** ** *******
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A/Guangdong/2361/2009_H1N1_CY0      GGATAGGGAGAACTAAAAGCATTAGTTTAAGAAACGGTTTTGAGATGATT 1050
A/Malaysia/5112/2009_H1N1_CY05      GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATT 1050
A/Nonthaburi/102/2009_H1N1_CY0      GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATT 1050
A/Khon_Kaen/INS451/2010_H1N1_C      GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATT 1050
A/Bangkok/INS491/2010_H1N1_         GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATT 1050
A/Bangkok/INS500/2010_H1N1_CY0      GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATT 1050
A/Bangkok/INS427/2010_H1N1_CY0      GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATT 1050
A/Haishu/SWL110/2010_H1N1_HM14      GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATT 1050
A/Mexico/InDRE797/2010_H1N1_CY      GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATT 1050
A/California/07/2009_H1N1_HM13      GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATT 1050
A/Thailand/CU-H2358/2010_H1N1_      GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAAAGGTTTTGAGATGATT 1050
NA__clinical_sample_No.14_          GGATAGGGAGAACTAAAAGCATTAGTTCAAGAAAAGGTTTTGAGATGATT 1050
A/Bangkok/INS477/2010_H1N1_CY0      GGATAGGGAGAACTAAAAGCTTTAGTTCAAGAAAAGGTTTTGAGATGATT 1050
A/American                          GGATAGGAAGAACTAAAAGTACTAGCTCAAGGAGTGGGTTTGAGATGATT 1050
                                    ******* ***********   *** * *** *  ** ************

A/Guangdong/2361/2009_H1N1_CY0      TGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
A/Malaysia/5112/2009_H1N1_CY05      TGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
A/Nonthaburi/102/2009_H1N1_CY0      TGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
A/Khon_Kaen/INS451/2010_H1N1_C      TGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
A/Bangkok/INS491/2010_H1N1_         TGGGATCCGAACGGATGGACTGGAACAGACAATAACTTCTCAGTAAAGCA 1100
A/Bangkok/INS500/2010_H1N1_CY0      TGGGATCCGAACGGATGGACTGGAACAGACAATAACTTCTCAGTAAAGCA 1100
A/Bangkok/INS427/2010_H1N1_CY0      TGGGATCCGAACGGATGGACTGGAACAGACAATAACTTCTCAATAAAGCA 1100
A/Haishu/SWL110/2010_H1N1_HM14      TGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
A/Mexico/InDRE797/2010_H1N1_CY      TGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
A/California/07/2009_H1N1_HM13      TGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
A/Thailand/CU-H2358/2010_H1N1_      TGGGATCCAAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
NA__clinical_sample_No.14_          TGGGATCCAAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
A/Bangkok/INS477/2010_H1N1_CY0      TGGGATCCAAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCA 1100
A/American                          TGGGACCCCGATGGATGGACAGAGACGGACAACAGTTTCTCTGTGAAGCA 1100
                                    ***** **  * ******** *  ** ***** *  *****  * *****

A/Guangdong/2361/2009_H1N1_CY0      AGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/Malaysia/5112/2009_H1N1_CY05      AGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/Nonthaburi/102/2009_H1N1_CY0      AGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/Khon_Kaen/INS451/2010_H1N1_C      AGATATCGTAGGAATAAATGAATGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/Bangkok/INS491/2010_H1N1_         AGATATCATAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/Bangkok/INS500/2010_H1N1_CY0      AGATATCATAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/Bangkok/INS427/2010_H1N1_CY0      AGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/Haishu/SWL110/2010_H1N1_HM14      AGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTCC 1150
A/Mexico/InDRE797/2010_H1N1_CY      AGATATTGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/California/07/2009_H1N1_HM13      AGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/Thailand/CU-H2358/2010_H1N1_      AGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
NA__clinical_sample_No.14_          AGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/Bangkok/INS477/2010_H1N1_CY0      AGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTC 1150
A/American                          AGACATTGTTGCAATAACTGATTGGTCAGGATATAGCGGAAGTTTTGTTC 1150
                                    *** **  * * ***** *** ***************** ******** *

