การกระจายตัวของเชื้อไวรัสไข้หวัดใหญ่ที่ดื้อต่อยาต้านเชื้อไวรัสในโรงพยาบาลพระมงกุฎเกล้า

นางสาวมิญช์ คร้ามอยู่

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ระดับโมเลกุลทางจุลชีววิทยาทางการแพทย์และวิทยาภูมิคุ้มกัน ภาควิชาเวช
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Distribution of Anti-viral resistant influenza viruses in Phramongkutklao Hospital

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Transfusion Medicine and Clinical Microbiology
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มิญช์ คร้ามอยู่ : การกระจายตัวของเชื้อไวรัสไข้หวัดใหญ่ที่ดื้อต่อยาต้านเชื้อไวรัสในโรงพยาบาลพระมงกุฎเกล้า (Distribution of Anti-viral resistant influenza viruses in Phramongkutklao Hospital) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. เขมาภรณ์ บุญบำรุง, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: Damon W. Ellison, ดร. ชลธิชา คลังทอง, 116 หน้า.

เชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ A pandemic 2009 H1N1 เชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ A H3N2 และเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ B เป็นเชื้อไข้หวัดใหญ่ที่ก่อโรคในประเทศไทย ยาที่ใช้ในการรักษาปัจจุบันมี 2 กลุ่มคือ กลุ่ม Adamantane ยับยั้งการทำงานของ M2 channel ในกลุ่มเชื้อไวรัสสายพันธุ์ A เท่านั้น และกลุ่ม Neuraminidase inhibitor (NAIs) ยับยั้งการทำงานของเอนไซม์ Neuraminidase ซึ่งสามารถใช้รักษาได้ทั้งสองสายพันธุ์ เนื่องจากเชื้อไวรัสไข้หวัดใหญ่ชนิด A พบการดื้อต่อยา Adamantane มากกว่าร้อยละ 99 ทำให้ NAIs เป็นยากลุ่มเดียวที่องค์การอนามัยโลกแนะนำให้ใช้ในการรักษา ยา Oseltamivir เป็นยารับประทานในกลุ่ม NAIs ที่มีการใช้อย่างแพร่หลายมากที่สุด แต่เริ่มมีการพบเชื้อไวรัสไข้หวัดใหญ่ที่ดื้อต่อยาชนิดนี้ในกลุ่มประเทศยุโรปและมีการแพร่กระจายไปในหลายภูมิภาคทั่วโลก โดยวิธี Pyrosequencing เป็นวิธีที่รวดเร็วและสามารถตรวจวิเคราะห์จำนวนตัวอย่างที่มีปริมาณมากได้ เหมาะสำหรับการตรวจคัดกรองเพื่อหายีนบ่งชี้ที่สัมพันธ์กับความไวต่อยาต้านไวรัส ในขณะที่ วิธี Neuraminidase inhibitor (NAI) assay เป็นวิธีการตรวจหาการดื้อต่อยาต้านไวรัสแบบ phenotypic ด้วยการเพาะเลี้ยงเชื้อ รายงานการเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ดื้อต่อยาต้านไวรัสในประเทศไทยยังคงมีจำนวนน้อย การศึกษานี้จึงออกแบบเพื่อทำการศึกษาการกระจายตัวของเชื้อไวรัสไข้หวัดใหญ่ที่ดื้อต่อยาต้านไวรัสในเขตกรุงเทพฯ ประเทศไทย โดยเปรียบเทียบผลระหว่างวิธี pyrosequencing และวิธี NAI assay จากการทดลองพบว่าเชื้อไข้หวัดใหญ่ตามฤดูกาลชนิด A ทั้งหมดมียีนบ่งชี้ที่ดื้อต่อยาในกลุ่ม Adamantane แต่ยังคงไวต่อยาต้านไวรัสในกลุ่ม Oseltamivir และผลของการตรวจวิเคราะห์ทั้งสองวิธีพบว่า ยากลุ่ม Oseltamivir สามารถต้านเชื้อไวรัสได้อย่างมีประสิทธิภาพในตัวอย่างเชื้อไวรัสไข้หวัดใหญ่ทุกสายพันธุ์ ถึงแม้ว่าอุบัติการณ์เชื้อไวรัสไข้หวัดใหญ่ดื้อต่อยา Oseltamivir ในประเทศไทยจะพบอัตราที่น้อย แต่การสำรวจและเฝ้าระวังการดื้อต่อยา Oseltamivir ในเชื้อไวรัสไข้หวัดใหญ่ยังคงมีความจำเป็นต่อการแนะนำในการใช้ยาเพื่อรักษาผู้ป่วยต่อไป

ภาควิชา	เวชศาสตร์การธนาคารเลือดและจุลชีววิทยาคลินิก	ลายมือชื่อนิสิต ----------
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สาขาวิชา	วิทยาศาสตร์ระดับโมเลกุลทางจุลชีววิทยาทางการแพทย์และวิทยาภูมิคุ้มกัน	ลายมือชื่อ อ.ที่ปรึกษาร่วม ----------
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MIN KRAMYOO: Distribution of Anti-viral resistant influenza viruses in Phramongkutklao Hospital. ADVISOR: KHAEMAPORN BOONBUMRUNG, Ph.D., CO-ADVISOR: DAMON W. ELLISON, Ph.D., CHONTICHA KLUNGTHONG, Ph.D., 116 pp.

Influenza A pandemic 2009 H1N1, influenza A H3N2, and influenza B have all been in circulating in Thailand. The data indicate increasing influenza like illness as reported by the Thai National Influenza Center every week. Adamantane an M2 channel blocker and Neuraminidase inhibitors (NAIs) are two classes of antiviral that are available for medical health care. Influenza A resistance to Adamantane drugs is greater than 99% worldwide leaving NAI agents as the only class which the World Health Organization (WHO) recommends for treatment. Oral Oseltamivir is the most commonly used agent worldwide, but many European countries have found Oseltamivir resistance emerging and spreading around the world. Pyrosequencing is a rapid and high throughput method for screening gene markers associated with antiviral susceptibility whereas, the NAI assay is a tool for monitoring phenotypic antiviral resistant by cell culture. Antiviral resistant influenza virus surveillance reports are limited in Thailand. This study was designed to determine the distribution of antiviral resistant influenza virus in Bangkok, Thailand by comparing the results of pyrosequencing and the NAI assay. All seasonal influenza A samples tested had genetic markers consistent with known resistance markers to Adamantane but were still susceptible to Oseltamivir. Both genotypic and phenotypic results showed that Oseltamivir was effective against all influenza viruses in this study. Although antiviral resistance surveillance of influenza virus in Thailand has found a very low percentage of resistant strains the use of Oseltamivir to treat influenza patients should still be monitored and continued surveillance of influenza for resistance in recommended.

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Field of Study:	Molecular Science of Medical Microbiology and Immunology	Co-Advisor's Signature	.....
		Co-Advisor's Signature	.....
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CHAPTER I

INTRODUCTION

Influenza is respiratory infectious disease that cause mild to severe illness(1). The disease can occur in tropical and subtropical regions (2). The number of morbidity and mortality of influenza in Thailand had increased from 2013(3-6). Only influenza A and B are the determinant of epidemics of respiratory and widespread of pandemic(7). Genetic reassortant of influenza A can express pandemic by virus infect to variety of mammal species whereas Influenza B only infect to human and can cause only epidemic(8). There are two antiviral drug groups for influenza therapy and prophylaxis(9). Adamantane is M2 ion channel blocker. At the present, this drug is not recommended for using because adamantane resistance has spread in world wild with central nervous system side effects (10). Therefore neuraminidase inhibitors (NAIs) group is the only class of antivirals recommended by World Health Organization (WHO) for treatment of influenza A and B infectious(11). There are incidences of NAI resistance at low level reported since 2008(12). Pyrosequencing was developed for detect antiviral resistance of influenza virus(13). For mutation gene detection, pyrosequencing is suitable to detect antiviral resistance surveillance with real-time read out, easily programmed and high throughput(14). Sanger Sequencing is the first generation of DNA sequencing method that need complication step for sequencing result(15). Then the method can confirm the identify mutant gene of this studies(16). To observe Oseltamvir resistant influenza virus by phenotypic method, Neuarminidase inhibitor (NAI) assay is available with virus isolation preparation(17). The result of NAI assay shows as IC50 value that refer to the NAI concentration could inhibit 50% of the NA enzyme activity of virus. Because NAIs is only drugging that against influenza virus in Thailand, the antiviral resistance of influenza virus surveillance is needed for health public. This study would observe NAIs resistant of influenza virus in Bangkok, Thailand for applied the data for carefully and best influenza treatment in local area.

CHAPTER II

LITERATURE REVIEW

2.1 Influenza

Influenza virus is the causative agent of Influenza. The signs and symptom of seasonal influenza virus by influenza type A and type B are well known (18). The respiratory symptoms begin suddenly which lead to mild to severe illness, in the most severe cases death can occur (1). In 6%-33% of seasonal influenza virus infected patients will have mild illness without fever and/or asymptomatic infection (19). The influenza virus transmission from asymptomatic infectious patient is unpredictable, 36% of baseline and convalescent serum samples collected from patients who had serologic evidence of 2009 H1N1 virus infection did not find virus or report illness (19). Children who have influenza virus infection commonly have otitis media, nausea, vomiting (20) and some of them maybe have gastrointestinal symptom such as abdominal pain, and diarrhea. An incubation period of 1 to 5 days before influenza symptoms occur is normal (average 2 days). In general the fever persist for 3 to 5 days but dry cough and malaise may exist for several weeks(21). Moreover influenza can include sinusitis, primary viral pneumonia, secondary bacterial pneumonia, coinfections with other viral and bacterial pathogen exacerbation of underlying cadiac or pulmonary disease, myositis including rhabdomyolysis, neurologic problems such as seizures, acute encephalitis, and postinfectious encephalopathy, the aspirin use associated symptom (Reye's syndrome), and myopericaditis. The complications due to other illnesses during an influenza infection can lead to death (22) influenza illness is

normally a self-limiting infection and is resolved after 3-7 days. The beginning symptoms in young children may mimic bacterial sepsis with high fever (23). In 6%- 20% of influenza infectious in children who reported febrile seizures require hospitalization. Study of laboratory confirmed hospitalized influenza associated pneumonia in children compare with hospitalized influenza infectious children without pneumonia reported that the children hospitalized with influenza associated pneumonia had higher risk for intensive care admission, respiratory failure, and death than the group without pneumonia. The influenza associated pneumonia was associated significantly with young children under 5 years old with asthma. Young health people are also at risk for severe illness from influenza. There is one study reporting that 50% (19 of 38) of previous healthy adults had severe viral pneumonia caused by influenza; 29% (11 of 38) had a coactive or secondary bacterial pneumonia, 63% (24 of 38) need intensive care admission for a median of 11 days, and 45% (17 of 38) died. The 2009 H1N1 pandemic strain of influenze seems to be associated viral pneumonia clinical syndrome and in some cases led to shock and respiratory failure (24, 25). Infection with any influenza virus strain can be associated with bacteria pneumonia and other bacterial coinfections. In 20%- 38% of 2009 H1N1 infectious patients who required intensive care admission had suspected or identified *Staphylococcus aureus*, *Streptococcus pneumonia, Streptococcus pyogenes*, or other virulent bacteria for secondary or concomitant bacterial pneumonia. Severe illness can be cause by secondary bacteria pneumonia or bacterial infection. A major reason of morbidity and mortality is exacerbation of underlying comorbidities such as asthma, chronic obstructive pulmonary disease (COPD) or cardiac disease. In 50%-80% of adults and children requiring hospitalization there were one or

more underlying comorbidities found (24, 25). The risk of severe illness is greater in pregnant and postpartum woman (within 2 weeks of delivery) for hospitalization or death (24-27). Population based studies have been used to study hospitalized children with laboratory-confirmed seasonal influenza. Even though most of them had a short hospitalization under 2 days, there was a 4%-11% chance of children to be hospitalized with laboratory confirmed cases and need treatment in the intensive care unit of which 3% required mechanical ventilation (28). Influenza infection is complicated and difficult to differentiate from other respiratory pathogens on the basis of signs and symptoms alone (20) (Table 1).

Table 1 The differentiation of influenza symptoms from cold symptoms as published by Centers for Disease Control and Prevention

Signs and Symptoms	Influenza	Cold
Symptom onset	Abrupt	Gradual
Fever	Usual; lasts 3-4 days	Rare
Aches	Usual; often severe	Slight
Chills	Fairly common	Uncommon
Fatigue, weakness	Usual	Sometimes
Sneezing	Sometimes	Common
Stuffy nose	Sometimes	Common
Sore throat	Sometimes	Common
Chest discomfort, cough	Common; can be severe	Mild to moderate; hacking cough
Headache	Common	Rare

The prevalence of other respiratory pathogens and the level of influenza in the community is the reason for the variable of sensitivity and positive predictive value of clinical definitions (20). The estimated positive predictive value of a clinical case definition of influenza which has acute onset of cough and fever in generally healthy older adolescents and adults who lived in the areas with confirmed influenza virus circulation for laboratory-confirmed influenza virus infection varies around 79%-88%. The report of laboratory- confirmed influenza in children aged under 5 years old found that only 28% (22 of 79) of hospitalized children and 17% (47 of 274) of outpatient children were positive for influenza virus (23). The probability of experiencing general influenza signs and symptom such as fever and cough in young children was less. Study conducted during the winter influenza season have reported that children aged 5-12 years had predictive value of fever and cough together 71%-83%, while the children younger than 5 years had found the symptoms 64% predictive for influenza. In another study with children presenting with fever or symptoms of acute respiratory tract infection during influenza season demonstrated that there is 70% (55 of 79) chance of under 6 months being hospitalized with laboratory-confirmed influenza presented with fever and cough symptoms. If both symptoms are found in 6 months to 5 years hospitalization was 91% (74 of 81) (23). Some young people who become infected by 2009 H1N1 influenza virus had a predominance of atypical presentations involving primary dehydration, irritability and/or, poor oral intake. In studies of older patients it has been suggested they present with unclear clinical case definitions when assessed by clinical symptoms. In 30% of nonhospitalized elderly patients age >60 years and have the following symptoms fever, cough, and acute onset showed a positive

predictive value of influenza. In 53% of 56 elderly hospitalized patients age >65 with chronic cardiopulmonary disease, a combination of fever, cough, and illness of less than 7 days had confirmed for influenza virus infection. The missing influenza like- illness (ILI) symptoms were ineffective in ruling out influenza. The study reported only 44-51% presented with typical ILI symptoms. Cough was not predictive of laboratory- confirmed influenza virus infection in vaccinated elder patients with chronic lung disease, even if they had fever and/or fever and myalgia both. The clinical presentation of influenza C virus is mild illness and indistinguishable from the common cold(21). Therefore, patients who have respiratory symptoms or fever during influenza season should still consider influenza illness in the absence of laboratory confirmation (20).

2.2 Influenza virus

Influenza virus is a member of the Orthomyxoviridae family (29). There are three types that can be the cause of respiratory syndrome, influenza A, influenza B and influenza C (30). Only influenza A and B cause epidemics of respiratory illness and widespread of pandemics (7). The viruses have 7-8 segments of negative-sense single-stranded RNA contained within an enveloped virus particle that are either spherical or filamentous in morphology (31). Influenza A infection is linked to more severe illness than influenza B and C and is found worldwide. Influenza A can infect a variety of mammals such as humans, horses, pigs, ferrets, cats, and dogs including avian influenza strains. Then co-infections with multiple influenza A viruses in one host can result in genetic re-assortment of influenza A and the emergence of a new virus capable of causing a pandemic. Influenza B only infects humans (Man) and does not distribute into subtypes, but can divide to lineages and strains. There are two lineages of influenza B circulating at present; B/Yamagata and B/Victoria (8). Influenza C is a mild infection that does not cause epidemics (29).

Transmission of influenza can spread by respiratory secretions of infectious person which contain up to 10^5 virus particles/ml. Coughing, sneezing, and even talking can cause infectious aerosols to be secreted. Moreover, infectious secretions can spread with respiratory mucosa by direct contact or indirect contact. The influenza virus is able to attach and infect Columnar epithelium cells of the upper respiratory tract and initiated a new infection. Influenza virus has an incubation period of 1 to 4 days before the onset of clinical disease, infected hosts can transmit the virus to others shortly after symptom appear (18).

There are two surface glycoproteins; haemagglutinin(HA) and neuraminidase(NA) in the host-derived lipid membrane which forms the virus envelope(30). Influenza A are classified into subtypes by 15 haemagglutinin subtypes (H1-H15) and 9 neuraminidase subtypes (N1-N9) whereas influenza B has one subtype of haemagglutinin and one of neuraminidase. Only 3 haemagglutinin subtypes (H1, H2, and H3) and 2 neuraminidase subtypes (N1 and N2) have established stable circulation in the human population since 1918. HA facilitates

entry of the virus into host cells through attachment to sialic acid receptors (32). The HA molecule of influenza virus will bind neuraminic acid (sialic acids) on the surface of host cells to initiate attachment. HA from different strains of Influenza recognizes different receptors in different species. Sialic acid residues with an α2,6 linkage is the target of HA from human virus whereas HA molecules from avian viruses selectively bind sialic acid residues with an α2,3 linkage (33). To become biological active, a precursor molecule (HA glycoprotein) will exist and cleavage by cellular proteases of human host that are found in human respiratory tract activate the HA (18). Influenza virus is internalized through Receptor-mediated endocytosis and then the acidification of the endosome induces a conformational change in the HA molecule that triggers fusion of the viral and endosomal membranes and releases the contents of the virion into cytoplasm. During budding the HA contained in the lipid membrane of the budding virus particals binds to the cell surface sialic acid, the NA glycoprotein serves to cleave glycosidic linkages to sialic acid and releases virus particle alowing virus to spread to another host epithelial cell (30).

Influenza A and B are the most prevalent influenza infections found in humans. Evolution of influenza A genes has developed two to three times faster than the corresponding genes in influenza B viruses (34). Influenza A virus has genetic and antigenic variation of HA and NA protein(35). Since 1918, there have only been three haemagglutinin subtypes (H1, H2, and H3) and two neuraminidase subtypes (N1 and N2) circulating in the human population. For influenza B viruses, only one subtype of HA and NA are recognized. The first time influenza A H1N1 subtype was found was between 1918 and 1957. The genomes of previously circulating human virus and avian virus had reassorted and emerged to cause a pandemic in 1957 in Asian and 1968 in Hong Kong (36). The preceding subtype in 1957 and 1968 were replaced by the new subtype while the reappearance of influenza A H1N1 in 1977 did not replace the preceding H3N2 subtype. At the present, there are two influenza A subtypes; H1N1 and H3N2 in circulation with influenza B.

Influenza A pandemic H1N1 has circulated in human, avian and swine populations. The antigenic structure of H1 and the rate of accumulation of mutations in HA and NA genes are similar to H3 (37). Influenza B has two lineages from HA genes which diverged in early 1970. The two lineages have predominated at different times. Influenza B/Victoria/2/87 was the predominate virus in 1987 and B/Yamagata/16/88 appeared in 1988.

The appearance of the influenza virion is roughly spherical and surrounded by an envelope. As the virus multiplies it incorporates lipid membrane from host cell into their outer layer. The lipid membrane consists of protein linked to sugars called glycoproteins. Haemagglutinin (HA) and Neuraminidase (NA) are the major glycoproteins in the envelope of the virus and determine the subtype of influenza virus. The ratio of HA to NA on the surface of the virus is four to one (Figure1-2) (38). The Human immune system responds to these spikes and is able to generate protection against infection. The matrix protein of virus is located below the lipid membrane and is referred to as M1. M1 Functions to be strength the virion. Ion channel protein M2 or AM2 for influenza A is the viral protein that is embedded in lipid membrane and is the target of antiviral adamantanes and NA protein is the target of antiviral neuraminidase inhibitors (39). The Influenza B protein only shows slight differences from the influenza A proteins. RNA segment 7 of influenza B virus encodes the BM2 protein unique in the influnenza B life cycle (40). AM2 consist of the following domains a 24-residue N-terminal extracellular domain, a single internal hydrophobic domain of 19 residues that acts as a transmembrane (TM) domain and forms the pore, and a 54-residue cytoplasmic tail (41). By contrast the BM2 protein consists of a 7-residue ectodomain, a 19-residue TM domain, and an 82-residue cytoplasmic tail (42). AM2 and BM2 function as homotretramers but use different amino acid residues to create the two channels; AM2 (residues 37–41) and BM2 (residues 19–23) (43). The high proton selectivity of A/M2 is expressed by histidine residue 37 and the channel gate is conferred by tryptophan 41 (44). In similar roles BM2 channel has histidine residue 19 and tryptophan 23 acting as the channel gate (45). Although influenza B contains the M2 protein , adamantane drug group is not effective against influenza B virus (46).