A/Guangdong/2361/2009_H1N1_CY0      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Malaysia/5112/2009_H1N1_CY05      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Nonthaburi/102/2009_H1N1_CY0      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Khon_Kaen/INS451/2010_H1N1_C      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Bangkok/INS491/2010_H1N1_         AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Bangkok/INS500/2010_H1N1_CY0      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Bangkok/INS427/2010_H1N1_CY0      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Haishu/SWL110/2010_H1N1_HM14      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Mexico/InDRE797/2010_H1N1_CY      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/California/07/2009_H1N1_HM13      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Thailand/CU-H2358/2010_H1N1_      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
NA__clinical_sample_No.14_          AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/Bangkok/INS477/2010_H1N1_CY0      AGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTT 1200
A/American                          AGCACCCAGAACTGACAGGACTAGACTGCATGCGACCTTGCTTTTGGGTT 1200
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A/Guangdong/2361/2009_H1N1_CY0      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/Malaysia/5112/2009_H1N1_CY05      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/Nonthaburi/102/2009_H1N1_CY0      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/Khon_Kaen/INS451/2010_H1N1_C      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGTGGGAG 1250
A/Bangkok/INS491/2010_H1N1_         GAGCTAATCAGAGGGCGGCCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/Bangkok/INS500/2010_H1N1_CY0      GAGCTAATCAGAGGGCGGCCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/Bangkok/INS427/2010_H1N1_CY0      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/Haishu/SWL110/2010_H1N1_HM14      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/Mexico/InDRE797/2010_H1N1_CY      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/California/07/2009_H1N1_HM13      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/Thailand/CU-H2358/2010_H1N1_      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
NA__clinical_sample_No.14_          GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/Bangkok/INS477/2010_H1N1_CY0      GAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAG 1250
A/American                          GAGCTAATCAGAGGAAGACCCAAGGAGAATACAATCTGGACCAGTGGAAG 1250
                                    ** ***********  * ***** ***** ********** ** ** **

A/Guangdong/2361/2009_H1N1_CY0      CAGCATATCCTTTTG 1265
A/Malaysia/5112/2009_H1N1_CY05      CAGCATATCCTTTTG 1265
A/Nonthaburi/102/2009_H1N1_CY0      CAGCATATCCTTTTG 1265
A/Khon_Kaen/INS451/2010_H1N1_C      CAGCATATCCTTTTG 1265
A/Bangkok/INS491/2010_H1N1_         CAGCATATCCTTTTG 1265
A/Bangkok/INS500/2010_H1N1_CY0      CAGCATATCCTTTTG 1265
A/Bangkok/INS427/2010_H1N1_CY0      CAGCATATCCTTTTG 1265
A/Haishu/SWL110/2010_H1N1_HM14      CAGCATATCCTTTTG 1265
A/Mexico/InDRE797/2010_H1N1_CY      CAGCATATCCTTTTG 1265
A/California/07/2009_H1N1_HM13      CAGCATATCCTTTTG 1265
A/Thailand/CU-H2358/2010_H1N1_      CAGCATATCCTTTTG 1265
NA__clinical_sample_No.14_          CAGCATATCCTTTTG 1265
A/Bangkok/INS477/2010_H1N1_CY0      CAGCATATCCTTTTG 1265
A/American                          CAGCATTTCCTTTTG 1265
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Alignment protein of NA gene influenza viruses by clustal W

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CLUSTAL 2.1 multiple sequence alignment


A/Malaysia/5112/2009_H1N1_          QIGNIISIWISHSIQLGNQNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Nonthaburi/102/2009_H1N1_         QIGNIISIWISHSIQLGNQNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Bangkok/INS427/2010_H1N1_         QIGNIISIWISHSIQLGNQNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Guangdong/2361/2009_H1N1_         QIGNIISIWISHSIQLGNQNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Haishu/SWL110/2010_H1N1_NA        QIGNIISIWISHSIQLGNQNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Mexico/InDRE797/2010_H1N1_        QIGNIISIWISHSIQLGNQNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Khon                              QIGNIISIWISHSIQLGNKNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/California/07/2009_H1N1_          QIGNIISIWISHSIQLGNQNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Bangkok/INS491/2010_H1N1_         QIGNIILIWISHSIQLGNQNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Bangkok/INS500/2010_H1N1_         QIGNIISIWISHSIQLGNQNQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Thailand/CU-H2358/2010_H1N1_      QIGNIISIWISHSIQLGNQSQIETCNQSVITYENNTWVNQTYVNISNTNF 50
NA                                  QIGNIISIWISHSIQLGNQSQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/Bangkok/INS477/2010_H1N1_         QIGNIISIWISHSIQLGNQSQIETCNQSVITYENNTWVNQTYVNISNTNF 50
A/American_black_duck/New_Brun      QIGNIISIWVSHSIQTGSKSHPETCNQSIITYENNTWVNQTYLNISNTNL 50
                                    ****** **:***** *.:.: ******:************:******:

A/Malaysia/5112/2009_H1N1_          AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/Nonthaburi/102/2009_H1N1_         AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/Bangkok/INS427/2010_H1N1_         AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/Guangdong/2361/2009_H1N1_         AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/Haishu/SWL110/2010_H1N1_NA        AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/Mexico/InDRE797/2010_H1N1_        AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/Khon                              AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/California/07/2009_H1N1_          AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGSKGDVFVIREPFISC 100
A/Bangkok/INS491/2010_H1N1_         AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/Bangkok/INS500/2010_H1N1_         AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/Thailand/CU-H2358/2010_H1N1_      AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
NA                                  AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/Bangkok/INS477/2010_H1N1_         AAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDVFVIREPFISC 100
A/American_black_duck/New_Brun      IAEQTVAPITLAGNSSLCPISGWAIYSKDNGIRIGSKGDVFVIREPFISC 100
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A/Malaysia/5112/2009_H1N1_          SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Nonthaburi/102/2009_H1N1_         SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Bangkok/INS427/2010_H1N1_         SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Guangdong/2361/2009_H1N1_         SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Haishu/SWL110/2010_H1N1_NA        SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Mexico/InDRE797/2010_H1N1_        SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Khon                              SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/California/07/2009_H1N1_          SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Bangkok/INS491/2010_H1N1_         SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Bangkok/INS500/2010_H1N1_         SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Thailand/CU-H2358/2010_H1N1_      SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
NA                                  SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/Bangkok/INS477/2010_H1N1_         SPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRF 150
A/American_black_duck/New_Brun      SHMECRTFFLTQGALLNDKHSNGTVKDRSPYRTLMSCPVGEAPSPYNSRF 150
                                    * :********************:*************:**.******** 

A/Malaysia/5112/2009_H1N1_          ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Nonthaburi/102/2009_H1N1_         ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Bangkok/INS427/2010_H1N1_         ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Guangdong/2361/2009_H1N1_         ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Haishu/SWL110/2010_H1N1_NA        ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Mexico/InDRE797/2010_H1N1_        ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Khon                              ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/California/07/2009_H1N1_          ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Bangkok/INS491/2010_H1N1_         ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Bangkok/INS500/2010_H1N1_         ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Thailand/CU-H2358/2010_H1N1_      ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
NA                                  ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/Bangkok/INS477/2010_H1N1_         ESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNIL 200
A/American_black_duck/New_Brun      ESVAWSASACHDGISWLTIGISGPDNGAVAVLKYNSIITDTIKSWRNNIL 200
                                    ************** .******************** .************** 

A/Malaysia/5112/2009_H1N1_          RTQESECACVNGSCFTVMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Nonthaburi/102/2009_H1N1_         RTQESECACVNGSCFTVMTDGPSDEQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Bangkok/INS427/2010_H1N1_         RTQESECACVNGSCFTVMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Guangdong/2361/2009_H1N1_         RTQESECACVNGSCFTVMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Haishu/SWL110/2010_H1N1_NA        RTQESECACVNGSCFTVMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Mexico/InDRE797/2010_H1N1_        RTQESECACVNGSCFTVMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Khon                              RTQESECACVNGSCFTVMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/California/07/2009_H1N1_          RTQESECACVNGSCFTVMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Bangkok/INS491/2010_H1N1_         RTQESECACVNGSCFTVMTDGPNDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Bangkok/INS500/2010_H1N1_         RTQESECACVNGSCFTVMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Thailand/CU-H2358/2010_H1N1_      RTQESECACVNGSCFTIMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
NA                                  RTQESECACVNGSCFTIMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/Bangkok/INS477/2010_H1N1_         RTQESECACVNGSCFTIMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNY 250
A/American_black_duck/New_Brun      RTQESECACINGSCFTIMTDGPSNGQASYKIFKIEKGKVVKSVELNAPNY 250
                                    ********.******.*****.: *******:*****:*****:***** 

A/Malaysia/5112/2009_H1N1_          HYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Nonthaburi/102/2009_H1N1_         HYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Bangkok/INS427/2010_H1N1_         HYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Guangdong/2361/2009_H1N1_         HYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Haishu/SWL110/2010_H1N1_NA        YYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Mexico/InDRE797/2010_H1N1_        YYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Khon                              HYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/California/07/2009_H1N1_          HYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Bangkok/INS491/2010_H1N1_         HYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Bangkok/INS500/2010_H1N1_         HYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Thailand/CU-H2358/2010_H1N1_      YYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
NA                                  YYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/Bangkok/INS477/2010_H1N1_         HYEECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGD 300
A/American_black_duck/New_Brun      HYEECSCYPDASEVMCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGVFGD 300
                                    :*********:**: ****************************:*** 
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Amino acid at position 274-276