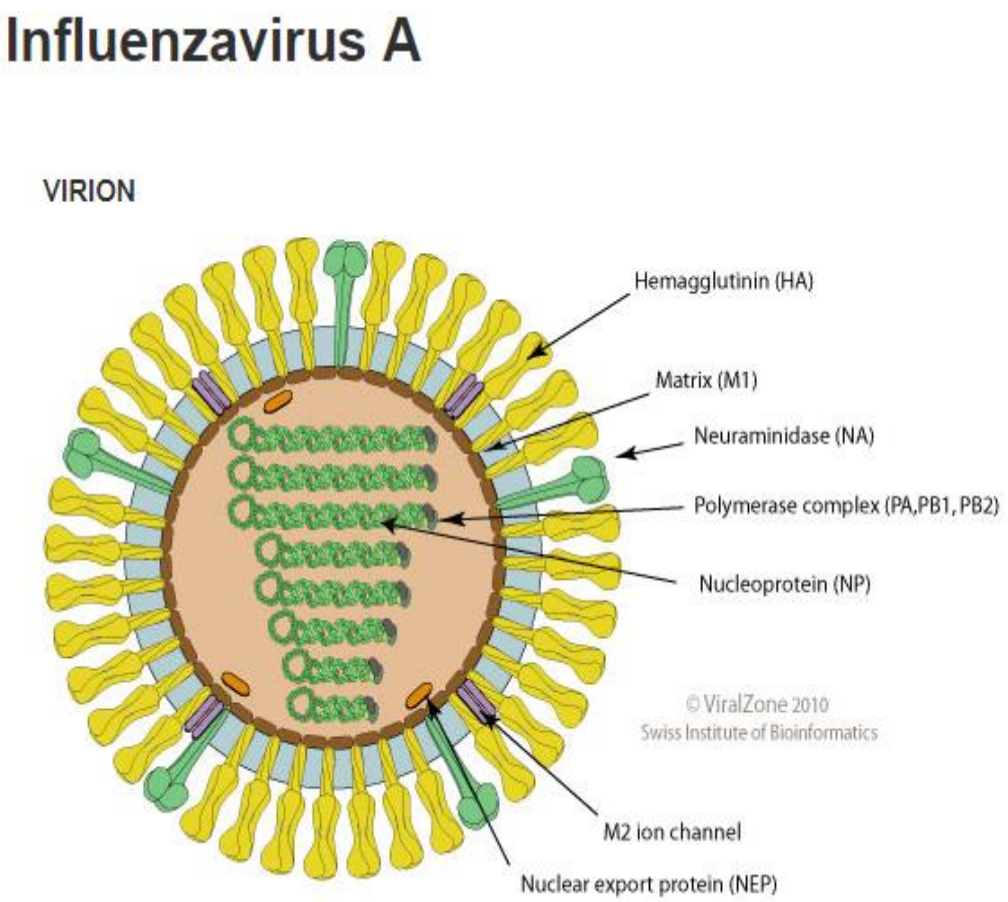


Figure 1 The structure of influenza A virus with HA and NA protiens. AM2 ion channel appears on Flu A only for antiviral adamantane target site(47)

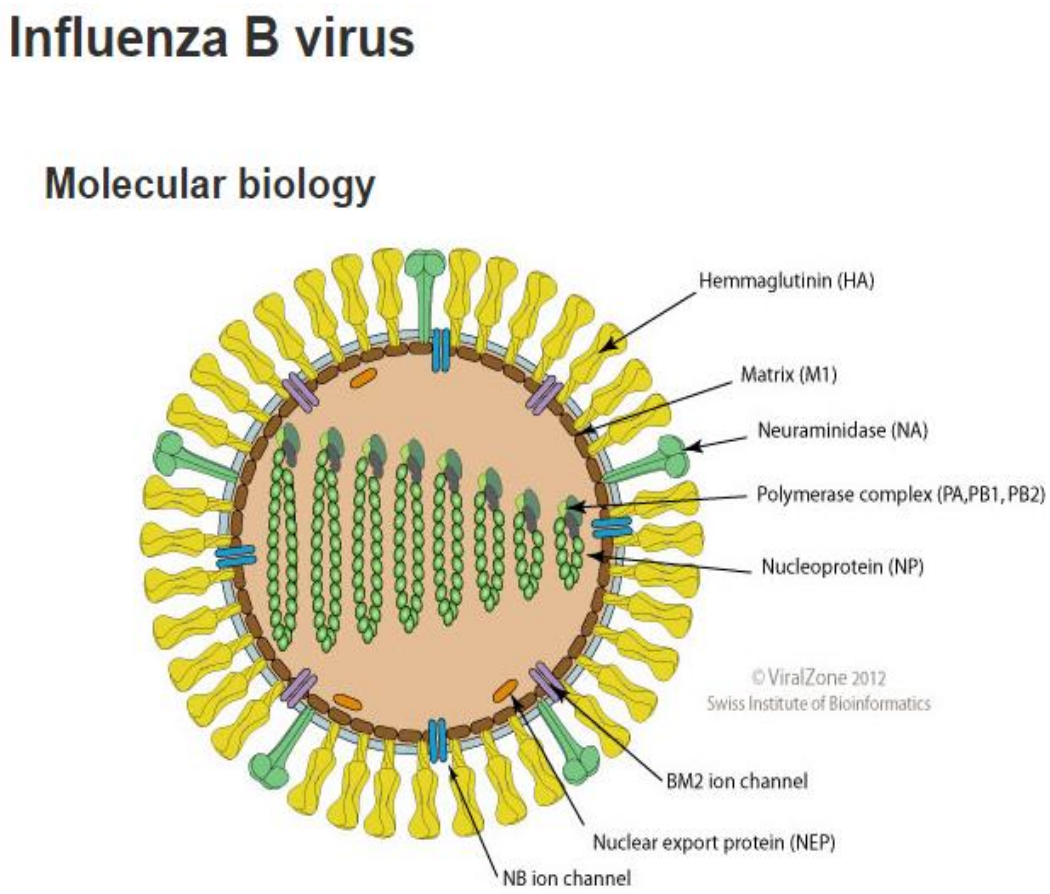


Figure 2 influenza B structure and the BM2 ion channel which has a homologue function as AM2 but antiviral adamantane cannot inhibit this flu type(47)

2.3 Pandemic and epidemic of influenza

There are currently 16 different known haemagglutinin (HA) proteins and 9 Neuraminidase proteins rearrangement of these protein can lead to new strains with ability to cause worldwide pandemics.. There are two mechanisms that influenza virus accumulate mutations antigenic drift and shift (26).

2.3.1 Antigenic drift

Antigenic drift occurs through the normal mutation process of influenza virus as an accumulation of changes overtime to the HA and NA proteins. During viral replication, point mutations occur in HA and NA genes and create a new variant virus strain. People can get infected through their lifetime and the previous human antibody cannot protect against the new strains that is the reason why the influenza vaccines must has updated each year (21, 26).

2.3.2 Antigenic shift

Antigenic shift is a genetic reassortment between HA or NA from one animal species with another. An HA protein or a combination of HA and NA proteins of Influenza type A replaced by significantly different HA and NAs. A pandemic is caused because there is not imunity to the new virus in the human population. . The new strain can be easilty transmitted human to human, leading to severe impact on the worldwide population. There are at least 3 types of antigenic shift mechanisms (Figure3) (21, 26).

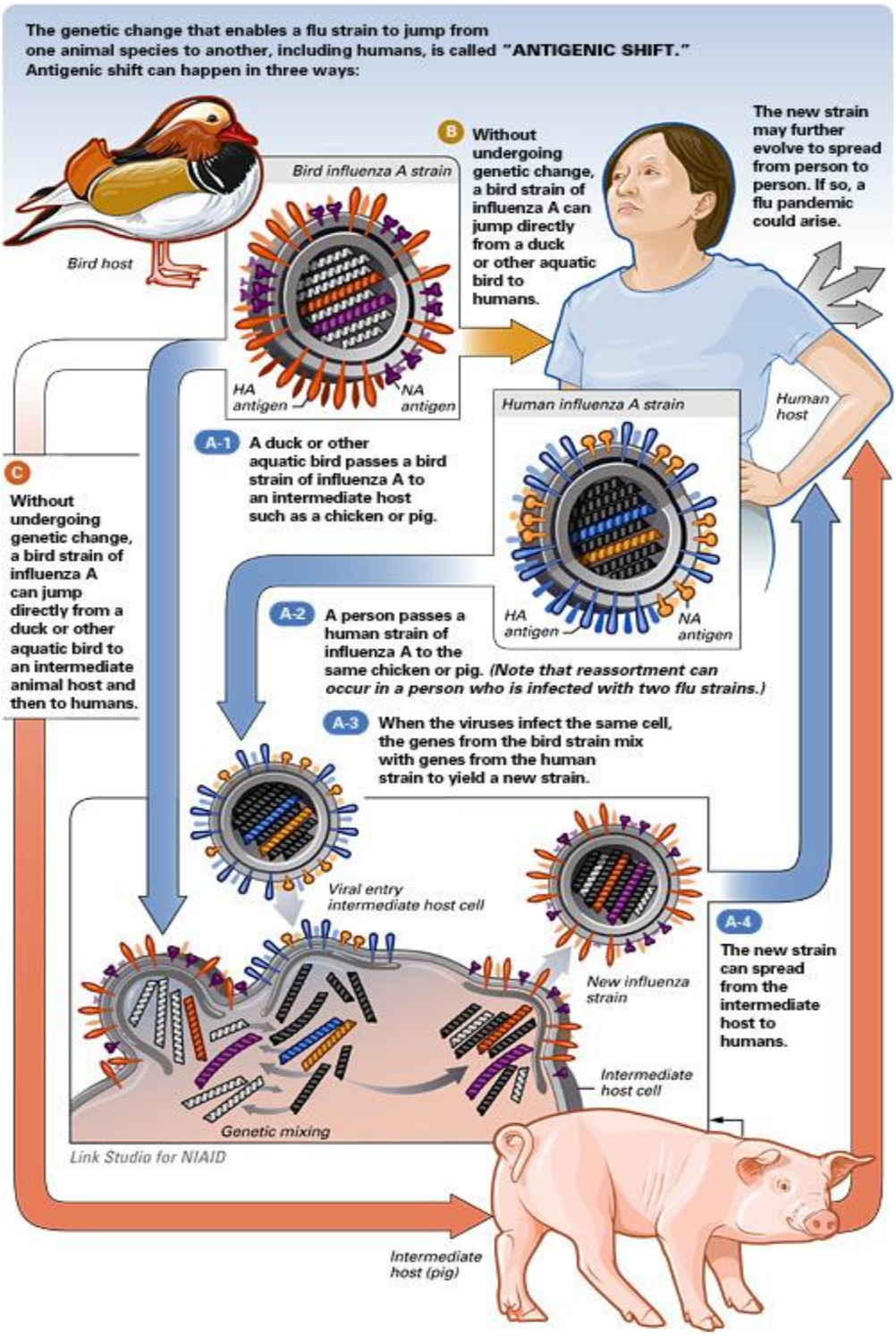


Figure 3 Diagram of antigenic shift from National Institute of Allergy and Infectious Diseases (NIAID)

Antigenic shift 1

The genetic reassortment can occur between nonhuman and human influenza viruses. The first host, aquatic bird, passes a bird strain of influenza A to intermediate host, chicken or pig. While the same intermediate host is infected with a human strain of influenza A. Then a new strain of influenza can occur by the genes from the bird strain mix with the genes from the human strain. The new strain can spread from intermediate host to human.

Antigenic shift 2

Influenza viruses from other animal such as birds or pigs transmit directly to human without undergoing genetic change.

Antigenic shift 3

Nonhuman viruses pass from one type of animal through an intermediate animal host to human without undergoing genetic change. For example, aquatic bird strain can transmit directly to a pig, intermediate host, and relay to human.

2.3.3 Seasonal influenza

Every year the epidemic of seasonal influenza can occur in tropical and subtropical regions by circulating virus strains. When people increase the ability for efficient and sustained human to human transmission, an influenza pandemic will occur and spread globally. A pandemic actually happens when a new influenza A emerges because the humans do not have immunity to the new virus. The characteristics of a flu pandemic are a rapid spreading around the world. A world's population will require some medical care but the nations do not have the staff, facilities, equipment, and hospital beds enough for the number of people who get the pandemic flu. In April 2009, H1N1 (swine flu) was first diagnosed. A new influenza virus spread rapidly across the United States and the world. 74 countries were affected by the pandemic and it has been reported that 18,000 case of H1N1 occurred in the United States in June 2009. Between April 2009 and April 2010, the Centers for Disease Control and Prevention (CDC) estimates that 43 million to 89 million people had H1N1 and between about 8,870 and 18,300 deaths. H3N2 was first detected in Hong Kong. In 2010, the first identified H3N2 was detected in swine

in the U.S.(48). The number of H3N2 infectious increased from 2011 to 2014 (H3N2 predominance season). In 2014- 2015, influenza B has increased in part of the country. The influenza positive test reported to CDC that the national summary of 2013 – 2014 season showed the number of positive test in each influenza types: 46.25% of infectious were influenza H3N2, 29.45% influenza B, and 22.73% influenza A, 1.58% unsubtyped (Figure 4-5).

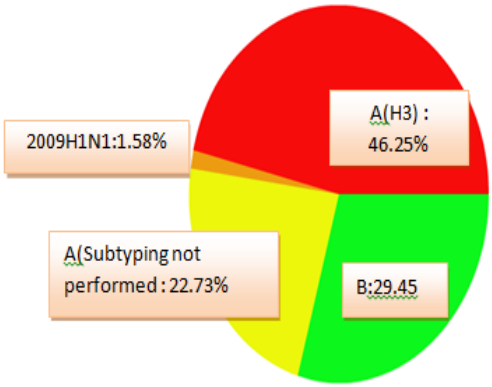


Figure 4 Influenza positive tests reported to CDC and influenza like illness activity, National summary of 2013 – 2014 season, week ending Sep 27, 2014
Report by: U.S. WHO/NREVSS Collaborating Laboratories and ILINet

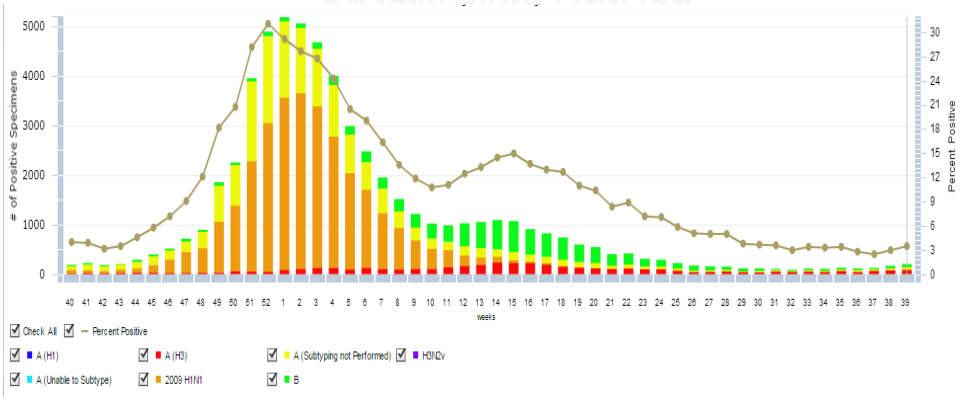


Figure 5 The graph shows the number of influenza positive tests in 2013-2014. The percent of positive test was increased in the season. The bar chart represents the number of influenza subtypes and the line shows the percentage of positive influenza (4)

Since January 1 to December 28, 2014, Bureau of epidemiology had reported that there was 71,761 cases of influenza illness patients and 81 led to death(4). The number of morbidity and mortality had increased from 2013 that had influenza infectious patients 43,867 cases. (3). The Central region of Thailand had the highest amount of infectious patient in 2014 (Figure6-7).

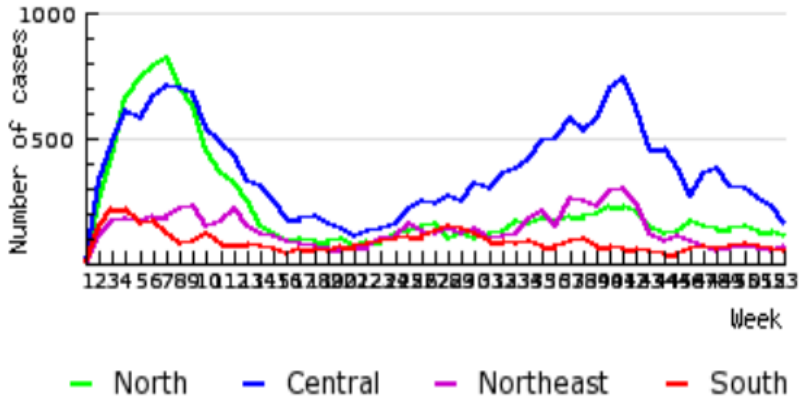


Figure 6 Distribution of influenza infectious patients by week in each region of Thailand in 2013 (49)

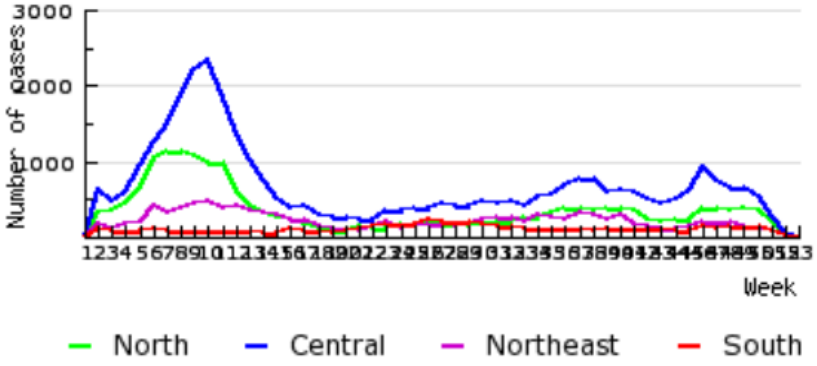


Figure 7 Distribution of influenza infectious patients by week in each region of Thailand in 2014 (4)

The most morbidity appeared in 0 – 4 year olds , 404.03 case per one hundred thousand people, next are 5-14 years old, 25-34 years old, 15 -24 years old and 35 – 44 years old in that order (50). The number of influenza patients is increasing every year (5, 6). Therefore, annual surveillance of antiviral resistant of influenza virus is necessary for antiviral drugs used in prevention and treatment of influenza.

2.4 The indications of Treatment

Treatment decisions for influenza infections is based on symptoms, disease severity, and the duration of illness. However, patients with previous good health or vaccination can required hospitalization, all were recommended to use antiviral treatment for every state of progression of disease (20). Severe influenza patients should get early antiviral treatment for the best outcome. There are many reports of early initiations of antiviral treatment fewer than 5 days after onset in severe illness. Early initiation treatment significantly reduced morbidity and mortality due to influenza infection (24, 25). The delayed initiation of treatment is not recommended for patients. There is no need to delay antiviral treatment until results of confirmatory diagnostic tests or specimens are collected. A full treatment course with antiviral drugs is recommended in patients who have suspected influenza even if the results of screening tests are negative unless the clinical description is not consistent with influenza. The CDC has criteria describing individuals at high risk of influenza complications. First is children who are younger than 5 years old. All children ages younger than 5 years are susceptible to complications from influenza but the highest hospitalization and mortality rate occurs in infants under 6 months. Second are elderly individuals older than 65 years old. Third, patients with underlying conditions such as chronic pulmonary, asthma, cardiovascular disease, renal disease, hepatic disease, hematological disease, metabolic disorders, diabetes mellitus, neurologic disease, neurodevelopment condition disorder such as disorder of brain, spinal cord, peripheral nerve, and muscle like cerebral palsy, epilepsy, stroke, mental retardation, moderate to severe developmental delay, muscular dystrophy and spinal cord injury. Fourth is an immunosuppressed patient from medication or HIV infection. Fifth is women who are within 2 weeks after delivery or recently pregnant. Sixth, people who have received long-term aspirin therapy and are younger than 19 years old. Seven, people who are of the following nationalities American Indian, and Alaskan Natives. Eighth, people who are obese defined as having a body mass index of 40 or more. Ninth are residents of nursing homes or other chronic-care facilities.

Neuraminidase inhibitors have been used for antiviral treatment for outpatients who are at high risk from underlying the conditions (51). The Centers for Disease Control and The world Health Organization both have recommended using neuraminidase inhibitor agents for suspected or confirmed influenza virus infections (52). The best time to start treatment is within 48 hours of influenza illness onset. This will provide the greatest benefit for all patients. To determine the most appropriated treatment and get new updates for antiviral resistance profiles of the circulating virus, clinicians should study the information of influenza virus in local, state, and national during influenza season (53). The treatment will be more difficult if seasonal influenza A (H1N1) virus strains show Oseltamivir resistance as previously described during the epidemic in 2007 – 2009 or H3N2 and 2009 H1N1 virus strains have neuraminidase inhibitor resistance more common occur in community (54).

The Oseltamivir resistance should be included for clinician decision, if the resistance has exited to the circulation of influenza in each strain. After illness, the treatment should be provide as soon as possible. There are many studies support that the antiviral treatment has greatest benefit if the treatment has started within 48 hours of illness onset(55). Because secondary bacteria infection complication such as pneumonia always occurs in influenza complication with patients at high risk, antibiotic therapy plus antiviral has been recommended for influenza suspected patients with community acquired pneumonia. Direct antibiotic for especially bacteria pathogens that connect with influenza such as *Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus aureus* including methicillin – resistant (MRSA) was recommended for the hospitalized (56). Any underlying of respiratory illness with required hospitalization should be recognized as influenza to a possible cause of illness in influenza season and require an influenza confirming test with antiviral treatment. All ventilators such as fans, blowers, coolers, and air conditioners, including the fluid management, should be attended for prevention and treatment of second bacterial pneumonia in severe influenza patients. Even if the outpatients do not have complicated symptoms with suspected or confirmed influenza and don't have information about if they are in high risk of disease or not, the treatment can be considered on the basis of clinician judgment andcan be initiated within 48

hours. Treatment for patients with uncomplicated influenza is not necessary, but will get more benefit if they get early short-term antiviral treatment (53). Same as the treatment of patients who start to recover, they do not need to start any treatment. Informing of knowledge of influenza activity in circulation should be available for antiviral drug treatment decision. Unnecessary antiviral treatment can be given if the treatment was taken for patients with respiratory illness in low season of influenza.

The treatment of patients with severe influenza is complicated. There is no clinical trial of antiviral strategies of oseltamivir and zanamivir in serious or life – threatening influenza to support licensure. All samples are outpatients who were previously healthy with uncomplicated illness. CDC Health Update Regarding Treatment of Patients with Influenza with Antiviral Medications in Health Alert Network of the official CDC Health Update suggested that Oseltamivir has clinical benefits although the treatment initiated after 72 hours of illness by a randomized placebo controlled study (57). However, antiviral treatment can help to reduce the risk of influenza in some cases of patients who have initiated treatment later than 48 hours of getting illness symptoms. There are observational studies reported about the antiviral treatment still keeping advantages for hospitalized patients with delayed treatment (up to 4 – 5 days after the symptoms of influenza condition onset (26, 58-60). The antiviral treatment by clinicians should be changed depending on the situation to give the best effect of treatment for patients. For instance, the patients who have prolonged illness need to extend treatment longer than 5 days by guide regarding.

The evaluation of the effectiveness of larger dose of antiviral for severe influenza illness treatment does not have any report. For example, an intensive care unit was required for a viral pneumonia patient. Moreover,the enteric absorptions with severe illness patients was adequate in one study report (61). Neuraminidase inhibitors have great effect for influenza patients with another type and those who have current guidance of treatment focused on influenza illness patients with severe illness or high risk of complications. These agents can be used with any previous healthy outpatient who does not have a high risk of suspicion or confirmed influenza.

The duration of symptoms in healthy persons with uncomplicated influenza can be reduced by neuraminidase inhibitors in about 1 day if the treatment is given within 48 hours of illness onset but for treatment after 48 hours starting may possible to offer for some benefit (58). There are 3 prescriptions of neuraminidase inhibitors. These were approved by the U.S. Food and Drug administration (FDA) and recommended for use in the United States during the 2014 – 2015 of influenza season (53). First, oral Oseltamivir was allowed to be used for treatment on infants that have age 2 weeks and older and be chemoprophylaxis to prevent influenza in people 1 year and older. The approved document was from FDA. To use oral Oseltamivir for treatment of influenza in infantsyounger than 14 days old and to be chemoprophylaxis in infants 3 months to 1 year of age, it does not have any part in FDA- approval but it was also recommended by the CDC and American Academy of pediatrics. On the other hand, there are some studies that did not recommend use of Oseltamivir for chemoprophylaxis in children younger than 3 months under the Critical judged situation. The second treatment is inhaled zanamivir whichwas approved by FDA to use with persons 7 years and older and for prevention of influenza in persons 5 years and older.