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A/Malaysia/5112/2009_H1N1_          NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRNGFEMI 350
A/Nonthaburi/102/2009_H1N1_         NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRNGFEMI 350
A/Bangkok/INS427/2010_H1N1_         NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRNGFEMI 350
A/Guangdong/2361/2009_H1N1_         NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISLRNGFEMI 350
A/Haishu/SWL110/2010_H1N1_NA        NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRNGFEMI 350
A/Mexico/InDRE797/2010_H1N1_        NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRNGFEMI 350
A/Khon                              NPRPNDKKGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRNGFEMI 350
A/California/07/2009_H1N1_          NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRNGFEMI 350
A/Bangkok/INS491/2010_H1N1_         NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRNGFEMI 350
A/Bangkok/INS500/2010_H1N1_         NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRNGFEMI 350
A/Thailand/CU-H2358/2010_H1N1_      NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISSRKGFEMI 350
NA                                  NPRPNDKTGSCGPVSSNGANGIKGFSFKYGNGVWIGRTKSISSRKGFEMI 350
A/Bangkok/INS477/2010_H1N1_         NPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSFSSRKGFEMI 350
A/American_black_duck/New_Brun      NPRPNDGTGSCGPVSSNGAYGVKGFSFRYGNGVWIGRTKSTSSRSGFEMI 350
                                    ****** .********** *:*****:************ * *.*****

A/Malaysia/5112/2009_H1N1_          WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Nonthaburi/102/2009_H1N1_         WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Bangkok/INS427/2010_H1N1_         WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Guangdong/2361/2009_H1N1_         WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Haishu/SWL110/2010_H1N1_NA        WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Mexico/InDRE797/2010_H1N1_        WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Khon                              WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/California/07/2009_H1N1_          WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Bangkok/INS491/2010_H1N1_         WDPNGWTGTDNNFSVKQDIIGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Bangkok/INS500/2010_H1N1_         WDPNGWTGTDNNFSVKQDIIGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Thailand/CU-H2358/2010_H1N1_      WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
NA                                  WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/Bangkok/INS477/2010_H1N1_         WDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWV 400
A/American_black_duck/New_Brun      WDPDGWTETDNSFSVKQDIVAITDWSGYSGSFVQHPELTGLDCMRPCFWV 400
                                    ***:*** *** **:****:.*.:*********************:******

A/Malaysia/5112/2009_H1N1_          ELIRGRPKENTIWTSGSSISF 421
A/Nonthaburi/102/2009_H1N1_         ELIRGRPKENTIWTSGSSISF 421
A/Bangkok/INS427/2010_H1N1_         ELIRGRPKENTIWTSGSSISF 421
A/Guangdong/2361/2009_H1N1_         ELIRGRPKENTIWTSGSSISF 421
A/Haishu/SWL110/2010_H1N1_NA        ELIRGRPKENTIWTSGSSISF 421
A/Mexico/InDRE797/2010_H1N1_        ELIRGRPKENTIWTSGSSISF 421
A/Khon                              ELIRGRPKENTIWTSGSSISF 421
A/California/07/2009_H1N1_          ELIRGRPKENTIWTSGSSISF 421
A/Bangkok/INS491/2010_H1N1_         ELIRGRPKENTIWTSGSSISF 421
A/Bangkok/INS500/2010_H1N1_         ELIRGRPKENTIWTSGSSISF 421
A/Thailand/CU-H2358/2010_H1N1_      ELIRGRPKENTIWTSGSSISF 421
NA                                  ELIRGRPKENTIWTSGSSISF 421
A/Bangkok/INS477/2010_H1N1_         ELIRGRPKENTIWTSGSSISF 421
A/American_black_duck/New_Brun      ELIRGRPKENTIWTSGSSISF 421
                                    *********************
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NA (clinical sample No.14) sequence with reference strain (A/Thailand/CU-H2358/2010(H1N1) H275Y)

NA gene analysis, the whole sequence of NA gene was performed. However the chromatogram of nucleotide sequence was not complete only the amplifying product of 1,265 bp was sequenced by ABI 3130 DNA sequencer (Perkin-Elmer Cetus, USA). The sequence of nucleotide was edited and analyzed by DNA baser program. The nucleotide sequence of NA gene is the following;

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CLUSTAL 2.1 multiple sequence alignment

A/Thailand/CU-H2358/2010_H1N1_      ATGAATCCAAACCAAAAGATAATAACCATTGGTTCGGTCTGTATGACAAT 50
NA                                  --------------------------------------------------

A/Thailand/CU-H2358/2010_H1N1_      TGGAATGGCTAACTTAATATTACAAATTGGAAACATAATCTCAATATGGA 100
NA                                  ----------------------CAAATTGGAAACATAATCTCAATATGGA 28
                                                          ****************************

A/Thailand/CU-H2358/2010_H1N1_      TTAGCCACTCAATTCAACTTGGGAATCAAAGTCAGATTGAAACATGCAAT 150
NA                                  TTAGCCACTCAATTCAACTTGGGAATCAAAGTCAGATTGAAACATGCAAC 78
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      CAAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATGT 200
NA                                  CAAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATGT 128
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      TAACATCAGCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGA 250
NA                                  TAACATCAGCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGA 178
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      AATTAGCGGGCAATTCCTCTCTCTGCCCTGTTAGTGGATGGGCTATATAC 300
NA                                  AATTAGCGGGCAATTCCTCTCTCTGCCCTGTTAGTGGATGGGCTATATAC 228
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      AGTAAAGACAACAGTATAAGAATCGGTTCCAAGGGGGATGTGTTTGTCAT 350
NA                                  AGTAAAGACAACAGTATAAGAATCGGTTCCAAGGGGGATGTGTTTGTCAT 278
                                     **************************************************

A/Thailand/CU-H2358/2010_H1N1_      AAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCAGAACCTTCTTCT 400
NA                                  AAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCAGAACCTTCTTCT 328
                                    **************************************************


A/Thailand/CU-H2358/2010_H1N1_      TGACTCAAGGGGCCTTGCTAAATGACAAACATTCCAATGGAACCATTAAA 450
NA                                  TGACTCAAGGGGCCTTGCTAAATGACAAACATTCCAATGGAACCATTAAA 378
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      GACAGGAGCCCATATCGAACCCTAATGAGCTGTCCTATTGGTGAAGTTCC 500
NA                                  GACAGGAGCCCATATCGAACCCTAATGAGCTGTCCTATTGGTGAAGTTCC 428
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      CTCTCCATACAACTCAAGATTTGAGTCAGTCGCTTGGTCAGCAAGTGCTT 550
NA                                  CTCTCCATACAACTCAAGATTTGAGTCAGTCGCTTGGTCAGCAAGTGCTT 478
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      GTCATGATGGCATCAATTGGCTAACAATTGGAATTTCTGGCCCAGACAAT 600
NA                                  GTCATGATGGCATCAATTGGCTAACAATTGGAATTTCTGGCCCAGACAAT 528
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      GGGGCAGTGGCTGTGTTAAAGTACAACGGCATAATAACAGACACTATCAA 650
NA                                  GGGGCAGTGGCTGTGTTAAAGTACAACGGCATAATAACAGACACTATCAA 578
                                     **************************************************

A/Thailand/CU-H2358/2010_H1N1_      GAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAATGTGCATGTG 700
NA                                  GAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAATGTGCATGTG 628
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      TAAATGGTTCTTGCTTTACCATAATGACCGATGGACCAAGTGATGGACAG 750
NA                                  TAAATGGTTCTTGCTTTACCATAATGACCGATGGACCAAGTGATGGACAG 678
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      GCCTCATACAAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGT 800
NA                                  GCCTCATACAAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGT 728
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      CGAAATGAATGCCCCTAATTATTACTATGAGGAATGCTCCTGTTATCCTG 850
NA                                  CGAAATGAATGCCCCTAATTATTACTATGAGGAATGCTCCTGTTATCCTG 778
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      ATTCTAGTGAAATCACATGTGTGTGCAGGGATAACTGGCATGGCTCGAAT 900
NA                                  ATTCTAGTGAAATCACATGTGTGTGCAGGGATAACTGGCATGGCTCGAAT 828
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      CGACCGTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACAT 950
NA                                  CGACCGTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACAT 878
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      ATGCAGTGGGATTTTCGGAGACAATCCACGCCCTAATGATAAGACAGGCA 1000
NA                                  ATGCAGTGGGATTTTCGGAGACAATCCACGCCCTAATGATAAGACAGGCA 928
                                    **************************************************
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A/Thailand/CU-H2358/2010_H1N1_      GTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGAGTAAAAGGATTTTCA 1050
NA                                  GTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGAATAAAAGGATTTTCA 978
                                    ********************************** **************