The third is intravenous peramivir which was announced as approved on December, 2014 to treat acute uncomplicated influenza patients 18 years old and older (58). The current Adamantanes was not recommended for use in treatment or prevention of influenza because the prevalence of high levels of agents resistant to Adamantine in modern days. Neuraminidase inhibitor agents do not have reports about the shortage nationally, but they have been followed and reported locally. Oral suspension can be compounded by a pharmacy from Oseltamivir capsule if there is difficulty locating commercial Tamiflu® with following the information from health care professionals regarding compounding and oral suspension from Oseltamivir 75 mg capsules (62). This compounded suspension should not be used for availability or when the FDA-approved Tamiflu® for oral suspension is commercially available. Influenza infection is not treated by antibiotics so, the inappropriate use of antibiotics can be reduced by early diagnosis of influenza. If patients have some suspected symptoms of bacterial infection, the patients should

be treated with suitable antibiotic treatment because bacterial infection can make symptoms like influenza or get bacterial infectious as complication infections(63). Furthermore, pneumococcal vaccine was recommended for adults who are 65 years old or more. The side effects with oral Oseltamivir have occurred in some cases that included a slightly increased risk of nausea and vomiting over placebo. Adults who received Oseltamivir had occurred 10% of nausea and 9% of vomiting. In children, there are occurances of children who have taken placebo in controlled clinical trials. There have been found 4% nausea and 9% vomiting. Moreover, the nausea and vomiting symptoms will be increased by oseltamivir taken with food.

It was not recommended to use inhaled Zanamivir with presence of bronchospasms and underlying airway diseases such as asthma, chronic obstructive, or pulmonary disease. However, inhaled zanamivir was not reported to cause adverse event increase over placebo in clinical trials. The most common of intravenous peramivir or intramuscular peramivir was diarrhea with people who received the agent (58).

The regulation of treatment should be applied to suit for the clinical occasion. Patients who have prolonged influenza symptoms should get treatment longer than 5 days. The effectiveness of larger doses of antivirals to treat severe influenza disease does not have any report to evaluation, but there is some data that suggestsdoubling the dose of Oseltamivir is a great strategy to treat severe illness patients with influenza A (H5N1). The doubling of the dosage has good toleration and advantage. Systematic absorption of the gastric tube has effect in some critically ill patients with influenza H1N1 or H5N1. This information can apply to another severe influenza with other viruses (53). Gastric stasis or bleeding decreasesthe ability of medication absorption which adds to the problem of administration. So parenteral medications might be more fit for the influenza infectious patient who has gastric problems. There are no official experiments or approval of the FDA to support the advantages of this clinical way of treatment. To obtain a greater understanding of treatment, clinical trials of experiments are needed. National Institutes of Health has information of patients in clinical trials of experiments of intravenous antiviral and combination antiviral treatments for

clinicians who are interested and want to consult in this roll. Patients with antiviral – resistant influenza virus will not respond to antiviral medications treatments. There are reports of Oseltamivir resistance within 1 week of treatment beginning in immunocompromised patients with 2009 H1N1 virus infection who received Oseltamivir treatment. Immunocompromised patients should receive infection control measures to reduce the risk for spreading Oseltamivir – resistant viruses. IND or other protocols can provide investigational parenterally administered products including paramivir and zanamivir in clinical trial. There are reports of reducing the likelihood of infection in samples with influenza A virus by using investigational zanamivir (53).

Intravenous zanamivir has been used with success in clinical setting so it is recommended for severely ill patients who are highly suspected or confirmed for Oseltamivir resistant 2009 H1N1 virus infection. The drug should be delivered to sites of infection in pneumonia or extrapulmonary disease patients that concern severe respiratory illness because inhalation zanamivir cannot be used with intubated patients. The use of unlicensed nebulized formulation of zanamivir in limited experiments was acceptable, but the clogging in ventilator tubing called for care in applying the nebulized preparation of licensed powder formulation contained in the disc inhaler (20).

Postexposure chemoprophylaxis needs the factor of local physician decision and recommendation such as the risk of influenza complication, the type, and duration of contact with influenza infectious for treatment to patient. After recent exposure, patients should be treated by postexposure chemoprophylaxis with antiviral within 48 hours. Local preference of chemoprophylaxis instruction for patients who have high risk of complication progression is important in limited antiviral medication available area. Sometimes CDC or local public health authorities can suggest the first directed at treatment by antiviral medication resources and using the antiviral chemoprophylaxis only in confident limited situation. Although chemoprophylaxis with antiviral medication has effect for morbidity result , that does not mean antiviral medication can replace to influenza vaccination when the vaccine available.

Lowers dose of chemoprophylaxis does not take out the risk for influenza infectious patients who was received antiviral chemoprophylaxis so influenza will come back when the drug was stopped. For controlling the disease , vaccine is recommended if there are available. Because influenza virus might be progress to resistant to antiviral drug while the virus still be found in the influenza infectious patients who had been receiving chemoprophylaxis, they should be supported to get medical evaluation same like a febrile respiratory illness suggestive of influenza development (64). Antiviral agents that were suggested for influenza A (2009 H1N1), influenza A (H3N2) and influenza B virus infection are oseltamivir or zanamivir. Early treatment for patients who have had been suspected influenza virus infectious is important. If clinical signs and symptoms of influenza had developed in people who got risk factors for influenza complications with household or close contact confirmed or suspected influenza person and occupational exposure health care staff, they should be gotten some advises about early sign and symptom of influenza and suggestion for contact their health care provider immediately to receive evaluation and possible early treatment. An appropriate alternative of early recognition of illness and treatment can be provided by using clinical judgment regarding situation in health care provider (65).

There is community studies of healthy adults administered antiviral medications during influenza virus activity supported that both oseltamivir and zanamivir had similar efficacy in preventing febrile and laboratory confirmed influenza illness. 92% of reduction in influenza illness showed in a 6 weeks study of oseltamivir medication among nursing home residents and 83% of zanamivireffection in 4 weeks study among community-dwelling persons at higher risk for influenza complications in average 60 years old of patients was showed. But severely immunocompromised person influenza prevention by antiviral agent effect was unfamiliar (66). There is a small nonrandomized study directed in a stem cell transplant unite recommended that oseltamivir can prevent pneumonia progression in influenza virus infected patients. However the duration of time of using antiviral should be careful, long term of chemoprophylaxis might be bring for antiviral resistant in influenza virus. For treatment or prevention of infectious patient or high

risk complication patients such as influenza infectious in severely immunosuppressed patient, physician or health care provider should refer to CDC or other health authorities' recommendation for prioritizing treatment of mild to severe influenza infectious patients or persons at high risk for complication. Actually duration of postexposure chemoprophylaxis is total less than 10 days after the showing of influenza symptom of person who got contact with influenza (20). Gastrointestinal symptom complication with oseltamivir chemoprophylaxis actually fails to complete a course and affects for antiviral resistant in influenza virus. Some study of primary school children and secondary school children who were treated with oseltamivir showed that they have low percentage of full course completed antiviral treatment. Stopping chemoprophylaxis before medication complete was the most common in gastrointestinal adverse event. The duration of community influenza activity has effect to the duration of pre exposure chemoprophylaxis. There is not data support about influenza antiviral enduring more than 6 weeks. The great toleration of zanamivir is 28 days and oseltamivir is 42 days (67).

Although almost of all influenza A and B virus have been susceptible to oseltamivir since September 2009 during 2008-2009 influenza season, oseltamivir resistance of seasonal influenza A (H1N1) circulating was discovered. From the incident, clinicians have more reasons for influenza virus testing in patient and were more careful to selection of antiviral agent to treatment and prevention. The results of influenza diagnosis testing affected to recommendations for antiviral treatment complication. In clinical routine, testing for antiviral resistance and influenza A virus subtype are not available. Oseltamivir and zanamivir have provided for treatment when oseltamivir resistant virus was not getting in situation. Nowadays antiviral resistant of influenza virus still have been changed so medical provider should follow CDC influenza surveillance report and updated antiviral treatment guidance usually. Age group, purpose for using, and medical conditions have impact for vary dosage recommendations. Duration of treatment was recommended for 5 days. Severe illness of hospitalized patients or persons with immunosuppression might be gotten longer treatment (53). Centers for Disease Control and Prevention had

recommendation about antiviral medication for treatment and chemoprophylaxis of influenza.

There are many advantages of early antiviral treatment especially within 48 hours of influenza illness onset. Early treatment can be decreased the duration of fever and illness symptoms so it can reduce some case to has shorten of hospitalized duration in patients. The risk of otitis media in young children, pneumonia, respiratory failure and some of complication symptoms risk might be reduced by early taken drug. Clinical trials and observational data supported that the number of death in influenza hospitalized patient can become lower by early treatment (65).

Ministry of public health of Thailand had published clinical practice guideline for influenza for physicians and health care staffs to treatment and look after influenza infectious patient or people who get risk for infection. The guideline associated with incident and medical resource in different of each local health care center such as distribution of antiviral drug, prevention instrument, and prevalence of disease in local and mental status(68).

If patient has pneumonia suspected symptom, they should be taken to chest x-ray in every case. Checking for severe of influenza is necessary. Only pneumonia or admitted patient case, the clinician has to send specimen for influenza virus detection. The indicators of admitted patient to hospital are intravenous infusion needed patient, closely clinical care needed patient, patient of high risk for severe symptoms that cannot follow in outpatient status or physician dependent.

2.5 Antiviral influenza virus treatment

2.5.1 Adamantane (M2 Protein inhibitor)

There are two types of agents in this drug group, amantadine (Symmetrel) and rimantadine (Flumadine). Both of them have antiviral activity effects to only influenza A by ion channel function of M2 protein inhibition. M2 protein is an acid activated ion channel that is only found in influenza A is necessary for the virus replication. The antiviral agent has many functions such as uncoating inhibition, disassembly of the virion during endocytosis and increasing the lysosomal pH which inhibits virus-induced membrane fusion of viral. The drugs have half life time about 11-15 hours in a normal person and prolonged life in patients with renal impairment. In elderly persons, the drugs have decreased about twofold in plasma elimination. Furthermore, these drugs can change haemagglutinin maturation during viral assembly in H7 virus subtype. However, M2 blocker agents are not recommended for treatment and prevention of influenza virus at present time because there is wide spread drug resistance in circulating influenza virus. Amino acid substitution in transmembrane portion of M2 protein has changed so all M2 inhibitors do not have an effect to the virus (69). The pore at Serine-31 position is a direct target of Adamantane binding to block proton passage. When the structure of the amino acid change to Asparagines, there is an effect to reduce capacity of drug binding and lead to M2 blocker drug resistance(70). S31N is the predominant mutation point of influenza virus that was found in Adamantane drug resistant H1N1, H3N2 and H5N1 isolated from humans, birds and swines (71). Resistant activity can appear rapidly in 30% of Adamantane treated patients within 2 – 4 days after taking the drug (69).

There were experiments showing CNS and dose of drugs association that had appeared in young adults who had taken > 200 mg of drug and elderly persons who had taken 100 mg of drug. Symptoms of minor central nervous system complaints such as anxiety, difficulty concentrating, insomnia, dizziness, headaches, jitteriness and gastrointestinal problems are the most common side effects of Adamantane. This drug can induce patients who had been treated by Amantadine to have antimuscarinic effects, orthostatic hypotension and congestive heart failure,

confusion, disorientation, mood alterations, memory disturbances, delusions, nightmares, ataxia, tremors, seizures, coma, acute psychosis, slurred speech, visual disturbances, delirium, oculogyric episodes and hallucinations. All of those symptoms always happen in elderly persons and those with renal failure and serious CNS side effect.

2.5.2 Neuraminidase inhibitors

At the present, two antiviral drugs have used against influenza: the Adamantanes and the Neuraminidase inhibitors (NAIs). Amantadine and rimantadine are antivirals in the adamantanes group (72). Viral ion-channel protein M2 is the target of their activity (73). These drugs are ineffective against influenza type B(74). Their activity is affective against type A influenza virus only (72). In 2006, CDC opposed the use of this class of drug(75). Therefore neuraminidase inhibitors are the only class of antivirals recommended by the WHO for therapeutic and prophylaxis of Influenza A and B infections (11).

The majority of antiviral influenza drugs are Oseltamivir and Zanamivir, the agents had have been approved since 1999 in USA and other countries (76). These are the NAI's drugs currently used worldwide against influenza (72). Oseltamivir is more effective and practical than Zanamivir because Oseltamivir is the orally administered drug whereas Zanamivir is inhalation (76). While the emergence of the pandemic of influenza in 2009, the Emergency Use Authorization (EUA) has published work on applling the parenteral drug Peramivir to treat hospitalized patients with already known or suspected influenza A (H1N1) pdm09 infection (77) Peramivir is only available in Japan and Korea. Laninamivir is an inhalation drug that has been developed only in Japan (72) and it is entering to phase II studies in the United States (9).

The main of influenza surface antigen is hemagglutinin (HA) and the neuraminidase (NA) (78) that stimulates the humoral immune system. HA protein is accountable for virus attachment to the sialic acid receptors on the host cell that makes a function of the catalytic activity of the NA which cleaves off the terminal N

acetyl neuraminic acid (Neu5Ac) on the sialic acid (72). NA destroys the sialic acid, HA receptor, placed on the mucin of respiratory mucus, the enzymatic activity of NA plays in releasing progeny virion from host cell and helping viral to spread throughout the upper respiratory system in the final step of the influenza virus replication cycle (Figure8) (72).

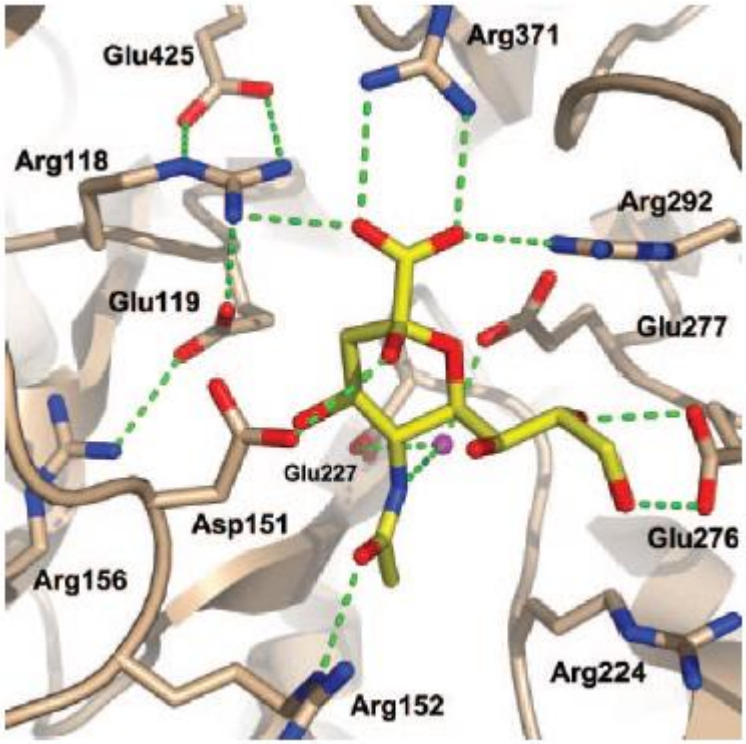


Figure 8 The picture shows influenza virus NA and sialic acid complexed. NA residues (R118, D151, R152, R224, E227, E276, R292, and R371) were drawn together with sialic acid (yellow). The green dotted lines are Hydrogen bonds and the magenta sphere is a water molecule(79).

Influenza A and B virus have around 100 copies of NA that is less diverse than HA which has 400 copies(80). The antiviral development used NA as an effective target against the influenza virus both types A and B. The arrangement of NA on the surface of virus is tetrameric (81). Previous study of the structure of NA lead to the design and synthesis of the series of mimic NA enzyme's substrates to compete for the binding and blocking the active site. The NAIs were designed on the basis of an early transition state analogue of sialic acid, 2,3-dehydro-2-deoxy-N-acetylneuraminic

acid (DANA). This model prompted the increase in the affinity of the compound to achieve a better capacity of binding the latter (Figure9) (72). The first NA crystal structures were solved in 1960 (74). Accordingly, this prevents the cleavage of the natural substrate and makes the progeny virions fail to release which reduces the spread of infected cells. (72). Oseltamivir and Zanamivir have been approved by the US Food and Drug Administration (78). In The comparison with the initial sialic acid transition analogue DANA, Oseltamivir has a 4-amino group and a 6-glycerol by a hydrophobic pentyl on either side chain (81). In contrast, Zanamivir has a 4-guanidino group (82). Both of them are the guidelines for antiviral treatment and prophylaxis worldwide from WHO (12).

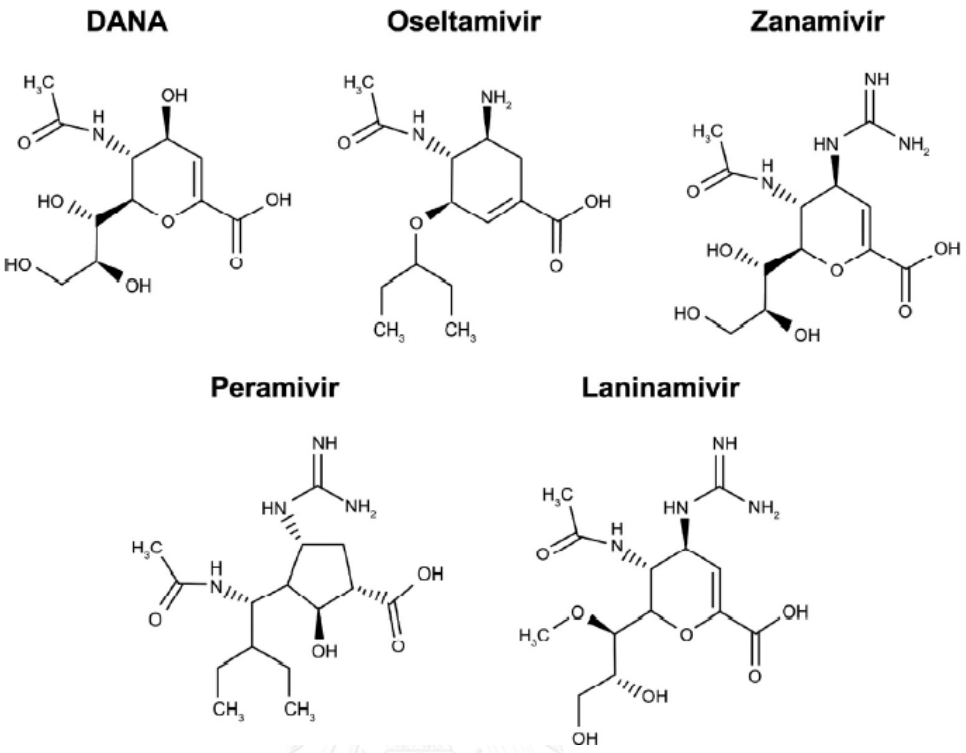


Figure 9 Neuraminidase inhibitor's structures are based on the structure of the 2,3-didehydro analog of the N-acetyl-neuraminic acid (DANA). The bioavailable prodrug of Oseltamivir is an ethyl ester that is converted into the active carboxylate by hepatic esterases. Zanamivir is a 4-deoxy-4-guanidino analog of DANA. Peramivir is a cyclopentane derivative with a guanidyl group and a lipophilic chain. Laninamivir is the active product of the esterified octanoate . The difference in molecule interactions on enzyme active sites refers to their antiviral activity efficiency.

2.6. NAIs Resistances

Previous reports refer the in vitro studies found the resistant NA inhibitors viral variants (76). The incidence of NAI resistance in a global multicentre study have been reported between 2008 and 2012 (12). This condition was found in influenza infected patients who were treated and untreated by NAIs drug (83). Oseltamivir is the most commonly used therapeutic drug of the day with many reports about the resistance of this agent (76). The different interactions at the NA enzyme site between Oseltamivir and Zanamivir make the mutations more resistant to Oseltamivir than Zanimivir (84). To accommodate for Oseltamivir drug binding, the NA must rearrange by the rotation of amino acid E276 and bond with R224 because the agent has a large hydrophobic side chain (85, 86). Children and immunocompromised patients have the highest incident of Oseltamivir resistance (87-89). They do not have the appropriate immune response against the virus that may lead to prolonged virus persistence in these patients (90, 91). The active sites of NA (N1, N2, and B neuraminidase) are conserved in different influenza viruses (78). The different sites lead to the different model of the NAIs that make the different options for development of resistance (92, 93). The majority of amino acids changing direct or indirectly alter the shape of active sites (72). The type of NAIs used take to a differential resistance mutation (94). A recent study found the specific subtype of them. The variant of mutation in the active site can separate the categories of NA resistant to 3 types.

2.6.1 N1 neuraminidase

The histidine to tyrosine amino acid change at position 274 (H274Y) is the most common mutation found in the Oseltamivir resistance of the seasonal H1N1, highly pathogenic H5N1, and A (H1N1)pdm09 virus (95-97). The high rate of resistance to Oseltamivir was reported during the 2007- 2008 influenza season worldwide (98). Moreover, the changing of isoleucine to arginine at position 222 (I222R) and serine to asparagines at position 246 (S246N) are reported shortly after the outbreak of the 2009 pandemic A/H1N1 virus(99, 100). These mutation reports refer to the reducing of susceptibility to multiple NAIs (78). The other variant NA mutation is I117V, isoleucine to varine (101). I222R, S246N and I117V have been synergistically combined with H274Y and they elevate the levels of resistance to the NAIs (72, 102, 103). The reduced sensitivity to Oseltamivir in the infected patients with pathogenic A(H5N1) viruses and seasonal A(H1N1) creates an asparagine to serine change at position 294 (N294S)(85, 104, 105). In NA inhibition assays, these mutations were associated with significant increase in IC_{50} value (106, 107). The amino acid positions at 222, 246, 274, and 294 interact with the hydrophobic pentoxil group at carbon ring position C6 of Oseltamivir, that is related with a hydrophobic pocket in the NA active site (108). It requires the reorientation of a glutamic acid residue at position 276 (E276) for binding the pentoxil group of Oseltamivir into the hydrophobic pocket (85, 109). Zanamivir is different. The C6-group of Zanamivir is a polar glycerol. It can bind to the active site which does not implicate with E276 orientation (85, 92, 109).