A/Thailand/CU-H2358/2010_H1N1_      TTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTC 1100
NA                                  TTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTC 1028
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      AAGAAAAGGTTTTGAGATGATTTGGGATCCAAACGGATGGACTGGGACAG 1150
NA                                  AAGAAAAGGTTTTGAGATGATTTGGGATCCAAACGGATGGACTGGGACAG 1078
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      ACAATAACTTCTCAATAAAGCAAGATATCGTAGGAATAAATGAGTGGTCA 1200
NA                                  ACAATAACTTCTCAATAAAGCAAGATATCGTAGGAATAAATGAGTGGTCA 1128
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      GGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGGCTGGATTG 1250
NA                                  GGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGGCTGGATTG 1178
                                    **************************************************

A/Thailand/CU-H2358/2010_H1N1_      TATAAGACCTTGCTTCTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGA 1300
NA                                  TATAAGACCTTGCTTCTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGA 1228
                                   **************************************************

A/Thailand/CU-H2358/2010_H1N1_      ACACAATCTGGACTAGCGGGAGCAGCATATCCTTTTGTGGTGTAAACAGT 1350
NA                                  ACACAATCTGGACTAGCGGGAGCAGCATATCCTTTTG------------ 1265
                                    ************************************

A/Thailand/CU-H2358/2010_H1N1_      GACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTTACCAT 1400
NA                                  -------------------------------------------------

A/Thailand/CU-H2358/2010_H1N1_      TGACAAGTAA 1410
NA                                  ----------
```

Result of real time RT-PCR

No.	Lab No.	Subtype	Segment	RNA/DNA	Date of collection	Extraction Date	comment		RNA No.		InfluA	H1 seasonal	H3 seasonal	Date of last real time RT-PCR	Date of last real time RT-PCR
							Keep at								
1	102600327	H1N1		RNA	17/9/53	18/10/2010	-80° c	60 µl	3	+				21/10/2010	
2	102600331	H1N1		RNA	17/9/53	18/10/2010	-80° c	60 µl	4	+				21/10/2010	
3	102600331	H1N1		RNA	17/9/53	18/10/2010	-80° c	60 µl	5	+				21/10/2010	
4	102600348	H1N1		RNA	17/9/53	18/10/2010	-80° c	60 µl	6	+				21/10/2010	
5	102600363	H1N1		RNA	17/9/53	18/10/2010	-80° c	60 µl	7	+				21/10/2010	
6	102600391	H1N1		RNA	17/9/53	18/10/2010	-80° c	60 µl	8	+				21/10/2010	
7	102600395	H1N1		RNA	17/9/53	18/10/2010	-80° c	60 µl	9	+				21/10/2010	
8	102260191			RNA	14/8/53	18/10/2010	-80° c	60 µl	10	-				21/10/2010	
9	102660201	H1N1		RNA	14/8/53	18/10/2010	-80° c	60 µl	11	+				21/10/2010	
10	102260203	H1N1		RNA	18/9/53	18/10/2010	-80° c	60 µl	12	+				21/10/2010	
11	102610111			RNA	18/9/53	18/10/2010	-80° c	60 µl	13	-	+		+	21/10/2010	30/12/2011
12	102610204	H1N1		RNA	19/9/53	18/10/2010	-80° c	60 µl	14	+				21/10/2010	
13	102620117			RNA	20/9/53	18/10/2010	-80° c	60 µl	15	-	+		+	21/10/2010	1/4/2012
14	102620230	H1N1		RNA	20/9/53	18/10/2010	-80° c	60 µl	16	+				21/10/2010	
15	1026300029	H1N1		RNA	20/9/53	18/10/2010	-80° c	60 µl	17	+				21/10/2010	
16	102630031	H1N1		RNA	20/9/53	18/10/2010	-80° c	60 µl	18	+				21/10/2010	
17	102630032	H1N1		RNA	20/9/53	18/10/2010	-80° c	60 µl	19	+				21/10/2010	
18	102630329	H1N1		RNA	20/9/53	18/10/2010	-80° c	60 µl	20	+				21/10/2010	
19	102630427	H1N1		RNA	20/9/53	18/10/2010	-80° c	60 µl	21	+				21/10/2010	
20	102630440	H1N1		RNA	20/9/53	18/10/2010	-80° c	60 µl	22	+				21/10/2010	
21	102630515	H1N1		RNA	20/9/53	18/10/2010	-80° c	60 µl	23	+				2211/2010 (2)	
22	102630521			RNA	20/9/53	18/10/2010	-80° c	60 µl	24	-	+		+	2211/2010 (2)	1/4/2012
23	102640332	H1N1		RNA	20/9/53	18/10/2010	-80° c	60 µl	25	+				2211/2010 (2)	
24	102640333	H1N1		RNA	21/9/53	18/10/2010	-80° c	60 µl	26	+				2211/2010 (2)	
25	102640377	H1N1		RNA	21/9/53	18/10/2010	-80° c	60 µl	27	+				2211/2010 (2)	
26	102640393	H1N1		RNA	21/9/53	18/10/2010	-80° c	60 µl	28	+				2211/2010 (2)	
27	102640409	H1N1		RNA	21/9/53	18/10/2010	-80° c	60 µl	29	+				2211/2010 (2)	
28	102650285	H1N1		RNA	22/9/53	18/10/2010	-80° c	60 µl	30	+				2211/2010 (2)	
29	102650294	H1N1		RNA	22/9/53	18/10/2010	-80° c	60 µl	31	+				2211/2010 (2)	
30	102650311	H1N1		RNA	22/9/53	18/10/2010	-80° c	60 µl	32	+				2211/2010 (2)	
31	102650340	H1N1		RNA	22/9/53	18/10/2010	-80° c	60 µl	33	+				2211/2010 (2)	
32	102650405			RNA	22/9/53	18/10/2010	-80° c	60 µl	34	-	+ cp 29.48			2211/2010 (2)	
33	102120146	H1N1		RNA	31/7/53	18/10/2010	-80° c	60 µl	35	+				2211/2010 (2)	
34	102660118	H1N1		RNA	23/9/53	18/10/2010	-80° c	60 µl	36	+				2211/2010 (2)	
35	102660350	H1N1		RNA	23/9/53	18/10/2010	-80° c	60 µl	37	+				2211/2010 (2)	
36	102660547			RNA	23/9/53	18/10/2010	-80° c	60 µl	38	-	+ (cp 31.05)			2211/2010 (2)	
37	102680189	H1N1		RNA	25/9/53	18/10/2010	-80° c	60 µl	39	+				2211/2010 (2)	
38	102700485	H1N1		RNA	27/9/53	18/10/2010	-80° c	60 µl	40	+				2211/2010 (2)	
39	102700503	H1N1		RNA	27/9/53	18/10/2010	-80° c	60 µl	41	+				2211/2010 (2)	
40	102700566	H1N1		RNA	27/9/53	18/10/2010	-80° c	60 µl	42	+				2211/2010 (2)	
41	102710011			RNA	28/9/53	19/10/2010	-80° c	60 µl	43	-	+			2211/2010 (2)	

No.	Lab No.	Subtype	Segment	RNA/DNA	Date of collection	Extraction Date	comment				InfluA	H1 seasonal	H3 seasonal	Date of test real time RT-PCR	Date of test real time RT-PCR
							Keep at								
42	102710392			RNA	28/9/53	19/10/2010	-80° c	60 µl	44	-	+ (cp 29.59)			2211/2010 (2)	
43	102720409	H1N1		RNA	29/9/53	19/10/2010	-80° c	60 µl	45	+				2211/2010 (2)	