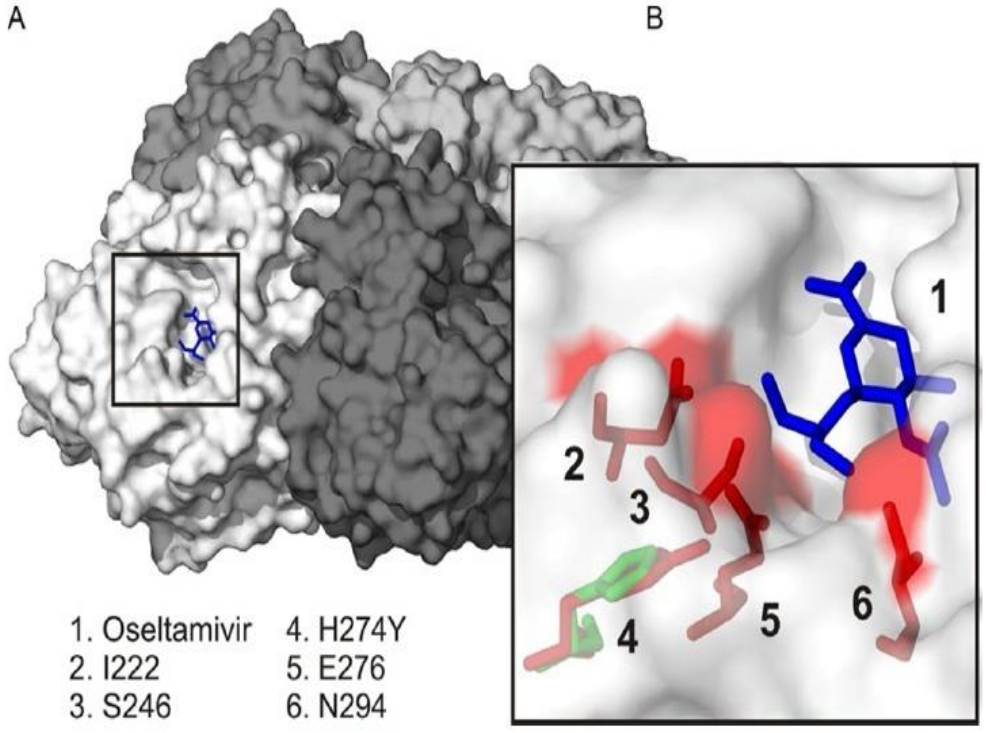


Figure 10 Oseltamivir resistance contacted with amino acid residues in N1 neuraminidase. 'A' show Oseltamivir carboxylate(blue) in complex with teteameric neuraminidase(gray). 'B' is an enlarged image of active site. 1-6 presented the association between NAI resistance, Oseltamivir and amino acid. These are the active sites of NA that Oseltamivir caboxylate is bound. At position 274 a histidine (H, green) to tyrosine (Y, red) change prevents reorientation of glutamic acid (E) side chain at position 276 when Oseltamivir carboxylate binds. Surface was made transparent to visualize otherwise hidden residues and colored red if a residue is a surface occupant.

2.6.2 N2 neuraminidase

The predominant mutations of seasonal A (H3N2) are the glutamic acid to valine change at position 119 (E119V) and the arginine to lysine change at position 292 (R292K) (78). In the case of E119V, a water molecule may accommodate in NA mutation that would interfere with the binding of Oseltamivir to the active site (72). E119V and R292K mutations are the cause of Oseltamivir resistance only (78). R292K change prevents the rotation of glutamic acid at position 276 like the resistance mechanism of H274Y (78). E119 do not have x-ray crystal structure to determine its exact resistance mechanism(78). Nevertheless, E119G, glutamic acid to glycine change, exposes that involves interaction with the C4 groups of oseltamivir (C4-amino) and zanamivir (C4-guanidino) (110). These mutations are less frequently detected in virus isolates than N1 neuraminidase (88).

2.6.3 B Neuraminidase

The cause of Oseltamivir-resistant B viruses is amino acid changes (78). The specific NA mutations that are only seen in influenza type B virus are E105K, R152K, D198N and R371K (111-113). The H274Y mutation with influenza B virus have been found in patients who do not have treatment (114). This excludes a 7 year old patient with cancer who was treated with Oseltamivir which had recovered from a N292S NA mutation (115). R152K mutation was identified in a zanamivir treated influenza B patient and has been reported in *in vitro* studies (112).

2.7 Testing for Influenza

Early treatment is important for influenza illness. The basis of the patient's disease such as severity and progression, age, underlying medical conditions, possibility of influenza, and time since onset of symptomssymtoms is important for making antiviral treatment decision for patients. To confirm the infectionus, there iswe have a rapid influenza diagnostic tests (RIDTs). It is an immunoassay that can identify nucleoproteinneucleoprotein antigens of influenza A and B virusal in respiratory specimens. Because its sensitivity is 50 – 70 %, a negative RIDT result does not mean a suspected influenza patient is not infected with the virusdo not infect with the viral. Then viral culture or molecular assay is a new choice of diagnosis test (58). Future, more molecular assays, are applied in many surveillances and monitoring about influenza antiviral drugs resistance. Although the isolation of viruses in cell cultures has many factors tofor be taken in condition, it is the gold standard for virus diagnosis. The result of standard isolation procedures is an average offor 4-5 days. Because of this,Then the cell culture method is limited to clinical use has limit to use in clinical(29). Molecular techniques can directly detect influenza A and B genetically in respiratory samples or viral isolation culture. The molecular method is suitable for rapid identification of human influenza and outbreak of pandemic. Reverse- transcription PCR (RT-PCR) is a technique used to amplify specific regions with RNA template. First reverse- transcribed is complementary DNA(cDNA) then undergoes amplification by PCR(29).

2.8 Pyrosequencing

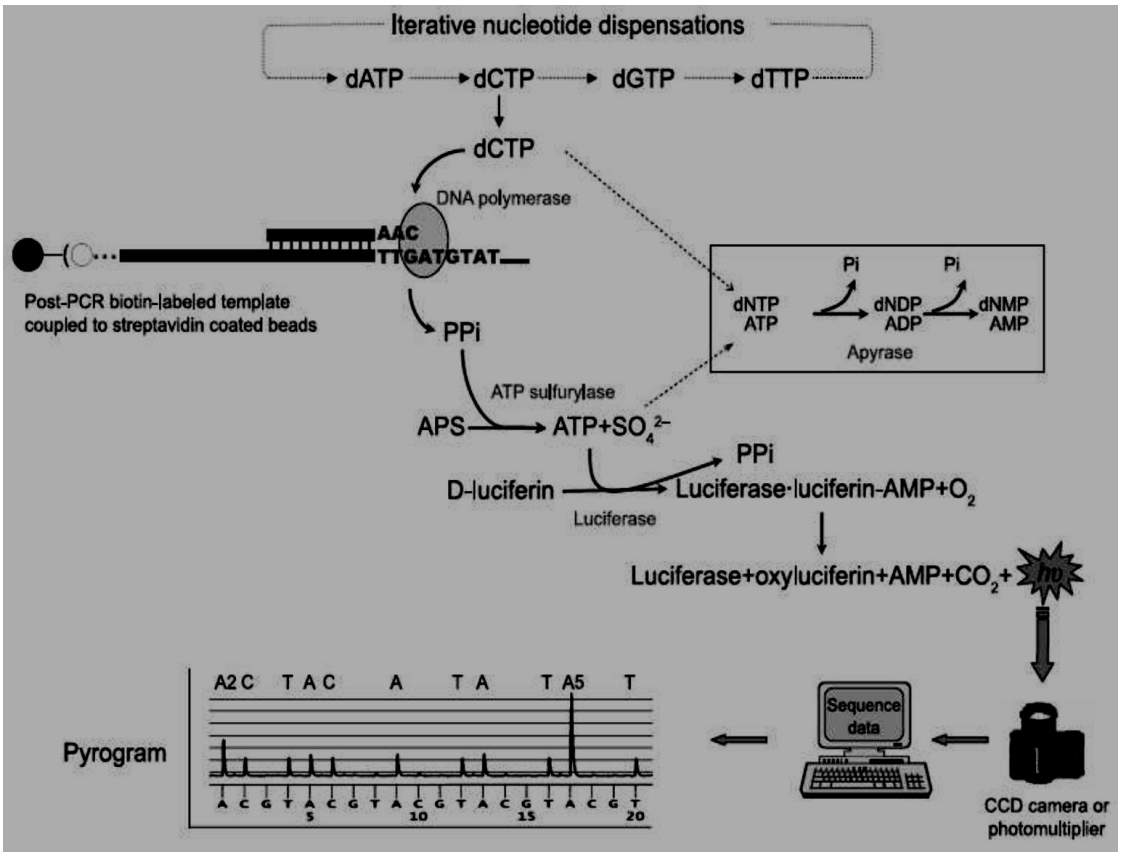
The first real-time sequencing detection by pyrophosphate derivative action had occurred in 1996 (116). Application of genetic disease detection, cancer diagnosis,and analysis had required more rapidity and high throughput method. Detection of DNA polymerase activity by an enzymatic luminometric inorganic pyrophosphate detection assay (ELIDA) had been developed for a real-time DNA sequencingapproach without electrophoresis (116).). There were older methods of sequencing detection such as sequencing by hybridization (117, 118), sequencing by mass spectrometry (119) and single molecule detection(120). Sequencing by synthesis is a procedure for sequence-base analysis with each nucleotide detection during DNA polymerase extension reaction running (121). A description of an enzymatic system application of incorporation DNA polymerase, ATP sulfurylase and luciferase to couple the release of pyrophosphate expressed light emission which was detected by luminometer in real-time was showed by Nyrén's study (122).

The first major improvement of pyrosequencing reaction in homogeneous phase in real-time had occurred with nonspecific signals with dATPαS inert effect for luciferase (116). The second improvement had appeared in 1998 through high catalytic activity of low amount of apyrase of efficiency degradation of unincorporated nucleoside triphosphates to nucleoside diphosphate (123). WHO collaborating Centre for the Surveillance, Epidemiology and Control of Influenza at Centers for Disease Control and Prevention (CDC), United states of America, had developed pyrosequencing assay for detecting antiviral resistance of Influenza virus (13). Pyrosequencing is the first subordinate of the conventional Sanger method. It is a DNA sequencing technology based on the sequencing-by-synthesis principle. Pyrosequencing is appropriate for single nucleotide polymorphism analysis and sequencing of short stretches of DNA (14). Pyrosequencing was developed by Royal Institute of Technology. Pyrophosphate that is released during primer-directed DNA polymerase catalyzed nucleotide incorporation had been detected by luminometric (124). The application of this technique is useful to study the detailed characterization of nucleic acids. Pyrosequencing uses a series of four enzymes to

accurately detect nucleic acid sequences during the synthesis. The enzymes in used is DNA polymerase, ATP sulfurylase, luciferase and apyrase with the substrates adenosine 5' phosphosulfate (APS) and luciferin are mixed with the sequencing primer that is hybridized to a single- stranded DNA biotin-labeled template. Product of PCR should be purified to a decreased substance and unincorporated nucleotide which would disturb the pyrosequencing reaction (125). There are two templates that are available for DNA preparation before sequencing reaction. First is solid-phase template preparation. This method uses Streptavidin-coated magnetic beads to prepare DNA template for pyrosequencing(125). Streptavidin-coated magnetic beads would immobilize the biotin-labeled DNA template with an annealed primer (116). v(125). However, the method had unstable baseline fluctuations. The DNA template was lost in washing step and difficult to auto (124). The second template is Liquid phase pyrosequencing. Apyrase is a crucial enzyme of this method which results from three enzyme of solid-phase. Real-time monitoring had occurred by annealing a sequencing primer to a single-stranded DNA template of sequencing reaction. Low amount of apyrase could efficiently degrade the unincorporated nucleoside triphosphate to nucleoside diphosphate and accordingly to nucleoside monophosphates in reaction system with high catalytic activity. The four enzyme system could remove the complicated step from solid-phase for the washing step and repetitive enzyme additions (14). Four deoxynucleotide triphosphates (dNTPs) are added into the reaction mixture. The mechanism starts with a nucleotide incorporation that releases the inorganic PPi by polymerase enzyme in a nucleic acid polymerization reaction. A quantity of the released inorganic pyrophosphate (PPi) in each nucleotide incorporation lead to an equimolar amount of incorporated nucleotide. In the presence of APS, the release PPi is quantitatively converted to ATP. The luciferase-mediated conversion, luciferin to oxyluciferin, is derived by the generate ATP. It produces the visible light in amount that are proportional to the amount of ATPs. The photon detection device detects the light in the luciferase-catalyzed reaction by a maximum of 560 nanometers wavelength such as a charge coupled device (CCD) camera or photomultiplier. In the reaction mixture, the non-incorporated dNTPs had been degraded with apyrase, a nucleotide-degrading

enzyme. The complete nucleotide degradation has a certain time interval of 65 seconds because the mechanism performed add dNTP one at a time. For this reason, if the added nucleotide is known, the sequences of template can be determined (125). The mechanism of pyrosequencing is shown in figure 11.

Figure 11The mechanism of pyrosequencing (14)



A peak signal from the generated light is observed in the program that agrees with the electropherogram in dideoxy sequencing. It is proportional to the amount of nucleotides incorporated; for example, a triple dATP incorporation generates a triple higher peak. The pyrogram on the screen shows the DNA strand that is extended by complementary nucleotides. In the pyrogram, the activities of DNA polymerase and ATP sulfurylase are showed on the slope of the ascending curve, the nucleotide degradation is showed on the slope of the descending curve, and the height of the signal displays the activity of luciferase. The performance with integrated software

which has many features for related SNP and sequencing analysis is base calling (the process of assigning bases to the peak. The automation of pyrosequencing is available. This technique is adapted for high-throughput analyses (125). It can analyze up to 96 samples with PyroMark Q96 and each sample sequence reading up to 100 nucleotide accurately (13). At the present, Pyrosequencing has been used in many applications for instant Single Nucleotide Polymorphism (SNP) genotyping (126). Identification of bacteria (127) , fungal, and viral typing. Furthermore, the method can also perform mutation detection. Pyrosequencing methods have been applied to detect the molecular markers of resistance to antiviral for seasonal influenza A (128) and influenza B (129). The primer and pyrosequencing protocol to detect the antiviral mutation originates from the Centers for Disease Control and Prevention (CDC)(13). The fast pyrosequencing with real-time read-out that is highly suitable for sequencing short stretches of DNA because this technique is executed in real time so nucleotide incorporations and base callings can be observed continuously for each of sample. The automated pyrosequencing can be easily programmed in the order of nucleotide dispensation and it can continuously study the pyrogram pattern that expose mutations, deletions and insertions. Many advantages of the pyrosequencing are high throughput, rapid speed, and simple combination with PCR protocols. Because it has real-time read-out method, pyrosequencing is highly suitable for sequencing short stretches of DNA (14). Sample preparation and single-strand DNA process is relatively rapid so this method is also less laborious than the conventional sequencing (14)

2.9 Sanger Sequencing

The First generation of DNA sequencing method is Sanger sequencing (130). During in vitro DNA replication, the process of selective incorporation of chain-terminating dideoxynucleotides makes the order of genetic result (131). Sanger sequencing method requires a single-stranded DNA template, a DNA polymerase, a DNA primer, normal deoxynucleoside triphosphates (dNTPs), and modified nucleotides (ddNTPs) that terminate DNA strand elongation. The formation of a phosphodiester bond between two nucleotides require 3'-OH group lacking in ddNTPs to cause the extension of the DNA strand to stop when a ddNTP is added. Four separate sequencing reactions of DNA sample contain all four of the standard dNTPs (dATP, dGTP, dCTP, and dTTP) the DNA polymerase, and only one of the four ddNTPs (ddATP, ddGTP, ddCTP, or ddTTP) for each reaction. To sequence analyzing after rounds of template DNA extension, gel electrophoresis is used for separated sizes of each of the four reactions in one of four separated lanes. The DNA sequence can be directly read by UV light or autoradiography. Automated sequencing machines can detect sequences of four reactions into one reaction run with radioactively or fluorescently labeled ddNTPs (15). This method is appropriate to identify mutant gene by insertions or deletions (16) and the molecular evolution of emerging virus studies (132).

2.10 Neuraminidase Inhibition Assay

Two classes of drug licenses are available for prevention and treatment of seasonal influenza(133). Adamantane (M2 ion channel blockers) were used for prophylaxis of influenza A but it has not been recommended nowadays with wild spread influenza A (H1N1 and H3N2) resistance data around the world (134). Neuraminidase inhibitors(NAIs) is the only antiviral drug that has affected at both influenza A and B (135). NAIs attack to envelope glycoprotein neuraminidase (NA) target which required for viral replication and successful establishment of infection (136). Oseltamivir has been approved by thefrom FDA to be administered orallyto use by administered orally. This drug was presentedhad presently in pre-post prophylaxis in Thailand for use against seasonal virus. After first report of Ooseltamivir-resistant seasonal influenza A (H1N1) by Norway to the World Health Organization (WHO) in January 2008, NAI susceptibility surveillance of influenza viruses need global antiviral resistance sSurveillance. To observe oOseltamivir resistant influenza virus, there is the NA enzyme assay (17). The principle of functional methods (NAI assay) is based on neuraminidase enzyme structural, the antigen and glycoprotein on the viral surface. A neuraminidase inhibitor is produced to tight interactions with the NA active site. It competes with neuraminic acid of virus for binding (137). NI assay detects a luminescent or fluorescent signal that is separated by an NA enzyme when the substrates have conjugated. There are different substrates between two assays of NI method. The 1,2-dioxetane derivative of neuraminic acid substrate is used in the chemiluminescent (CL) assay such as a NA-Star kit1, the first-generation and NA-XTD kit (138, 139). The fluorescent (FL) assay use 2'-o-(4-methylumbelliferyl)-N-acetyl-neuraminic acid substrate (MUNANA)(88) for example the NA-Fluor influenza neuraminidase. The NI assay shows the result as IC50 (the 50% inhibitory concentration) value that refers to the NAI concentration that inhibit 50% of the enzyme activity of the virus (140). The both kit of NAI assay requires virus propagation in cell culture or embryonated in a chicken egg in determining the IC50. The clinical specimen is insufficient to measure in this assay because it has a low viral content(140). Specimens were cultured in a Madin Darby

canine kidney (MDCK), then the virus would be aliquoted and stored at -70°C until used. Two passages of influenza virus in MDCK cells are strongly recommended to limit the number of NA inhibitor resistant viruses that can be unstable during cell culture passage. Serial virus two fold dilutions were prepared for standardized antigen to mix with ten fold dilution of drug. Each of virus dilution was added to 2 columns duplicate. Then the plate was shaked with a seal for 30 minutes at 37 degrees Celsius. After that, the substrate was added to each well including the blank row to make sure virus with drug and substrate mixed. The plate with substrate would react in the dark at 37 degree Celsius for 60 minutes with shaking. The reaction was added to a stop solution to all wells to terminate and read NAI result immediately.

IC50 values are calculated for the duplicates independently, and the mean IC50 taken as the final value by using sigmoidal curve fitting of point to point plotting with GrapPad Prism Software. NAI susceptibility reference panel provided for laboratories research by International Society for Influenza and others Respiratory Virus Disease – Antiviral Group (isirv-AVG) (Table2) (141).

Table 2 Evaluation of the panel of influenza viruses in Chemiluminescence NA enzyme inhibition assays from ISIRV organization

Chemiluminescence-based assays[b]

			Oseltamivir carboxylate IC_{50}	
Strain designation	(Sub)type	Genotype [c]	Range	Median
B/Perth/211/2001	B	WT	2 - 4	2.1
B/Perth/211/2001	B	D197E	10 - 30	22.4
A/Fukui/20/2004	A(H3N2)	WT	0.1 – 0.2	0.2
A/Fukui/45/2004	A(H3N2)	E119V	3 – 5	4.3
A/Mississippi/03/2001	A(H1N1)	WT	0.2 – 0.4	0.2
A/Mississippi/03/2001	A(H1N1)	H275Y	104 – 127	120.6
A/Perth/265/2009	A(H1N1pdm09)	WT	0.2 – 0.3	0.3
A/Perth/265/2009	A(H1N1pdm09)	H275Y	71 - 107	101.7

The clinical specimen is insufficient to measure in this assay because it has a low viral content (140). NAI assays are applied for the monitoring of antiviral resistant (142). For the routine monitoring, the method have to reliable and relatively simple (143). At the present, the NA enzyme assay has developed and adopted to be used around the world (144). NAI resistant monitoring is necessary for treatment efficacy and global spread of resistant influenza viral strains.

CHAPTER III

MATERIALS AND METHODS

3.1 Sample collection

January – December 2014, Influenza virus positive specimens that had detected by real time Reverse transcriptase polymerase chain reaction (RT-PCR) from acute respiratory patients in Phramongkutklao hospital were collected for monitor antiviral drug resistance by genotyping pyrosequencing method and phenotyping neuraminidase inhibitor assay. Positive RT-PCR samples was support from department of virology of AFRIMS. This project was approved by the ethics committees of the Walter Reed Army Institute of Research (Silver Spring, MD, USA) and the Royal Thai Army Medical Department (Bangkok, Thailand). The upper respiratory tract specimens was collected in added antibiotic and antimycotic virus transport medium. After collection, the specimen should be placed in 4 Celsius and be gotten in process as soon as possible. If the specimens cannot be in experiment within 48 hours, they could be kept in 70 Celsius to maintain condition. Because of less than 29 Cycle threshold (Ct) value of real time PCR refer to strong positive reactions demonstrative of abundant target nucleic acid in the specimen.

3.2 RNA extraction

QIAamp Viral RNA Extraction kit (QIAGEN, Germany) was used in this process. 79 virus transported medium (VTM) specimens were thawed at room temperature and mention specimen numbers on tube cap. 79 micro tubes were prepared by label specimen number. 140 µl of specimens was mixed with 560 µl of viral lysis buffer (Buffer AVL) and they had incubated at room temperature for 10 minutes. After that 560 µl of Ethanol (96-100%) was added to the specimens and mix them by pulse-vortexing for 10 – 15 seconds. The specimens was taken into short spin by centrifuge for remove drops from inside the lid. Next 630 µl of the solution was applied to the QIAamp Mini column that was in a 2ml collection tube. Then the

column was centrifuged at 8,000 round per minutes for 1 minute. The tube that contain the filtrate was discard and the QIAamp Mini column had place into a new clean 2 ml collection tube. The column tube cap was opened carefully and then repeat to added 630 µl of the solution again. After the QIAamp Mini column was centrifuged at 8,000 rpm, 1 minute, 500 µl of washing buffer 1 (AW1) was added and centrifuged at 8,000 rpm for 1 minute. The new clean 2 ml collection tube was provided to place QIAamp Mini column after centrifuged and the tube that contained the filtrate would be discard. 500 ml of washing buffer 2 (AW2) was added into the QIAamp Mini column and then centrifuged them at 14,000 rpm for 3 minutes. The QIAamp Mini column was changed to place into the new 2 ml collection tube and centrifuged 14,000 rpm again for 1 minute. For elution step, the QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube and added 65 µl of elution buffer. After the elution buffer was added, the QIAamp Mini column still incubated at room temperature for 1 minute. To collect RNA solution, the filtrate was collected by centrifuged at 8,000 rpm for 1 minute. The viral RNA can preserve for a year by freeze at -20 Celsius or -70 Celsius.