44	102730344			RNA	30/9/53	19/10/2010	-80° c	60 µl	46	-	+		+	2211/2010 (1)	1/4/2012
45	102730403	H1N1		RNA	30/9/53	19/10/2010	-80° c	60 µl	47	+				2211/2010 (1)	
46	102730442			RNA	30/9/53	19/10/2010	-80° c	60 µl	48	-	+			15/11/2010	
47	102740225	H1N1		RNA	10/1/2010	19/10/2010	-80° c	60 µl	49	+				15/11/2010	
48	102740245			RNA	10/1/2010	19/10/2010	-80° c	60 µl	50	-	+		+	15/11/2010	1/4/2012
49	102740423			RNA	10/1/2010	19/10/2010	-80° c	60 µl	51	-	+ cp 32.95			15/11/2010	
50	102770326	H1N1		RNA	10/4/2010	19/10/2010	-80° c	60 µl	52	+				15/11/2010	
51	102780006	H1N1		RNA	10/5/2010	19/10/2010	-80° c	60 µl	53	+				18/11/2010	
52	102780503			RNA	10/5/2010	19/10/2010	-80° c	60 µl	54	-			+	18/11/2010	1/4/2012
53	102790077			RNA	10/6/2010	19/10/2010	-80° c	60 µl	55	-	+ cp 30.21			18/11/2010	
54	102790315	H1N1		RNA	10/6/2010	19/10/2010	-80° c	60 µl	56	+				18/11/2010	
55	102790337			RNA	10/6/2010	19/10/2010	-80° c	60 µl	57	-	+		+	18/11/2010	1/4/2012
56	102790353	H1N1		RNA	10/6/2010	19/10/2010	-80° c	60 µl	58	+				18/11/2010	
57	102790428	H1N1		RNA	10/6/2010	19/10/2010	-80° c	60 µl	59	+				18/11/2010	
58	102790483	H1N1		RNA	10/6/2010	19/10/2010	-80° c	60 µl	60	+				18/11/2010	
59	102800315			RNA	10/6/2010	19/10/2010	-80° c	60 µl	61	-			+	18/11/2010	1/4/2012
60	102800386	H1N1		RNA	10/7/2010	19/10/2010	-80° c	60 µl	62	+				18/11/2010	
61	102810306	H1N1		RNA	10/7/2010	19/10/2010	-80° c	60 µl	63	+				19/11/2010	
62	102810463	H1N1		RNA	10/8/2010	19/10/2010	-80° c	60 µl	64	+				19/11/2010	
63	102810550			RNA	10/8/2010	19/10/2010	-80° c	60 µl	65	-	+		+	19/11/2010	1/4/2012
64	102840299			RNA	10/11/2010	19/10/2010	-80° c	60 µl	66	-	+		+	19/11/2010	1/4/2012
65	102840509	H1N1		RNA	10/11/2010	19/10/2010	-80° c	60 µl	67	+				19/11/2010	
66	102840669	H1N1		RNA	10/11/2010	19/10/2010	-80° c	60 µl	68	-	+ cp 34.16			19/11/2010	
67	102850366	H1N1		RNA	10/12/2010	19/10/2010	-80° c	60 µl	69	+				2211/2010 (1)	
68	102850415	H1N1		RNA	10/12/2010	19/10/2010	-80° c	60 µl	70	+				2211/2010 (1)	
69	102850443			RNA	10/12/2010	19/10/2010	-80° c	60 µl	71	-	+		+	2211/2010 (1)	1/4/2012
70	102820156	H1N1		RNA	10/9/2010	19/10/2010	-80° c	60 µl	72	+				2211/2010 (1)	
71	102800467			RNA	unknown	19/10/2010	-80° c	60 µl	73	-	+		+	2211/2010 (1)	1/4/2012
72	102860234			RNA	13/10/2010	19/10/2010	-80° c	60 µl	74	-	+		+	2211/2010 (1)	1/4/2012
73	102520462	H1N1		RNA	9/9/2010	19/10/2010	-80° c	60 µl	75	+				2211/2010 (1)	
74	102520466	H1N1		RNA	9/9/2010	19/10/2010	-80° c	60 µl	76	+				2211/2010 (1)	
75	102520456	H1N1		RNA	9/9/2010	19/10/2010	-80° c	60 µl	77	+				20/10/2010	
76	102520438	H1N1		RNA	9/9/2010	19/10/2010	-80° c	60 µl	78	+				20/10/2010	
77	102520386	H1N1		RNA	9/9/2010	19/10/2010	-80° c	60 µl	79	+				20/10/2010	
78	102520419	H1N1		RNA	9/9/2010	19/10/2010	-80° c	60 µl	80	+				20/10/2010	
79	102540165	negative		RNA	9/11/2010	19/10/2010	-80° c	60 µl	81	-				20/10/2010	
80	102540156	H1N1		RNA	9/11/2010	19/10/2010	-80° c	60 µl	82	+				20/10/2010	
81	102580508	H1N1		RNA	15/9/2010	19/10/2010	-80° c	60 µl	83	+				20/10/2010	
82	102580532	H1N1		RNA	15/9/2010	19/10/2010	-80° c	60 µl	84	+				20/10/2010	
83	102580475	H1N1		RNA	15/9/2010	22/11/2010	-80° c	60 µl	85	+				2211/2010 (2)	

No.	Lab No.	Subtype	Segment	RNA/DNA	Date of collection	Extraction Date	comment		RNA No.	[illegible]	InfluA	H1 seasonal	H3 seasonal	Date of last real time RT-PCR	Date of test real time RT-PCR
							Keep at	[illegible]							
84	102410230	H1N1		RNA	29/8/2010	19/10/2010	-80° C	60 µl	86	+				20/10/2010	
85	102860306	H1N1		RNA	13/10/2010	19/10/2010	-80° C	60 µl	87	+				20/10/2010	
									can't detect by real-time RT-PCR and recheck H1 agint						
									weakly positive flu A and H1						
									all color can't picture to show						

VITAE

VITAE

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