3.3 Reverse transcriptase polymerase chain reaction

cDNA synthesis and PCR amplification was perform by SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (QIAGEN, Germany) with specific primer that recommended from CDC and previous study. PCR master mix was prepared by follow the table of master mix component. To preparing of the PCR master mix based on “N” that is the number of reaction (Table3) with RT-PCR primer (Table4).

Table 3 PCR master mix component for preparing

Reagent	Volume(µl), N=1
Nuclease free water	16.5
2X Reaction Mix	25
Superscript IIIHiFiTaq	1
Forward primer (20µl)	1
Reverse primer (20µl)	1
Rnase inhibitor	0.5
Template RNA	5
Total volume	50

Table 4 RT- PCR primer for RNA interesting fragment amplification from CDC and previous study

Type of Influenza virus	Forward primer for RT-PCR	Reverse primer for RT-PCR
M2 for H1 and H3	Sw-M2-F670	Sw-M2-R900
NA for H1	Uni-sw-N1-B-F780mo1	Uni-sw-N1-B-R1273mo1
NA119/292 for H3	HuN2NA-F333-biot	Hun2NA-R914
NA151 for H3	HuN2NA-151-F423	Hun2NA-151-R567-biot
NA fragment 1 for Flu B	BNA-F301	BNA-R848-biot
NA fragment 2 for Flu B	BNA-F408	BNA-R848-biot
NA fragment 3 for Flu B	BNA-F962	BNA-R1274-biot

All of reagents was thawed, gently mixed by inversion, briefly centrifuged to collected all contents at the bottom of the tube and placed all of them in cooler rack. Each of primer set was labeled on 1.5 sterile microcentrifuge tubes. Each of master mix set was prepared in each PCR reaction tube. The last component to added is RNA templates. 5 µl of each RNA extracted samples was added into PCR reaction labeled tube in pre-PCR room. All of PCR reaction tubes containing RNA template would be transferred toamplification area where the Rotor-Gene Thermal Cycler machine (QIAGEN, Germanly) is set and was placed into the rotor wells. The Rotor-Gene system was run below the thermal cycler program (Table 5).

Table 5 Rotor-Gene Thermal program for reverse transcriptase RT-PCR

cDNA synthesis and pre-denaturation	Perform 1 cycle	50°C for 30 minutes
Taq inhibitor inactivation (Denaturation)	Perform 1 cycle	95°C for 2 minutes
PCR amplification: Denature Anneal/Extension	 Perform 45 cycles Perform 1 cycle	 95°C for 15 seconds 55°C for 30 seconds

After finish PCR running, the result of PCR product would be checked by Gel electrophoresis

3.4 Gel electrophoresis

RT-PCR product was checked by 1.5% Agarose Gel electrophoresis. The Agarose gel was prepared by measured 0.42 g of Agarose powder (Sigma-Aldrich, Singapore) and added to 100 ml flask. Then 30 ml of Tris-borate-EDTA (TBE) buffer was added to the flask. The agarose was melt in a microwave for several short intervals. The casting tray was prepared with the plastic template and comb. When the agarose solution cooled around 50-55 Celsius, the melt agarose solution was poured into the casting tray and let cooled until it is solid. After that the comb should be carefully pull out and pick up the gel to the electrophoresis chamber and added TBE buffer over the gel about 2-3 ml. To loading the gel, 1 µl of loading buffer was added to each of 5 µl PCR reaction and then 6 µl of each sample loading buffer mixture was pipette into separate wells in the gel. The DNA ladder standard was pipette 10 µl into at least one well of each row on gel. To running the gel, the lid was placed on the gel box for connecting the electrodes. The power supply was turned to 100 volts. Bubbles forming on each electrode would make sure that the current gel box is running and the correct direction could be checked by observing the movement of the blue loading dye. When the blue dye approached to the end of the gel, the gel was removed and observed the result by Gel documentation machine (Syngene, England).

3.5 Pyrosequencing

Run sample sheet of samples to be tested was prepared by Microsoft Exel and saved as a text (*.txt). Then the sample sheet text file would be imported into the PyroMark TM ID Platform (QIAGEN, Germany). After Run sample sheet had prepared, the Pyrosequencing Reaction would be set up. Pyrosequencing 96 sample Prep Thermoplate holder was placed upon the heat block and allowed the plate to heat to 82 Celsius. All of reagent such as the Streptavidin Sepharose TM beads, PyroMark TM Binding Buffer, Annealing Buffer, Denaturationn Solution, 1X wash Buffer and 70% Ethanol from 4 Celsius storage was removed to place at room temperature. The binding buffer solution was prepared by adding the following solution in conical tube and mix by vortex (Table6).

Table 6 The following of binding buffer solution per 1 reaction

Component	Volume per 1 Reaction
Binding Buffer	40 µl
MiliQ water	17 µl
SteptavidinSepharoseTM beads	3 µl
Final volume	60 µl

After binding buffer was prepared to suit with the number of reaction, the solution was aliquot 60 µl into each of well of a 96-well PCR plate. Then 20 µl RT-PCR product was added into the respective wells of 96-well plate containing binding buffer solution. The plate was covered with a plastic adhesive cover and placed on a plate shaker and shaken at 1,400 rpm for at least 10 minutes. Until begin the sample clean up steps with PyroMark TM Vacuum Prep workstation (QIAGEN, Germany), the plate was allowed to continue shaking. Annealing buffer solution was prepared by adding the following solution (Table7) with pyrosequencing primer (Table8) in 1.5 ml microcentrifuge tube and mix by vortex.

Table 7 The following of annealing buffer solution per 1 reaction

Component	Volume per 1 reaction
Annealing buffer	40 µl
Sequencing primer (100µM)	0.16 µl
Final concentration 0.45 µM	

Table 8 The sequencing primer for pyrosequencing

Type	Sequencing primer
H1N1 – M2	Sw-M2-F747-seq, GCG ATT CAA GTG ATC C
H1N1 – NA-H274	Uni-sw-N1-B-F804seq, GYT GAA TGC MCC TAA TT
H3N2 – M2-new	ACC CGC TTG TTG TTG CC/TG CGA A/GTA TC
H3N2 – NA-D151	AGT ACG TG/AA TAG GAC
H3N2 – NA-E119V	AGG TT/AC TC
H3N2 – NA-R292K	TTT CCA GTT GTC TC/TT GCA
FLU B – NA-E117A	TTG ATA ATA AGG GA/CA CC
FLU B – NA-R150	ACA G/AAA ACA AG
FLU B – NA-D197E	ATG GCC CC/TG/A ACA GT
FLU B – NA-I221T	AAA A/CAA C/AAT/C CCT AAG G
FLU B – NA-S249G	AGG TA/GT TA/GG TGA ATG
FLU B – NA-H273Y	AGT AAA AC/TA C/TA CT
FLU B – NA-R374K	GAA AA/GA ATG
FLU B – NA-G407	GAA TGA AAG AAC CTG/A GTT GGT AT

40 µl of the annealing buffer solution was aliquot into the respective wells of a 96-well clear plastic plate (Pyrosequencing 96 Plate low) and then the plate would be placed onto its position on the Vacuum Prep workstation (QIAGEN, Germany). The designated position on the Vacuum Prep Workstation should be put the plastic reagent trays on before fill 70% Ethanol, Denaturation solution, 1X Wash Buffer and distilled deionized water. After distilled deionized water was added into a

plastic reagent tray, the filters was put in the PyroMark TM Prep Tool and turned on at the Vacuum Prep Workstation and the vacuum pump. This will allow water to flush through and prime the filter probes on the Prep tool. Then the tool would be place in the plastic reagent tray until it was ready to use. Next the 96-well plate containing the PCR product bound to streptavidin SepharoseTM beads was removed from the shaker and placed on its respective position on the vacuum Prep Workstation. The plastic adhesive cover on the plate was removed gently to avoid splashing.

After that the Prep tool would be placed in the respective wells of the 96-well PCR plate until the all volume had been collected about 5-10 seconds. The visible Streptavidin SepharoseTM beads with immobilized amplicons captured on the tips of filter probes of the Prep tool will be perceivable. The Prep tool with Streptavidin SepharoseTM beads was transferred to the reagent tray containing 70% ethanol and flushed until the tray is almost emptied (15-25 seconds), then lift to drain the tool. This step allows any unbound amplicon or unincorporated reagents from the RT-PCR reaction to be washed off. Next the Prep tool was transferred to the reagent tray containing Denaturation solution and flushed until the tray is almost emptied (15-25 seconds), then lifted to drain the tool. This step allows for the denaturation of the double-stranded DNA amplicon containing a biotin-tagged, single-stranded DNA template. The complementary strand synthesized from the non-biotinylated primer will be wash away. Then the Prep Tool was transferred into the reagent tray containing 1X Wash Buffer and flushed until the tray is almost emptied (15-25 seconds) and lifted to drain the tool for 30-40 seconds. To release beads bound to the single-stranded template, the Prep tool was moved over the PSQ96 Plate low containing annealing buffer including sequencing primer but do not put into the plate and turned the vacuum off and placed the Prep tool to the 96-well PSQ96 Plate low. Next the Prep tool was move in circular motions (15-25 seconds) in the PSQ96 Plate low so the mixture on the plate should now appear cloudy. Then the PSQ96 Sample Prep Thermoplate holder was placed on the 89 Celsius heat block for 2 minutes. The plate should be not leaved on the heat block for more

than 2 minutes. After that the plate was removed from the heat block and cool down on the bench for 5 minutes.

For Pyrosequencing Using the PyroMarkTM Q96 ID Equipment and software (QIAGEN, Germany), the software was opened and “SQA” on the left side of program window was selected. “SQA Runs” icon was clicked to start a new run. To see the imported run, the “Import” folder was expanded and double clicks to open the file. The number of cartridge should be match with the “Instrument Parameters Code 000X”. For select a dispensation entry, the dispensation order that is under “Entry” in the “Setup” menu was selected. If the target specific entry is not in the Entry list, then create a new SQA entry. To create a new SQA entry, click on the “SQA Entries” icon and right click on the “SQA” folder and select “New Entry”. Under “Entry ID:”, type in the specific name. Under the “Dispensation order” tab, enter 5(CATG) in the “ Entered dispensation order:” box. The sequence should be visible under the “Expanded dispensation order:” box. The name of the entry should now appear in there well when the Pencil tool was clicked and highlighted all the wells that are to be used. To see the volumes of the enzyme(E), substrate(S), and nucleotides(A,C,G, and T) that need to be added to the cartridge, “Run” in a drop-down menu of the “View” tab was selected. Then the PyroMarkTM Gold reagent kit (QIAGEN) from 4 Celsius storage was removed. The enzyme and substrate were reconstruct in 620 µl deionized water and swirl to mixed (Do not vortex). The reconstituted enzyme and the substrate are stable at least 5 days at 4 Celsius, or for one freeze (-20 Celsius). The dispensation pins (needles) should be ensure that they are clean and not bent before use. Each channel in the cartridge was filled by the enzyme, substrate and nucleotides with the label facing the user. The volume should be loaded carefully to ensure that air bubbles are not created. The PSQ96 Plate Low containing the annealed DNA template and sequencing primer was load onto the PyroMarkTM Q96 ID instrument (QIAGEN, Germany). Then the cartridge that is containing enzyme, substrate, and nucleotides was loaded on the PyroMarkTM Q96 ID instrument, with the cartridge label facing towards the user. For start the pyrosequencing reaction, “PyroMarkTM ID” in the “Instrument” option menu was select and click “Run”. After

the substrate is added, the peak of substrate should appear to confirm that the enzyme and substrate reagent was working.

After the program was running, the Prep tool was cleaned by Placing the hand tool in the reagent tray that is containing distilled deionized water. The tool was flushed by turning on the vacuum until almost of the tray emptied. Then the vacuum was turn off. The reagent trays from the vacuum Prep workstation were removed and discarded excess solution. They were rinsed by distilled deionized water and then set them upon the paper towels on the bench to dry. When the pyrosequencing run was finished, the PSQ96 Plate Low would be removed and discarded from the PyroMarkTM Q96 ID instruments. The cartridge that was containing unused enzyme, substrate, and nucleotides was removed to discard the reagents. Then they were washing with distilled deionized water at least 3 times. All dispensation pins should be checked to make sure that they are cleaned by applying pressure to the top of the channels in the cartridge. When the SQA run was completed, the window was clicked “close”. The running from the import folder was reopened to reveal the SQA analysis window. “ALL” which is under the “Analyzed” option was selected to analyze all the samples in the run. To analyzed only selected wells, the wells you wish to analyze would be highlight and click “selected”. The icon for a completed run file is a blue check, and will replace the green icon, which denotes a run setup that has not yet been run. When analysis was done, the data was saved and the PyroMarkTM ID software was closed.

The Pyrosequencing instrument will generate sequence that will be analyzed and reported by IdentiFireTM software. All pyrogram of pyrosequencing results were printed and verified visually to described the amino acid markers of resistance to Oseltamivir (Table9).

Table 9. Amino acid markers of resistance to Oseltamivir

Type	Amino acicid Oseltamivir resistant marker on NA gene
Flu A pdm H1N1	H275Y
Flu A H3N2	E119V, D151N/G/E/V, R292K, N294S
Flu B	E117A, R150K, D197N/E,I221T, H273Y, R374K, S249G,G407S

3.6 Sequencing

The PCR product was purified by using QIAquick PCR Purification Kit. First, 5 volumes of Buffer PB that is used in DNA cleanup procedures and enables efficient binding of single- or double-stranded PCR products to the spin-column membrane to 1 volume of the PCR reaction and mixed. Second, a QIAquick column was placed in a 2 ml collecting tube. To bind DNA, the sample was applied to the QIAquick column and centrifuge at 13,000 rpm for 30 – 60 seconds until all the sample has passed through the column. The flow through solution was discard and the QIAquick column was placed back to the same tube. To washing, 0.75 ml of Buffer PE that is a wash buffer was added to the QIAquick column. Then the column was centrifuged at 13,000 rpm for 30 – 60 seconds. The flow through solution was discard and the QIAquick column was placed back to the same tube. The QIAquick column would be centrifuged once more in the provide 2 ml collecting tube at 13,000 rpm for 1 minute to remove residual wash buffer. Next the column was placed in a new clean 1.5 ml microcentrifuge tube. To DNA elution, 50 µl of Elution buffer was added to the center of the QIAquick column membrane and centrifuged the column at 13,000 rpm for 1 minute. For increased DNA concentration, add 30 µl elution buffer to the center of QIAquick membrane, let the column stand for 1 minute and then took the column to centrifuge. After that we sent PCR product with sequencing primer to AIT biotech company, Singapore for seqeuncing detection.

3.7 Influenza virus isolating using Madin-Darby canine kidney epithelial cell (MDCK cell)

The specimen for virus isolation was thawed. 0.2% Trypsin was prepared by added 100 mg of trypsin to 50 ml of Milli Q water. The solution was stirred gently until tripsin is dissolved and sterile by filtering through 0.2 µm pore size filter unit. Then the trypsin was aliquoted into appendrof tube, 0.2 ml each tube with labeled "0.2% trypsin". This solution can storage at -20 Celsius to 5 Celsius for 2 years. 3.75% Bovine serum albumin (BSA) solution was prepared by mixed 3.75 gm of BSA to 100 ml of Dulbecco's Modified Eagle Medium (DMEM) (Table 10). Next the solution would be swirled gently until BSA is dissolved before sterile by filtering through 0.2 µm pore size filter unit and aliquoted 27 ml in conical tube with labeled "3.75% BSA in DMEM". Preparation of DMEM Maintenance media (MM) for MDCK cell can make ready by compounded the component in table14. After that the labeled bottle was swirled gently to mix the solution and storage at 2-8 Celsius that has the shelf life 1 month or the earliest expiration date of reagents.

Table 10 The component of DMEM Medium for MDCK

Component	Volume (ml)
3.75% BSA	27
L-Glutamine	5
Penicillin-Streptomycin solution	5
Gentamicin	0.4
Fungizone	0.5
HEPES	12.5
DMEM	500

To prepared of DMEM MM for MDCK cell plus 2 µg/mg trypsin, the medium was made ready by mix the component in the table 16, then swirled the bottle that labeled "DMEM MM for MDCK + trypsin" gently to mix the solution and storage at 2-8 Celsius for use within 24 hours (Table11).

Table 11 The component of DMEM MM for MDCK + trypsin

Component	Volume(ml)
DMEM MM for MDCK	100
0.2% trypsin	100
Add trypsin freshly daily before use	

The MDCK cell which 1-2 day old in culture tubes was requested for an experiment equal to the number of specimen plus 3 tubes for controls. The culture tube would be placed in a rack. The cell was observed in flat surface. 1 to 2 centimeters from the end of the tube around the middle under inverted microscope (the density of cells increases towards the bottom and sides of the tube). The number of each specimen was label on the culture tube. The specimen would be thawed in cool water or leaved at room temperature then transfer tube to the biological safety cabinet. Maintenance media (DMEM) was pre-warmed at 35±1 Celsius by kept in incubator and protected from the light. For inoculation of specimen into MDCK cell, the first, Growth media in the culture tube was poured off in a waste container. Second, 2 ml of Phosphate buffered Saline was added in the tube. Third, the solution was poured off into the waste container. Forth, 1 ml of DMEM without trypsin was added in the tube. Fifth, the maintenance media was poured off into the waste container. Sixth, the specimen was mixed by using micropipette tip with filter. Seventh, 100 µl of specimen was added slowly and carefully to the flat side of the culture tube to avoid aerosols formation, and immediately rock the tube gently to avoid the aerosols formation so the inoculums covers the cell sheet. Touching the pipette shaft to the specimen and culture tube should be avoided because it may contaminate fresh culture tubes. Eighth, the cell in tube was incubated in 35±1 Celsius incubator for 30 minutes to 1 hour. Ninth, 1.5 ml of DMEM with trypsin (2 µg/ml) was added into the tube then the cap was closed tighten and the culture tube was incubated in 35±1 Celsius incubator. Three control tubes must be included in each assay. The first control tube is cell control (CC) that is a culture tube without washing, medium replacement or inoculation. The second

control tube is negative control that is a culture tube inoculated with virus transported medium instead of specimen. The cell control and negative control should be observed and was not found cytopathic effect (CPE). And the third control tube is positive control which inoculated with the reference viruses that should be found some degree of CPE (Table12).

Table 12 The result of cytopathic effect (CPE) of infected cell culture

Result of CPE recoding	The picture of observation
No CPE	No CPE observed
1+	25% CPE (approx 25% of all cells show CPE)
2+	50% CPE (approx 50% of all cells show CPE)
3+	75% CPE (approx 75% of all cells show CPE)
4+	100% CPE (all cells show CPE)

Contamination was determined by the appearance of bacterial or fungal growth in the culture tube. If the culture tube was found contamination, the supernatant of the tube was poured into a new microcentrifuge tube and centrifuged at 2,000 rpm for 2 minutes. Then the contaminated supernatant was filter with the 0.2 µm syringe filter and inoculated filtered supernatant into a fresh culture tube. The contaminated culture tube was discarded in biohazard container. The liquid waste in the waste container was disinfect by added 1:10 dilution of Bleach. The culture tube would be observed cells for cytopathic effect (CPE) and contamination by inverted microscope daily on day 2. On day 7, if the culture tube developed CPE to 75 – 100% (3 – 4+ CPE) or until the CPE stops progressing, it would be collected and transferred to harvest in T25 flask. However the culture tube without CPE for 6-7 days, it would be transferred to next culture tube passage. For those culture tubes that have 3-4+ CPE, cell suspension was poured into a new microcentrifuge tube and

centrifuged at 2,000 rpm for 2 minutes. The supernatant was collected in a new cryovial labeled with the specimen number, cell like, number of passage (P-1 to indicate 1st passage, P-2 to indicate 2nd passage and P-3 to indicate 3rd passage) and date of harvest. For those culture tubes that have no CPE, cell supernatant was poured directly to a labeled cryovial. The specimen was kept in 2-8°C refrigerator for Haemagglutination (HA) titration assay for 7 day. If the assay cannot take on time, the supernatant would be stored in a -70 Celsius freezer. Inoculation of the next passage would be done if HA titer is less than 8. If HA titer is more than 8, the specimen would be passed to T25 flask with a confluent monolayer of MDCK cells. The cells was checked for healthy and in a monolayer by using a inverted microscope at 40X magnification. The DMEM medium for cell growth was discarded from the flask and washes the cells 3 times with 6 ml of Phosphate Buffered Saline. Then the cells were washed by 5 ml of DMEM medium and removed with vacuum pipette. Next 100 µl of each specimen was inoculated into T25 flask. Allowing inoculums to adsorb by incubated at 37 Celcius for 30 minutes to 1 hour. After that 6 ml of DMEM plus trypsin was added to the flask and incubated at 35 Celcius with daily observation for cytopathic effect shown. When the cell culture in T25 flask showed 3 – 4+ of CPE, it would be harvested in the cryovial tube and kept at -70 Celcius for HA test assay. To harvesting the virus, the supernatant was transferred to the 10 ml plastic sterile tube and centrifuged at 3,000 rpm for 5 minutes to removed excess cell. Then the supernatant was collect in labeled cryovial tube and kept in freezer.

3.8 Haemagglutination titration of control viral isolates

The appropriated type of 96-well microtitre plate was choose and labeled plate oriented as in figure12.

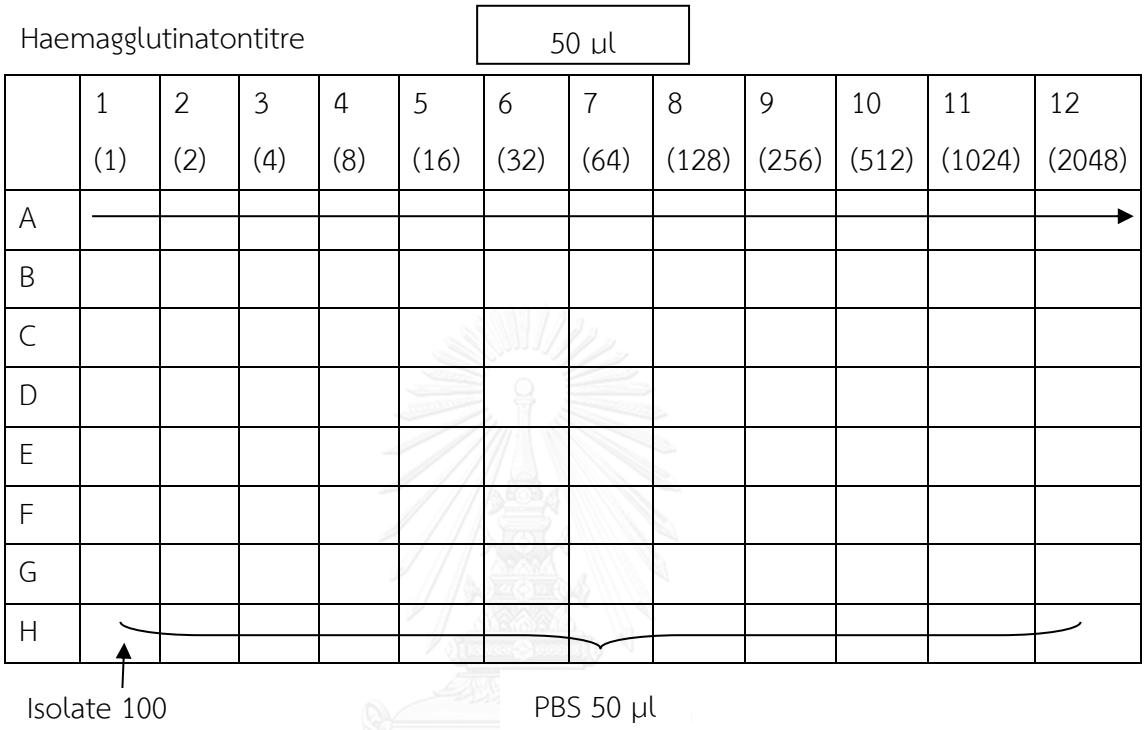


Figure 12 outline of haemagglutination titration of control antigens and viral isolates

50 µl PBS (PH 7.2) was added to wells 2 to 12 of each row (i.e. A2-A12; B2-B12; etc. up to H2-H12). 100µl of each different viral isolate was added to first wells of rows A-G (i.e. A1-G1 to allow for the testing of up to 7 different isolates). An red blood cell (RBC) control was prepared in row H (well H1) by adding 50 µl PBS. 50 µl from the first was transferred to successive wells of each row for making serial 2-fold dilution (i.e. A1 to A2; A2 to A3; etc. up to A11 to A12). The final 50 µl was discarded. 50µl of standardized RBCs would be added to each well.

The solution in the plate was mixed by laboratory shaker for 10 seconds or by manually agitating the plates thoroughly then the plate was covered and incubated at room temperature for 1 hour. The RBC control should be checked for complete settling of the cells. The result would be record and interpreted. The

haemagglutination titration end-point is defined as the highest dilution of virus that still causes complete haemagglutination. The haemagglutinationtitre is the reciprocal of this dilution.

3.9 Neuraminidase inhibitor assay

Control virus and virus isolates were thawed to use in the assay in a class II biosafety carbinet. The reagent was prepared at the room temperature. The use of both drug-sensitivity and drug-resistant control virus of appropriate influenza subtype allows for an assessment to be made of the drug susceptibilities of the virus isolates be tested. NA-XTD Kit (NovaBright™, United State) was used in the assay. Haemagglutination (HA) titre of virus isolates up to 8, so the HA titre of each isolates should be test before take in the experiment. If the HA titre is higher than 8, it would be diluted with NA-XTD assay buffer (AB) to prepare titre to be 8. If the titre is lower than 8, the supernatant of virus isolates should be repeated in the culture passage again to improve the number of virus for excess the HA virus activity. Each of virus isolates was prepare to 400µl (Table13).

Table 13 The following of preparing HA titre of virus for 400 µl

Previous HA titre	Dilution for titre 1:8	Volume of Virus (µl)	Volume of AB buffer (µl)
1:8	-	400	-
1:16	1:2	200	200
1:32	1:4	100	300
1:64	1:8	50	350
1:128	1:16	25	375
1:256	1:32	12.5	387.5
1:512	1:64	6.25	393.75

Neuraminidase inhibitors (NI) master stock was prepared by dissolved 19.3 mg of 25 mM of Oseltamivir carboxylate (D-tartrate salt, MW = 368.4) in 2 ml distilled water. The master stock was aliquoted and stored at 4°C. The NI master stock was used to prepare 500 µM working stock (WS) by dilute the 25 mM master stock 1:50.

The unused portion was discarded after the assay. After that the serial dilutions of working stock was prepared in NA-XTD assay buffer (AB) (Table14). Total volume of each NI dilution required is 25 µl/well of 96-well plate.

Table 14 The dilution of working stock to use in the assay

Dilution	Combine	Final volume(µl)	NI conc before assay (3x)	NI conc in final assay
1(1:25)	30 µl WS NI + 720 µl AB	750	20,000 nM	6,600 nM
2(1:5)	100 µl Dil 1 + 400 µl AB	400	4,000 nM	1,320 nM
3(1:5)	100 µl Dil 2 + 400 µl AB	400	800 nM	264 nM
4(1:5)	100 µl Dil 3 + 400 µl AB	400	160 nM	52.8 nM
5(1:5)	100 µl Dil 4 + 400 µl AB	400	32 nM	10.56 nM
6(1:5)	100 µl Dil 5 + 400 µl AB	400	6.4 nM	2.11 nM
7(1:5)	100 µl Dil 6 + 400 µl AB	400	1.28 nM	0.422 nM
8(1:5)	100 µl Dil 7 + 400 µl AB	400	0.256 nM	0.084 nM

Next the neuraminidase inhibition assay was performed by added 25 µl of appropriated NA dilution (dilution 3 to dilution 8) to desire wells in row A through F and added 25 µl of NA-XTD assay buffer to wells in row G and H. Then 25 µl of diluted reference virus (drug-sensitive control (SC) and drug-resistant control (RC)) would be added per well in column 1 and 2 (A to G) and 25 µl of diluted virus sample was added in column 3 to 12. The H row was No virus assay control, so 25 µl of NA-XTD assay buffer was added in H1 to H12 again. Total well volume is 50 µl before addition of dilute NA-XTD substrate (Table15). After that the lid was placed on the plate and the plate was incubate at 37°C for 20 minutes. While the plate was incubating, the NA-XTD substrate was diluted 1:1000 in sufficient volume for

immediate use (25 µl per well) at room temperature. For one 96-well plate, a total of 4 ml of diluted NA-XTD substrate would be prepared by mixed 4 µl of NA-XTD substrate + 4 ml NA-XTD assay buffer. Then 25 µl of diluted NA-XTD substrate was added to each well following by placed the lid on the plate and incubated for 30 minutes at room temperature. Then 60 µl of NA-XTD Accelerator was added to each well. The plate should be read immediately or up to 2 hours after adding NA-XTD Accelerator by PerkinElmer 2030 instrument (Finland) and determined the half maximal effective concentration (EC50) by GraphPad Prism 4 program.

Table 15 Plate layout for NAI assay

	1	2	3	4	5	6	7	8	9	10	11	12
A:Dil 3	SC	RC	Virus1	Virus2	Virus3	Virus4	Virus5	Virus6	Virus7	Virus8	Virus9	Virus10
B:Dil 4												
C:Dil 5												
D:Dil 6												
E:Dil 7												
F:Dil 8												
G:No Drug	▼	▼	▼	▼	▼	▼	▼	▼	▼	▼	▼	▼
H:No Drug-No virus				NA-XTD assay buffer 50 µl								

3.10 Statistic analysis

3.10.1 One- way anova

One- way anova tests whether the means of the measurement variable are the same for the different groups within two or more nominal variable groups. This tool be used for comparing the means of groups of measurement data(145).

3.10.2 Bonferroni's method

For multiple comparisons, Bonferroni is an instrument to make the significance level of a statistic test. In this study, we used Bonferroni corrected significance level of 0.05/10 = 0.005(146) with influenza virus type and subtype which circulated in PMK hospital.

3.10.3 Chi-square test

Chi-square statistic uses for testing relationships between categorical variables. This procedure was used between IC50 result and pyrosequencing result of influenza B.

CHAPTER IV

RESULTS

4.1 Distribution of influenza in Pramongkutklao hospital

Samples from patients influenza like illness who admitted in Phramongkutklao hospital between January to September 2014 were screened for influenza typing by reversed- transcriptase polymerase chain reaction. Criteria cut off of cycle threshold (Ct) value for selective sample to take in this experiment had referred from CDC 2014. Positive influenza virus will has cycle threshold less than or equal 38 cycles and strong positive of influenza virus group will has Ct value less than 30 cycles. Then we selected strong positive influenza clinical samples 79 samples that had been preserved in viral transportation medium (VTM). 22 (27.8%) of 79 (100%) samples are influenza A pandemic H1N1. 24 samples (30.4%) are influenza A H3N2 , and 33 samples (41.8%) are influenza B with undermined lineage (Table16).

Table 16 The number and percentages of each categories of seasonal influenza viruses in 2014

Subtypes	n	% of all samples
Influenza A (Pdm H1N1)	22	27.8
Influenza A (H3N2)	24	30.4
Influenza B	33	41.8
Total	79	100

4.2 Pyrosequencing

All clinical samples in VTM were extracted by QIAGEN Kit (Germany) and were amplified by specific correlated drug resistant position primers with reverse transcriptase PCR. Then PCR products were approved by gel electrophoresis.

4.2.1 Influenza A pandemic H1N1

After passing RT-PCR experiment with sw-M2-F670 primer and sw-M2-R900 mo1 primers, 22 bands of M2 gene of Influenza A pandemic H1N1 which product size had about 230 base pairs, appeared clearly on gel. By using Uni-sw-N1-B-F780-mo1 and Uni-sw-N1-B-R1273mo1 primers which have correlated NAI resistance region, 22 bands of NA gene products with 493 base pairs showed (Figure13).

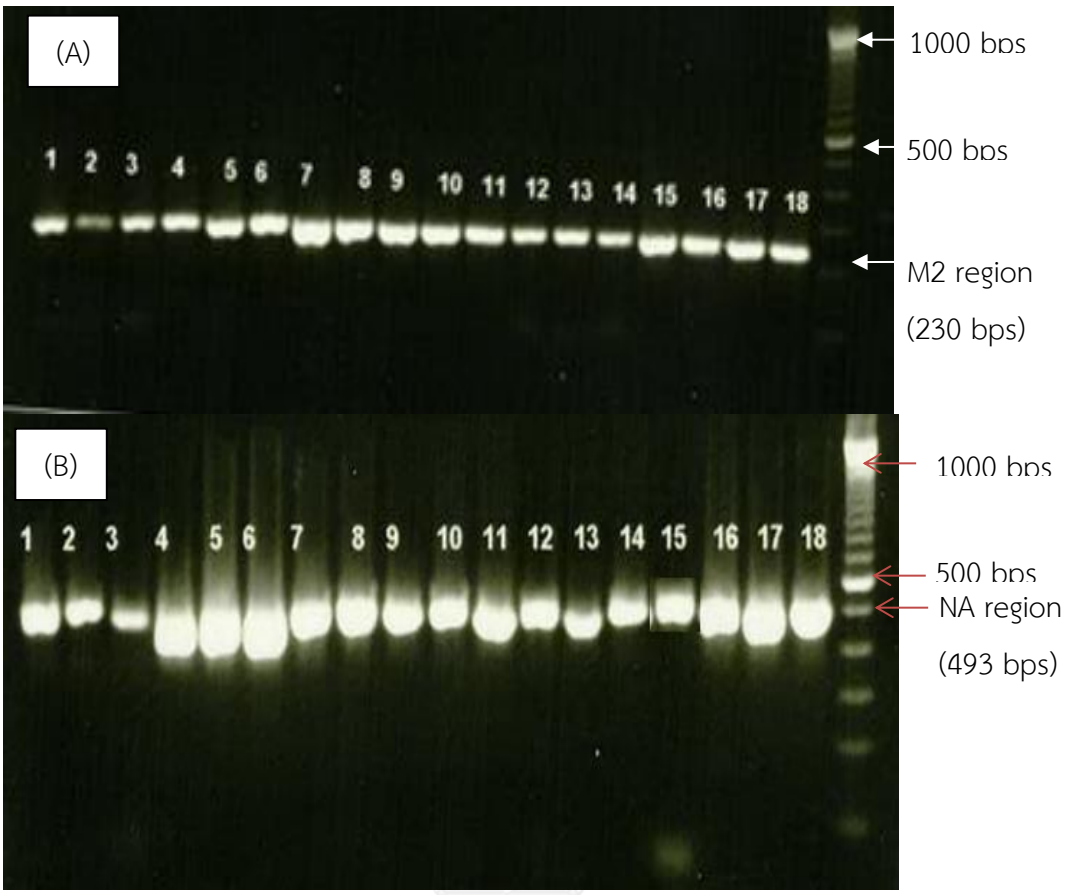


Figure 13 The examples of product bands of M2 region at 230 base pair size (A) and NA at 493 base pair size (B) region of influenza A pandemic H1N1 from cDNA amplification step by RT-PCR with specific primer.

By pyrosequencing, mutation points on position 31 of M2 gene and position 275 of NA gene were detected on the cDNA products of influenza A pandemic H1N1 that responded to 2 antiviral drug groups, adamantane and neuraminidase inhibitors. All of influenza A pandemic H1N1 samples in this experiment are resistant to M2 blocker antiviral drug group while 16 samples of this type showed susceptiabilityle to neuraminidase inhibitor at high percentage (94-100%) (Figure14). Another 6 samples still showed oseltamivir susceptibility (Table17).

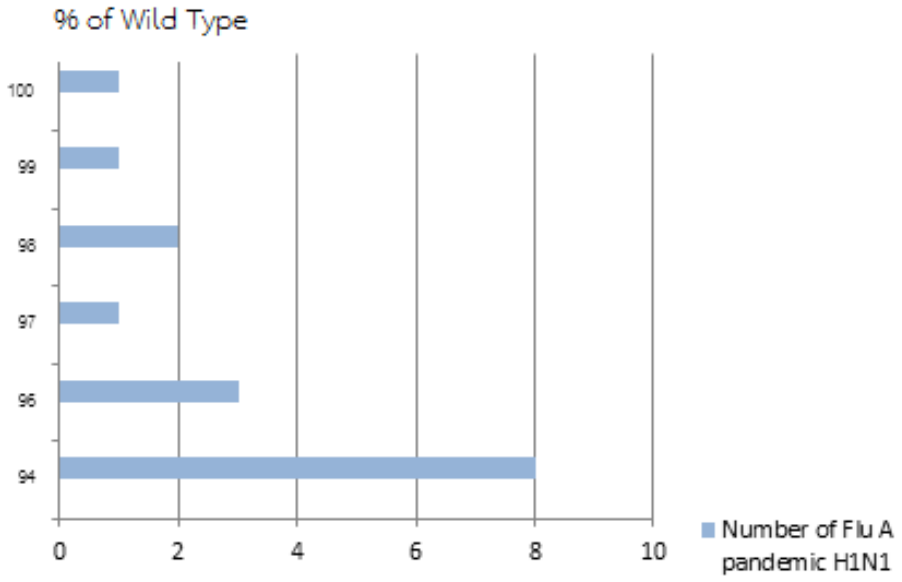


Figure 14 16 samples of pandemic H1N1 had high percentages between 94 to 100% of wild type virus that were susceptible to Neuraminidase inhibitor. 8 samples have 100% of wild type base point. 3 and 2 samples have 99% and 97% of susceptible base point. Another percentage (94, 96 and 98 %) has 1 sample of each level.

Table 17 Result of pyrosequencing of M2 and NA gene detection of pandemic H1N1 influenza virus. M2 gene detection by SQA program of pyrosequencing showed M2 resistance gene on position 31 of 22 samples. NA gene detection by AQ program of pyrosequencing had susceptible result to NAI agent with percentage.

NO	Specimen No.	Pyrosequencing		Result	
		M2 Position 31	NA Position 275	M2	NA
1	FLU-PMKA3098	Mutation	Wild Type	Resistance	Susceptible
2	FLU-PMKD059	Mutation	Wild Type	Resistance	Susceptible
3	FLU-PMKB0237	Mutation	Wild Type	Resistance	Susceptible
4	FLU-PMKD0633	Mutation	Wild Type	Resistance	Susceptible
5	FLU-PMKD0531	Mutation	Wild Type	Resistance	Susceptible
6	FLU-PMKD0568	Mutation	Wild Type	Resistance	Susceptible
7	FLU-PMKD0574	Mutation	Wild Type	Resistance	Susceptible
8	FLU-PMKA3020	Mutation	Wild Type	Resistance	Susceptible
9	FLU-PMKD0588	Mutation	Wild Type	Resistance	Susceptible
10	FLU-PMKA3058	Mutation	Wild Type	Resistance	Susceptible
11	FLU-PMKD0590	Mutation	Wild Type	Resistance	Susceptible
12	FLU-PMKD0596	Mutation	Wild Type	Resistance	Susceptible
13	FLU-PMKA3117	Mutation	Wild Type	Resistance	Susceptible
14	FLU-PMKA3153	Mutation	Wild Type	Resistance	Susceptible
15	FLU-PMKA3177	Mutation	Wild Type	Resistance	Susceptible
16	FLU-PMKA3200	Mutation	Wild Type	Resistance	Susceptible
17	FLU-PMKA3201	Mutation	Wild Type	Resistance	Susceptible
18	FLU-PMKD0612	Mutation	Wild Type	Resistance	Susceptible
19	FLU-PMKD0615	Mutation	Wild Type	Resistance	Susceptible
20	FLU-PMKA3244	Mutation	Wild Type	Resistance	Susceptible
21	FLU-PMKD0670	Mutation	Wild Type	Resistance	Susceptible

NO	Specimen No.	Pyrosequencing		Result	
		M2 Position 31	NA Position 275	M2	NA
22	FLU-PMKD0717	Mutation	Wild Type	Resistance	Susceptible

4.2.2 Influenza A subtype H3N2

After amplification method, 230 base pairs of 24 samples showed a M2 product band on the gel after being amplified with sw-M2-F670and sw-M2-R900 mo1 primer. For NA gene detection, there were 3 points of interest with correlation with oseltamivir resistant in influenza virus. At the position NA119 and NA292, the same primers for amplication were used. HuN2NA-F33-biot and Hun2NA-R914 were used for fragment amplification to detect gene mutation at NA119 and NA292. 18 samples of both positions had product band appear on size 581 base pair. 6 samples of H3N2 were found without PCR product. The third position of NA gene of influenza A H3N2 which contained a mutation was NA151. The fragment for detection of this position was amplified with HuN2NA-151-F423 primer and HuN2NA-151-R567-biot primer. 18 samples showed product on 144 base pairs size but the other 6 samples could not see any product on site (Figure15).

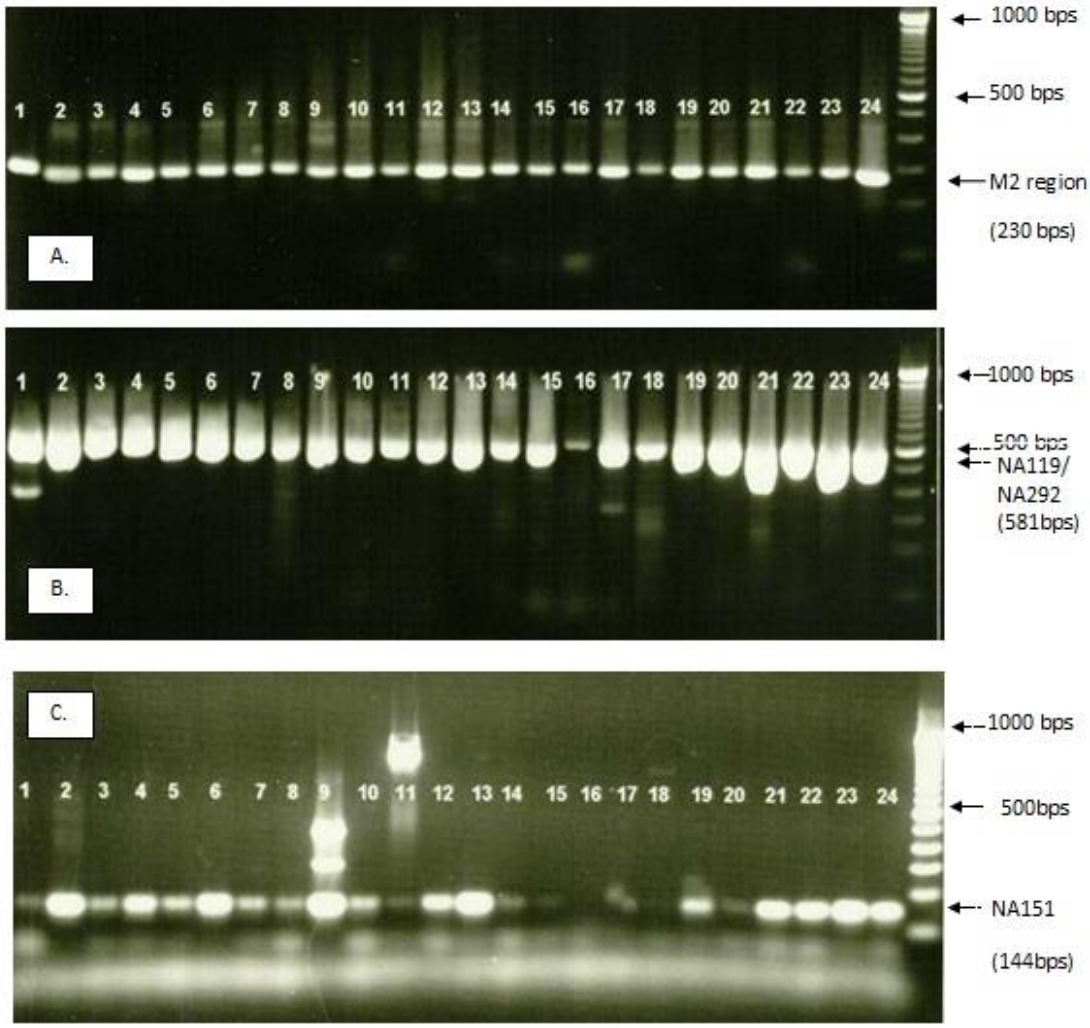


Figure 15 (A.) 24 product bands of M2 gene of influenza A H3N2 with 230 base pair size which were amplified by sw-M2-F670 primer and sw-M2-R90.0 mo1 primer. (B.) The example of NA119 and NA292 products bands on 581 base pair size that were amplified by same primer, HuN2NA-F33-biot and Hun2NA-R914. (C.) Product bands of NA151 position at 144 base pair size with RT-PCR amplification by HuN2NA-151-F423 primer and HuN2NA-151-R567-biot primer.

Pyrosequencing result of flu A H3N2 showed that all 24 samples were resistant to M2 blocker drug group. Moreover, wild type percentage on specific NA region correlation with NAI susceptability were found on 18 samples on the E119E and D151D positions as well as 16 samples of R292R wild type gene. On 151, residues presented variation population gene but they did not have any effect to IC50 (Figure16) (Table18).

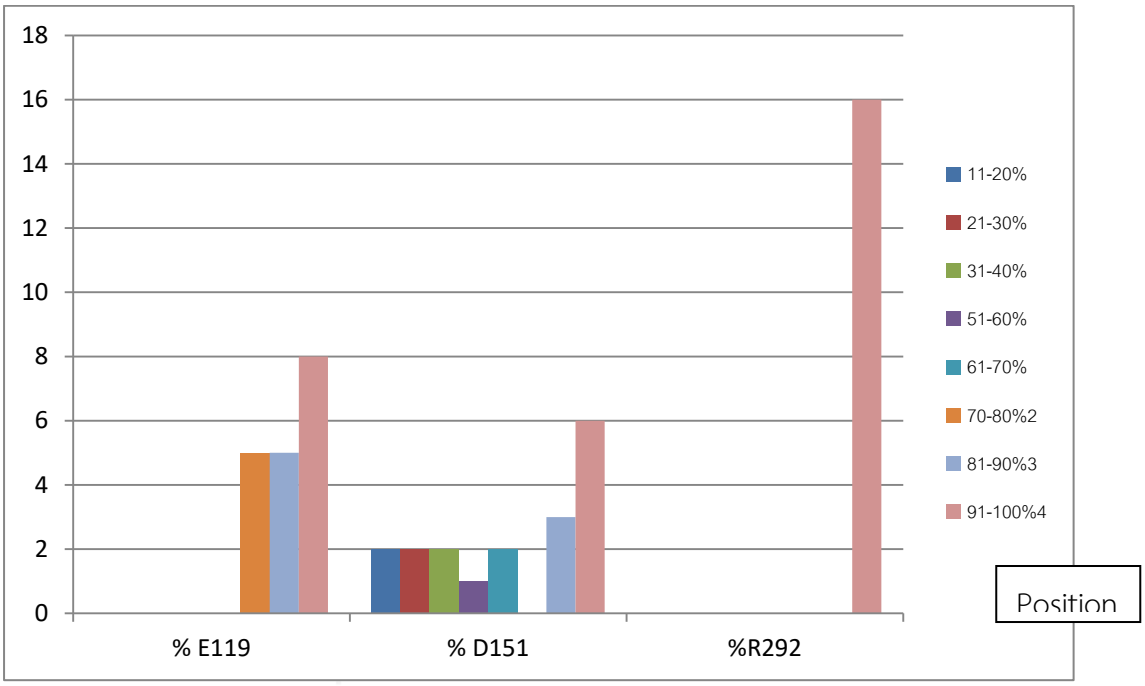


Figure 16 The number of samples which had low and high levels of percentage of wild type detected NA gene on each position.

Table 18 Result of pyrosequencing of M2 and NA gene detection of H3N2 influenza virus samples showed the result of resistance on M2 gene at position 31, Serine amino acid changed to Asparagine. For pyrosequencing detection on NA gene at position 119, 151 and 292, AQ program had analyzed sequences and reported to pyrogram with wildtype result.

No	**Specimen No**	Pyrosequencing				Result	
		M2	NA			M2	NA
		Position 31	E119	D151	R292		
1	FLU-PMKA2466	Mutation	Wild type	Wild type	Wild type	R	S
2	FLU-PMKA2485	Mutation	Wild type	Wild type	Wild type	R	S
3	FLU-PMKD0526	Mutation	Wild type	Wild type	Wild type	R	S
4	FLU-PMKA2593	Mutation	Wild type	Wild type	Wild type	R	S
5	FLU-PMKD0545	Mutation	Wild type	Wild type	Wild type	R	S
6	FLU-PMKA2731	Mutation	Wild type	Wild type	Wild type	R	S
7	FLU-PMKA2798	Mutation	Wild type	Wild type	Wild type	R	S
8	FLU-PMKA2810	Mutation	Wild type	Wild type	Wild type	R	S
9	FLU-PMKA2834	Mutation	Wild type	Wild type	Wild type	R	S
10	FLU-PMKA2840	Mutation	Wild type	Wild type	Wild type	R	S
11	FLU-PMKD0564	Mutation	Wild type	Wild type	Wild type	R	S
12	FLU-PMKA3039	Mutation	Wild type	Wild type	Wild type	R	S
13	FLU-PMKA3473	Mutation	Wild type	Wild type	undetermined	R	S
14	FLU-PMKA3707	Mutation	Wild type	Wild type	undetermined	R	S
15	FLU-PMKB0284	Mutation	Wild type	Wild type	Wild type	R	S
16	FLU-PMKD0693	Mutation	Wild type	Wild type	Wild type	R	S
17	FLU-PMKD0708	Mutation	Wild type	Wild type	Wild type	R	S
18	FLU-PMKD0722	Mutation	Wild type	Wild type	Wild type	R	S

Remark: R is Resistance, S is Susceptible

4.2.3 Influenza B

When products of RT PCR of influenza B virus were approved by gel electrophoresis, product bands of fragment 2 which were amplified by BNA-F408 and BNA-R848-biot primers to detect suspected mutation positions correlated with resistant or reduced susceptability to oseltamivir were found. There were 5 points of suspected positions on NA gene that were observed; R150, D197, I221, S249, and H273. The fragment 2 product appeared on the gel at 440 base pair size. Fragment 3 product amplified by BNA-F962 and BNA-R1274 biot to detect 2 suspected positions; R374 and G407 on fragment product size 312 base pairs. Both fragment products showed 33 bands of 33 influenza B samples. No bands were found on the The fragment 1 product, that was amplified by BNA-F301 and BNA-R848-biot to detect the E117 point (Figure17). From the alignment program, a 3'end mismatch between the BNA-F301 primer and the NA gene of influenza B was found. A new primer, BNA-F317, was designed and set up in the experiment. A new RT-PCR amplification result of fragment 1 had 33 samples appear on the gel after BNA-F317 primer was used with BNA-R848-biot primer (Figure18). Consequently, fragment 1 products were used to detect E117V. This position had 4 samples show 100% mutation with E117V and 29 samples susceptible to the NAI drug group. Fragment 2 gene detection resulted in 32 of 33 influenza B samples showing the wild type gene on the R150 position, 26 wild type on D197, 25 wild type on I221 and S249, and 21 wild type samples on position H273. The pyrosequencing result of fragment 3 had only G407 position because pyrosequencing specific primer for R374 had a mismatch at the 3'end. Then, the F1099 primer to detect R374 was replaced by anew primer and 22 samples of influenza B showed wild type genes. Some of samples could not be determined by the pyrosequencing program (Table19).

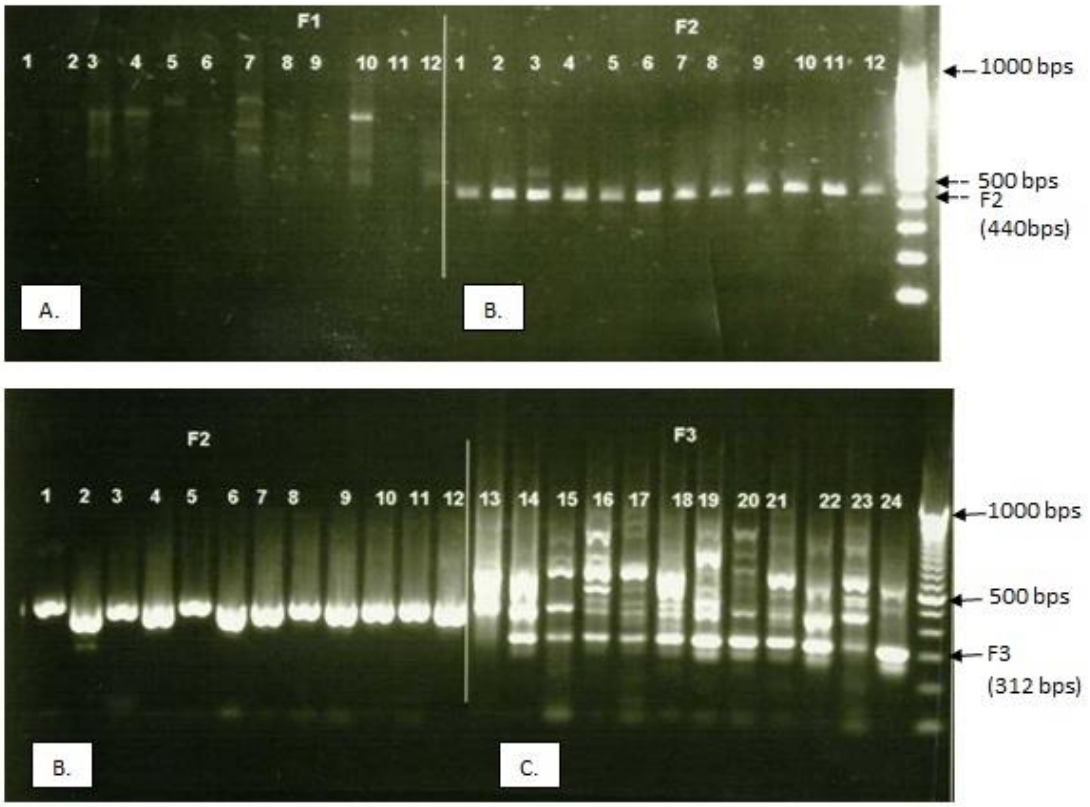


Figure 17 (A.) The picture showed that fragment 1 could not be amplified by BNA-F301, the old forward primer. (B.) On the example of fragment 2 product, there appeared a band on 440 base pair size after RT-PCR with BNA-F408 and BNA-R848-biot primer. (C.) Example product of fragment 3 that was amplified by BNA-F962 and BNA-R1274 biot primer.

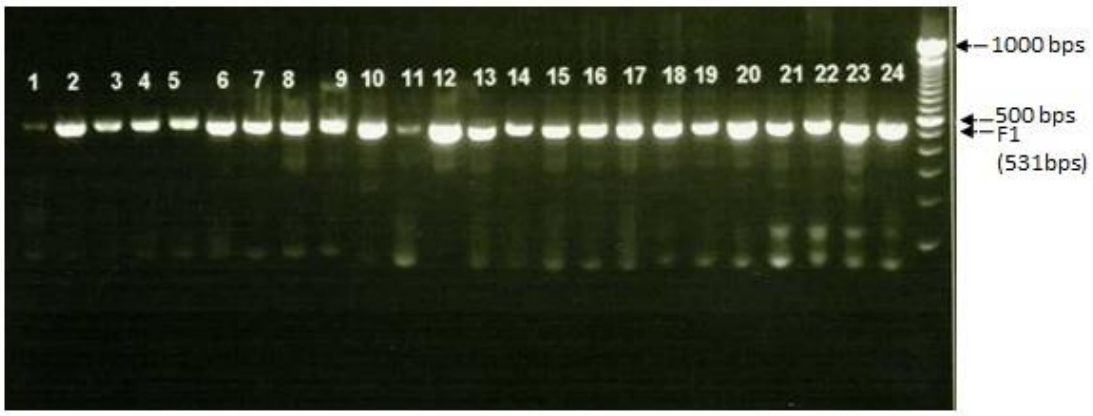


Figure 18 Picture of fragment 1 RT-PCR product after changing forward primer of fragment 1 amplification from BNA-F301 to BNA-F317, on 531 base pair size.

Table 19 Pyrosequencing result of influenza B on 8 suspected points that correlated with resistance or reduced susceptability to oseltamivir; E117, R150, D197, I221, S249, H273, R374 and G407. On the E117 position, 4 samples were found to have 100% of gene mutation.

No	Specimen No	Pyrosequencing of NA								Result
		E117	R150	D197	I221	S249	H273	R374	G407	
1	FLU-PMKA2994	WT	WT	WT	WT	WT	WT	U	WT	S
2	FLU-PMKA3335	WT	WT	U	WT	WT	U	U	WT	S
3	FLU-PMKA3209	WT	WT	WT	WT	WT	WT	WT	WT	S
4	FLU-PMKA2493	MT	WT	WT	WT	U	WT	U	WT	S
5	FLU-PMKA2928	WT	WT	WT	WT	WT	WT	WT	WT	S
6	FLU-PMKA3008	WT	WT	WT	WT	WT	U	WT	WT	S
7	FLU-PMKA3012	WT	WT	WT	WT	WT	U	U	WT	S
8	FLU-PMKA3014	WT	WT	WT	WT	WT	U	WT	WT	S
9	FLU-PMKA3044	WT	WT	WT	WT	WT	U	WT	WT	S
10	FLU-PMKA3062	WT	WT	WT	WT	WT	WT	WT	WT	S
11	FLU-PMKA3093	WT	WT	WT	WT	WT	WT	WT	WT	S
12	FLU-PMKA3103	WT	WT	WT	WT	WT	WT	WT	WT	S
13	FLU-PMKD0600	WT	WT	WT	WT	WT	WT	WT	WT	S
14	FLU-PMKA3125	MT	WT	WT	WT	WT	WT	U	WT	S
15	FLU-PMKA3131	WT	WT	WT	WT	WT	U	WT	WT	S
16	FLU-PMKD0603	WT	WT	WT	WT	WT	WT	WT	WT	S
17	FLU-PMKD0604	WT	WT	WT	WT	WT	WT	WT	WT	S
18	FLU-PMKA3143	WT	WT	WT	WT	WT	WT	WT	WT	S
19	FLU-PMKA3189	WT	WT	WT	WT	WT	WT	U	U	S
20	FLU-PMKA3219	WT	WT	WT	U	WT	WT	WT	WT	S

Remark: WT is wild type. MT is mutation. U is undetermined result. S is susceptible.

No	Specimen No	Pyrosequencing of NA								Result
		E117	R150	D197	I221	S249	H273	R374	G407	
21	FLU-PMKA3230	WT	WT	WT	WT	WT	WT	WT	WT	S
22	FLU-PMKD0617	WT	WT	WT	WT	WT	WT	U	U	S
23	FLU-PMKD0618	WT	WT	WT	WT	WT	WT	WT	WT	S
24	FLU-PMKA3236	WT	WT	WT	WT	WT	WT	WT	WT	S
25	FLU-PMKA3312	WT	WT	WT	WT	WT	WT	WT	WT	S
26	FLU-PMKA3313	WT	WT	WT	WT	WT	WT	WT	WT	S
27	FLU-PMKA3317	WT	WT	WT	WT	WT	WT	WT	WT	S
28	FLU-PMKA3327	WT	WT	U	U	U	U	U	WT	S
29	FLU-PMKD0648	WT	WT	U	U	U	U	WT	WT	S
30	FLU-PMKA3400	WT	U	U	U	U	U	U	U	S
31	FLU-PMKA3705	MT	WT	U	U	U	U	WT	WT	S
32	FLU-PMKD0702	WT	WT	U	U	U	U	WT	WT	S
33	FLU-PMKD0726	WT	WT	U	U	U	U	WT	WT	S

Remark: WT is wild type. MT is mutation. U is undetermined result. S is susceptible.

4.3 Sequencing

After 4 samples of influenza B were found to have 100% mutation on E117 position, an experiment was designed to confirm pyrosequencing result by selecting 1 sample (FLU-PMKA3125) from 2 experiments (pyrosequencing and NAI assay) that were clinical samples and isolated samples from T25 collection tube for sequencing. Sequencing results of both clinical FLU-PMKA3125 and isolated FLU-PMKA3125 showed the wild type on all NA resistant correlation positions (Figure19-21).

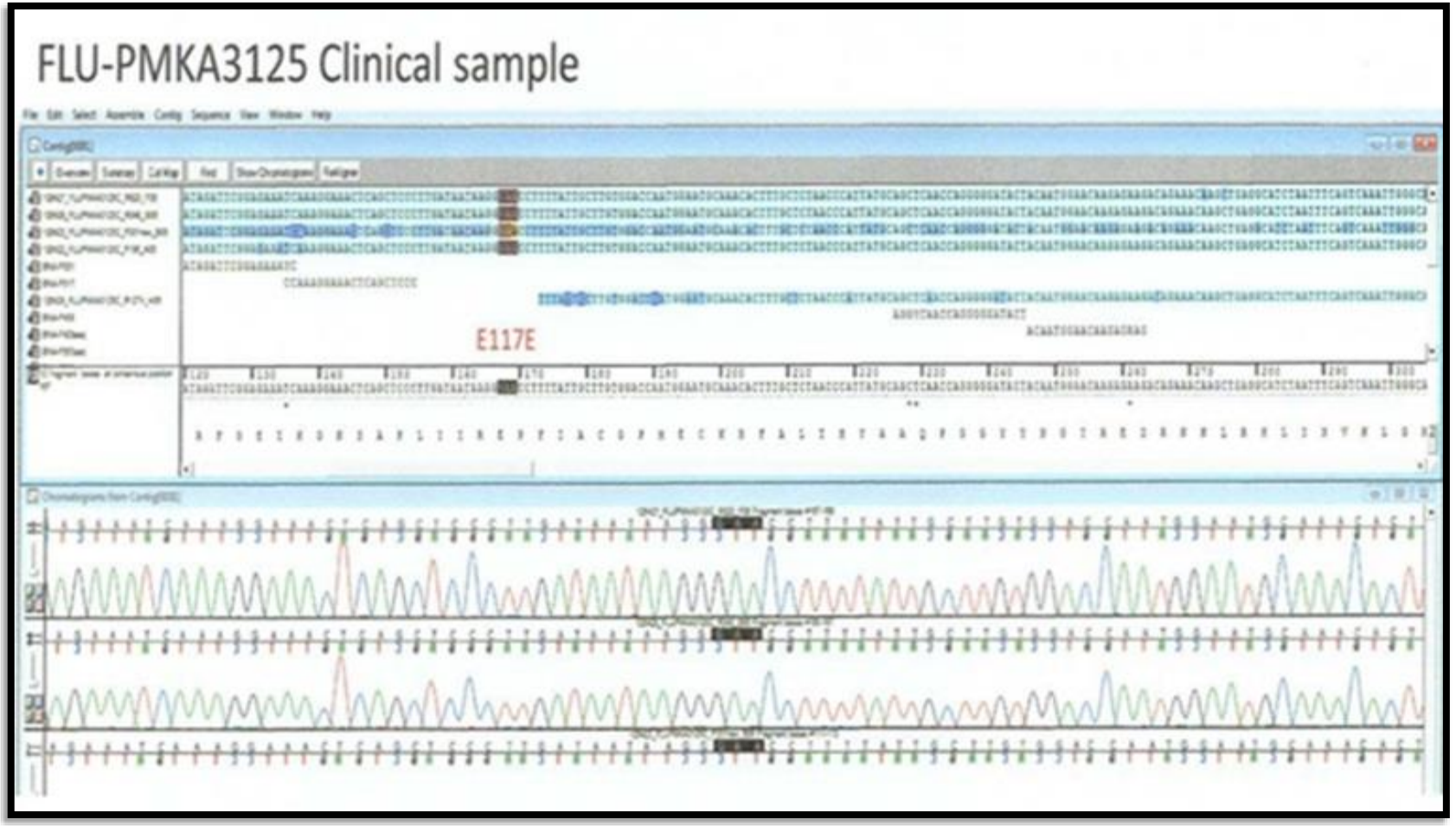


Figure 19 Sequencing alignment between Flu-PMKA3125 Clinical sample by Sequencher program show that there is GAA sequence on 117 position which Glutamic acid (E) had not changed.

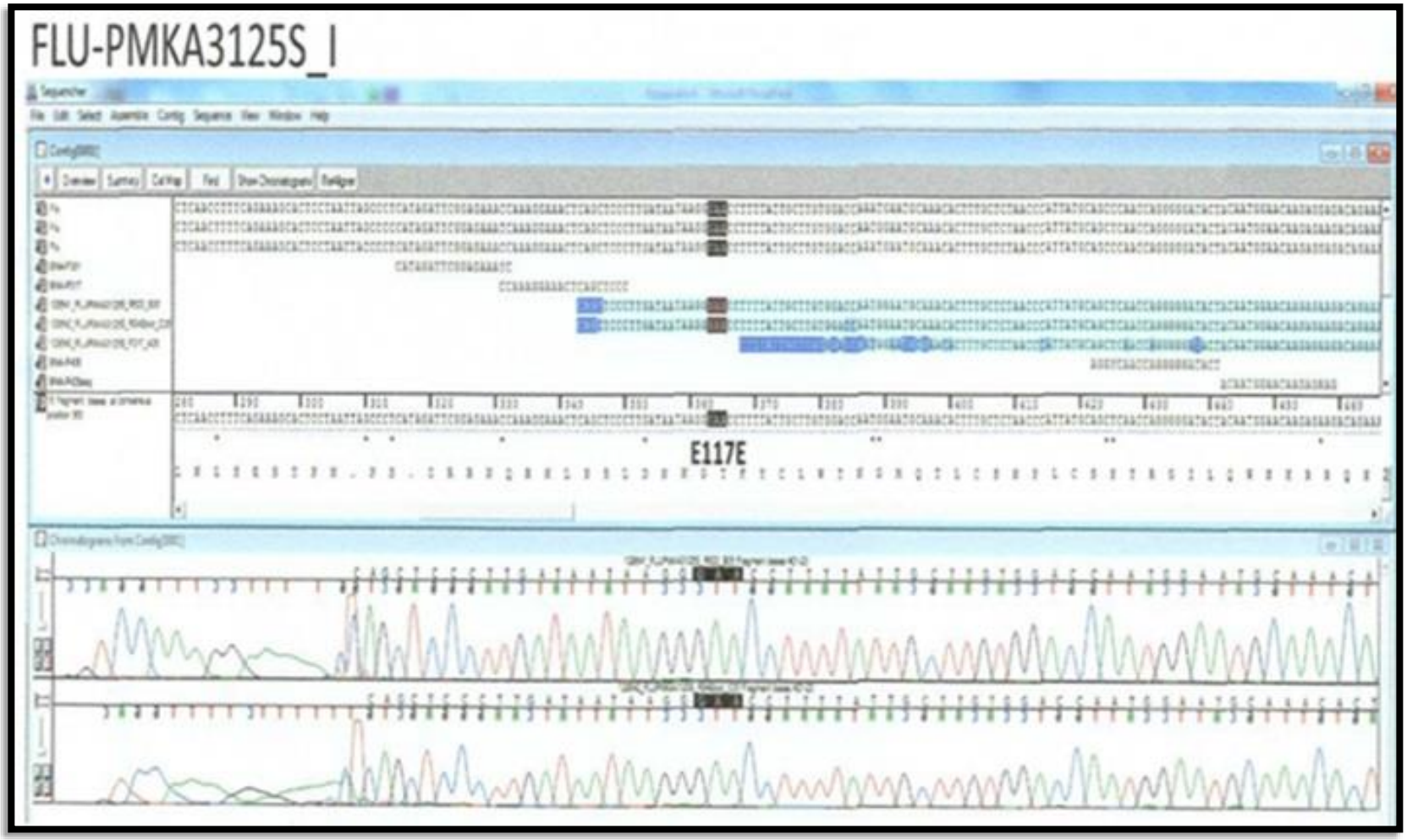


Figure 20 Short fragment of Flu-PMKA3125 was amplificated by BNA-F317 and BNA-R848 biot and then the product was taken for sequencing. The result of sequencing showed that there was not amino acid changing on E117 position

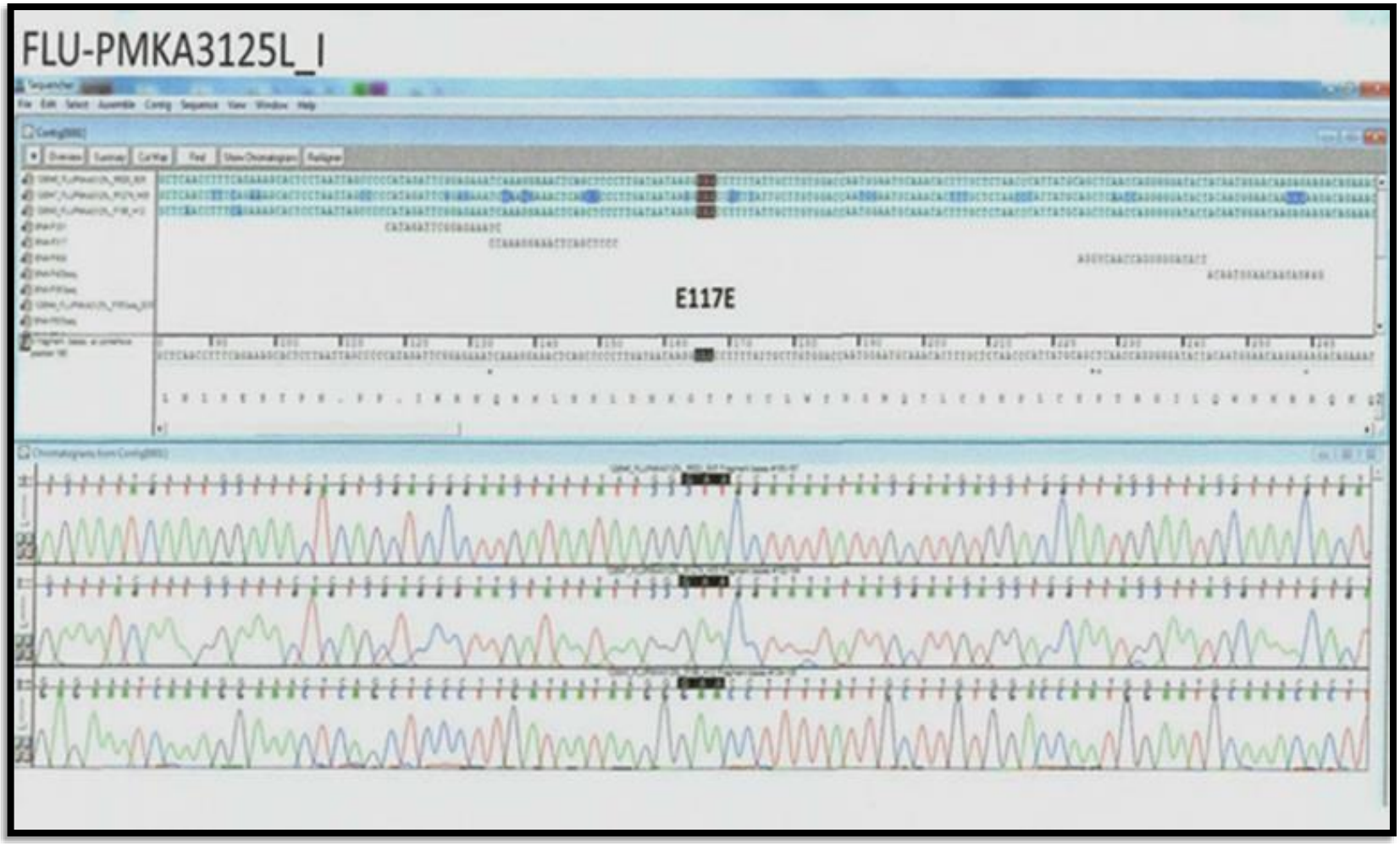


Figure 21 To confirm sequencing result, the large fragment was amplificated by F196 and R1274biot. The sequence showed wild type bases on E117 position

4.4 Neuraminidase inhibitor assay

From 79 clinical samples, 67 isolated samples remained from 3 passages of cell culture process. 16 samples of influenza A pdm H1N1, 18 samples of influenza A (H3N2) and 33 samples of influenza B were isolated and taken in phenotyping neuraminidase inhibitor assay (Table20).

Table 20 The number of positive influenza samples isolation in NAI assay.

Type	Neuraminidase inhibitor assay					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Influenza A (H1N1)	16	72.7%	6	27.3	22	100%
Influenza A (H3N2)	18	75.0%	6	25.0%	24	100%
Influenza B	33	100%	0	0	33	100%
Total	67	84.8%	12	15.2%	79	100%

Chemilumination neuraminidase inhibitor assay was the tool for analyzed Oseltamivir susceptibility of seasonal influenza virus and presented IC50 (the concentration of NAI agent that inhibited 50% of NA activity of virus) result by Graph Pad Prism Software. Influenza A pandemic H1N1 had mean of susceptibility to Oseltamivir drug at 0.44 ± 0.37 and had 95% confident interval of IC50 in the period 0.24 – 0.64. Mean value of IC50 of Influenza A (H3N2) and influenza B were 0.27 ± 0.20 and 2.61 ± 1.37 in the range of 95 % confident interval 0.17 – 0.37 (H3N2) and 2.12 – 3.09 (Flu B). The highest of IC50 was found in influenza B. (Table21).

Table 21 The number of isolated seasonal influenza virus in NAI assay experiment with mean of IC50 results. Data analyzed by one way ANOVA showed 95% confident interval of IC50 range of each influenza type. P- value showed < 0.001 means that at least two means are significantly different from one another.

Type	n	Mean of IC50	95% CI	P-value
Influenza A (H1N1)	16	0.44 ± 0.37	0.24 – 0.64	< 0.001*
Influenza A (H3N2)	18	0.27 ± 0.20	0.17 – 0.37	
Influenza B	33	2.61 ± 1.37	2.12 – 3.09	

By Bonferroni multiple comparisons method analysis, influenza B had an IC50 value significantly different from influenza A pandemic H1N1 and influenza A (H3N2). In Influenza type A, both subtype H1N1 and H3N2 are not significantly different in IC50 of oseltamivir value (Table27).

Table 22 Multiple Comparisons by Bonferroni's method showed that influenza B had the highest of oseltamivir concentration to inhibit 50% of NA activity of influenza virus significantly.

	Group I	Group II	Mean Different	Sig.
IC50	Pdm H1N1	H3N2	0.170	1.000
	Pdm H1N1	Flu B	-2.165	<0.001*
	H3N2	Flu B	-2.334	<0.001*

Both genotyping assay by pyrosequencing and phenotyping assay by neuraminidase inhibitor assay for monitoring oseltamivir resistant in seasonal influenza virus found sussceptability to oseltamivir completely in this experiment.

Correlation between percentage of E117 on fragment 1 of influenza B and EC50 value from influenza B NAI assay was analyzed by Chi-square Test for Independence experiment (Table23).

Table 23 Correlation coefficient of Chi-square test showed Asymp. Sig value is >0.05 that mean to between percentage of E117 has different from EC50 value of influenza B significantly.

	%WT	EC50
Chi-Square	8.758[a]	.000[b]
df	25	32
Asymp. Sig.	.999	1.000

CHAPTER V

DISCUSSION

There are three subtypes of influenza virus that occurred in the Thailand seasonal influenza epidemic. The epidemiological surveillance and investigation of influenza infection in Thailand were reported to have an increased number of influenza infectious patients in each year from data provided by the Department of Disease Control of Bureau of Epidemiology. The Influenza-like illness (ILI) patients, who were admitted to Phramonkutkloa hospital between January to September 2014, had identified to seasonal influenza as influenza A pandemic H1N1 (27.8%), influenza A H3N2 (30.4%) and influenza B (41.8%). The prevalence of influenza in Phramonkutkloa hospital was similar to the report of the Thai national influenza center(4). Almost all of the positive influenza virus in the outpatient department in 2014 were influenza B (147). The ratio of influenza A pandemic H1N1 and H3N2 were the same in 2014. The antigenic changes of the hemagglutinin (HA) in influenza A had frequently happened more than influenza B. The reassortment mechanism of influenza A virus had shown in 1957 and 1968 by pig pandemic influenza and then reemerged in 2009 as influenza virus pandemic 09 H1N1. There was another important influenza viral subtype that circulated in pigs, H3N2. Both swine influenza viruses H1N1 and H3N2 were easy to transmit to various hosts such as avian and human. The many subtypes of influenza A which occurred by point mutation on glycoprotein surface (hemagglutinin) were found around the world in every year. Therefore, it was important to follow the variation of influenza antigenic changes for prevention and treatment regularly. Although the basic structure of the hemagglutinin of influenza A virus resembles influenza B virus, the information of influenza B's hemagglutinin is not elucidate. The influenza B virus was classified in one group until 1970. Since 1980, there were categorized two lineages as Victoria lineage (B/Victoria/2/87) and Yamagata lineage (B/Yamagata/16/88) in circulation (129). During 1991 to 2000, Victoria lineage influenza virus was strictly found in only

East Asia (148). After 2001 the influenza B Victoria lineage virus was reported in cases outside East Asia and then continually spread to South and East Asia (149). Yamagata lineage virus had found a minority group in 1997 (148) . There was evidence that the mixed infection of two lineages of influenza B virus and the reassortment process were in co-circulation in the middle of 1990 (129, 149). The Victoria-like HA and Yamagata-like NA (VicHA-YamNA) were found in USA and Hong Kong, SAR, China in 1990s (148). Recently, there were two dominants in co-circulation, VicHA-YamNA and YamHA-YamNA, whereas VicHA-VicNA has no report (129). The department of medical science of Thailand had reported that almost of influenza B lineage was Victoria lineage influenza B virus in 2012. There were equal changes to both lineage of influenza B proportion in August to September, 2012. After November 2012, the Yamagata lineage had continually increased to be the most common lineage in epidemic cases of epidemic until 2015. In this study, a mix of lineages in the influenza B sample were found. The association of lineage gene had focused on NA gene which was incompletely present for dividing the lineage. There was evidence of the variable protein markers of multiple lineages with frequent reassortment in circulation (150). During the mixed infection, two lineages of influenza B could show the genetic reassortment that led to the situation of co-circulation by multiple lineages of influenza virus. This mechanism had impacted the human influenza evolution (149). After reasssortment in 2014, Victoria lineage had recurrent to be the main prevalent lineage of influenza B in 2016 (151). One of two lineages of influenza B presents dominant on the season epidemic, but it does not mean the other has disappeared from circulation. There is a hidden reassortmennt into virus genetics which may affect the fluctuation of lineage infection of influenza B. This situation of antiviral resistance in influenza virus is more concerning.

This research studied the association of the detection method between genotypic and phenotypic in the resistant influenza virus. Pyrosequencing is a developed tool for detecting the known and the suspect gene markers of NAI resistant influenza virus in seasonal influenza A pandemic 2009 H1N1, influenza A H3N2 and influenza B. The detection of Adamantane resistant in influenza A by using pyrosequencing was successfully applied to early detecting surveillance for wide

spreading resistance strains in 2005 until now (152). The results in this study showed that the Adamantane resistant via M2 blocker was widespread around the world including Thailand. All of influenza A pandemic H1N1 and influenza A H3N2 were composed of S31N gene (100%) which meant complete resistance to Adamantane. Moreover, pyrosequencing is the current method that used to prove and promptly to allow for surveillance NAI resistant association gene. The WHO had reported that H274Y mutation gene in pandemic 2009 H1N1 related to Oseltamivir-resistance with pyrosequencing protocol for global surveillance (153).

The influenza A pandemic H1N1 and influenza A H3N2 were not found with an Oseltamivir resistant gene in this study. There were many previous studies that used the pyrosequencing method for studing molecular marker gene association with NAI resistance. All of E119, D151, and R292 were the suspected positions which had reported the association with reduced susceptibility of Oseltamivir in influenza A H3N2 (152). In this study, we found the wild type on the specific NA region correlated with NAI susceptible. All of influenza A H3N2 samples (18) were found on the E119E and D151D positions. On the R292R position, there were detected at 16 of 18 samples and 2 of undetermined samples. Interestingly, at 151, residues were present in gene variation in population but they did not have an affect on EC50. The influenza B virus was concerned about developing a Oseltamivir resistance strain. The pyrosequencing markers for detected Oseltamivir resistance strains on influenza B virus were related with reducing susceptibility to NAIs by NA enzyme inhibition assay (129). The H275Y mutation of influenza A pandemic 2009 H1N1 was unofficially reported to be the suspected marker which referred to NAIs resistance but it had evidences of NA inhibition assay reducing the susceptible for NAIs (129).

The fragment 1 of influenza B virus, E117V, was found in 4 of 33 samples. By the way, all mutants on fragment 1 by pyrosequencing were susceptible to NAI antiviral drugs. The fragment 2 of influenza B virus on R150, D197, I221, S249 and H273 showed the wild type samples as following 32, 26, 25, 25 and 21 samples respectively. The fragment 3 showed only the G407 position for wild type. Another position, R374, was found mismatched at the 3'end. Then, the F1099 primer for detect R374 was redesigned to a F1099 new primer. Only 22 of 33 influenza B

samples were found as the wild type gene and the others were undetermined. Neuraminidase inhibition assay was the ordinary method for drug resistance observation with a complicated procedure of virus propagation in cell culture for preparing to the drug susceptibility testing (152).

The growing of the virus in cell culture by carefully processing at least 2 sub-passages was easy to contaminate and lose the EC50 value since there is insignificance in the clinical definition of cutoff EC50 value criteria to determine resistance virus. The NAI assay for susceptibility of drug detection must be combined with sequence analysis such as sequencing or pyrosequencing with specific known molecular markers to confirm the incident of elevated EC50 values (135).

There were correlations with EC50 value on NAI assay and NA gene marker on pyrosequencing of influenza A pandemic 2009 H1N1. The amino acid change on the 151 position of influenza A H3N2 had frequent phenotypic expression by reduced susceptibility of NAIs by chemiluminescent NAI assay(152). At the D151V position of influenza A H3N2, an increasing of 150-fold EC50 values in the more recent years was found (113). The NA gene of influenza A H3N2 in this study was detected to be a wildtype strain which showed the normal range of EC50 value. Some samples showed variation of population on 151 residues. In this study, the changing of D151 amino acid residue which did not correlate with NAI resistance was confirmed (152).

There are many NAI resistant associated mutation points that were marked to detect by pyrosequencing. E117 of influenza B virus was the position that had demonstrated Oseltamivir susceptibility reduction. In this study a mismatch RT-PCR and sequencing primer that had never been used to detect at 117 position by pyrosequencing in previous study was found. There was the variable at the sequence of targeted region on NA gene so, the primer should be revised to optimize the detection process for all influenza B (152). For the amplification and sequencing analysis of influenza B, the primer design must be updated regularly to be appropriate for the evolution of point mutation and reassortment in co-circulation of influenza B. The B/Illinois/03/2008 was detected at the E117 point mutation in unpublished data from the CDC showed Oseltamivir and Zanamivir susceptibility reduction. 100% gene mutation on position E117 in four influenza B samples was

found in this study after confirming one of the mutation samples by using Sanger sequencing. The result showed a wildtype gene at the suspected position with normal EC50 value.

Pyrosequencing is a suitable tool for monitoring viral variation in pandemic and epidemic outbreak with high performance that could detect minor populations in viral gene. This method could show the base variation which might be connected to mutation genes whereas, Sanger sequencing was limited by showing the major population on a gene fragment. However, there are some undetermined pyrosequencing results because of poor sample preparation for analysis resulting in the gap of base variation of influenza viral gene. Because of this, confirmation with Sanger's method and error analyzing program should closely follow. The undetermined results of influenza B were taken to Sanger sequencing method. All suspected positions consisted of wild type gene. The evaluation of influenza virus had changed every season in each year by following Centers for Disease Control and Prevention. Pyrosequencing is a great tool that provides high throughput, is rapid and flexible to design the assay for direct detection of the point interesting gene which relates to reassortment of co-circulation type/subtype or lineages or drug resistant associated marker. The training of professional and developing assay were needed for complex NAI resistant analysis. Even though variation of mutant population on suspected target sites of seasonal influenza virus in this sample group as found, Oseltamivir clearly remained to be efficient for treatment and prevention. Finally, the influenza annual report by Thailand national influenza center had reviewed increased morbidity and mortality of seasonal influenza every year without antiviral resistance of influenza incident. The incessant evolution change of influenza viral in circulation had express in prolong. Furthermore, the continual study of the gene development including antiviral resistance in influenza virus and follow up are necessary for type/suptypes selection of prevention vaccine and treatment recommendation in public health.

CHAPTER VI

CONCLUSION

There are 3 types of influenza virus in Thailand's circulation. Influenza All seasonal influenza A samples tested had genetic markers consistent with known resistance markers to Adamantane but were still susceptible to Oseltamivir. An Oseltamivir susceptible gene was found in every suspected position with some undetermined results in almost alll of Influenza B samples. At Position 117, mutation base in 4 samples were found. RT PCR primer for influenza B cDNA amplification from reference study, BNA-F301 was found mismatch at 3' end then was changed to BNA-317 for amplification work. F1099 pyrosequencing primer for R374 detection in influenza B had mismatch at 3' end. After primer replacement, 22 of 33 samples of influenza B could be detected well. Although some pyrosequencing results showed undetermined and one mutation point, Sanger sequencing showed wild type genes in all of them.

IC50 results from chemilumination NAI assay presented mean of susceptibility to Oseltamivir drug at 0.44 ± 0.37 and had 95% confident interval of IC50 in the period 0.24 – 0.64. Mean value of IC50 of Influenza A (H3N2) and influenza B were 0.27 ± 0.20 and 2.61 ± 1.37 in the range of 95 % confidence interval 0.17 – 0.37 (H3N2) and 2.12 – 3.09 (Flu B). IC50 of each types were in normal range of Oseltamivir susceptibility.

By Bonferroni multiple comparisons method analysis, influenza B had an IC50 value significantly different from influenza A pandemic H1N1 and influenza A (H3N2).

Both genotyping assay by pyrosequencing and phenotyping assay by neuraminidase inhibitor assay for monitoring oseltamivir resistant in seasonal influenza virus found complete susceptibility to oseltamivir in this experiment.

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APPENDIX

APPENDIX A

Appendix A-1: Composition and preparation for RNA extraction

Carrier RNA

Buffer AVE	310	µl
Lyophilized carrier RNA	310	µg

Dissolve the carrier RNA thoroughly, divide it into conveniently sized aliquots, and store it at –30 to –15°C. Do not freeze–thaw the aliquots of carrierRNA more than 3 times.

Addition of carrier RNA to Buffer AVL

A-Table 1. Volumes of Buffer AVL and carrier RNA–Buffer AVE mix required for the QIAamp Viral RNA Mini procedure.

No. samples	Vol. Buffer AVL (ml)	Vol. carrier RNA–AVE (µl)	No. samples	Vol. Buffer AVL (ml)	Vol. carrier RNA–AVE (µl)
1	0.56	5.6	13	7.28	72.8
2	1.12	11.2	14	7.84	78.4
3	1.68	16.8	15	8.40	84.0
4	2.24	22.4	16	8.96	89.6
5	2.80	28.0	17	9.52	95.2
6	3.36	33.6	18	10.08	100.8
7	3.92	39.2	19	10.64	106.4
8	4.48	44.8	20	11.20	112.0
9	5.04	50.4	21	11.76	117.6
10	5.60	56.0	22	12.32	123.2
11	6.16	61.6	23	12.88	128.8
12	6.72	67.2	24	13.44	134.4

Buffer AW1

Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle and in Table 2. Buffer AW1 is stable for 1 year when stored closed at room temperature (15–25°C), but only until the kit expiration date.

A-Table 2. Preparation of Buffer AW1

Kit cat. no.	No. of preps	AW1 concentrate	Ethanol	Final volume
52904	50	19 ml	25 ml	44 ml
52906	250	98 ml	130 ml	228 ml

Buffer AW2

Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle and in Table 3. Buffer AW2 is stable for 1 year when stored closed at room temperature (15–25°C), but only until the kit expiration date

Table 3. Preparation of Buffer AW2

Kit cat. no.	No. of preps	AW2 concentrate	Ethanol	Final volume
52904	50	13 ml	30 ml	43 ml
52906	250	66 ml	160 ml	226 ml

Appendix A-2: Kit component of SuperScript® III One – Step RT-PCR system with platinum® Taq High Fidelity (Table 3)

A-Table 3 Component of SuperScript® III One – Step RT-PCR system with platinum® Taq High Fidelity

Component	25 reaction kit
SuperScript®III RT/Platinum®Taq High Fidelity Enzyme Mix	25 ml
2x Reaction Mix (a buffer containing 0.4 mM of each dNTP, 2.4 mM $MgSO_4$	1 ml
5-mM Magnesium Sulfate	500 µl

Appendix A-3: Preparing gel for Gel electrophoresis

TBE electrophoresis buffer (10X)

Table4 TBE electrophoresis buffer (10X) preparation

Reagent	Quantity (for 1 L)	Final concentration
Tris base	121.1 g	1 M
Boric acid	61.8 g	1 M
EDTA (disodium salt)	7.4 g	0.02 M

Prepare with RNase-free H2O. Dilute 100 mL to 1 L to make gel running buffer. Store for up to 6 mo at room temperature.

Appendix A-4: Prepare solution for pyrosequencing method

Binding buffer

Binding buffer	40	µl
MiliQ water	17-27	µl
Streptavidin Sepharose™ beads	3	µl
(Final volume	60-70	µl)

Adding the following solution in conical tube and mix by vortex.

Annealing buffer

Annealing buffer	40	µl
Sequencing primer (100 µM)	0.18	µl
(Final concentration 0.45 µM)		

Appendix A-5: Prepare reagents for NAI assay

25 mM master stock of Oseltamivir

Oseltamivir	19.3	mg
Distilled water	2	ml

Working stock

Dilute the 25 mM master stock 1:50

25 mM master stock	50	µl
H_2O	2,450	µl

Serial dilutions of Neuraminidase inhibitors in NA-XTD™ Assay Buffer

Table5 Serial dilutions of Neuraminidase inhibitors in NA-XTDTM Assay Buffer for NAI assay. Total volume of each NI dilution required is 25 µl/well

Dilution	Combine	Final volume(µl)	NI conc before assay (3x)	NI conc in final assay
1(1:25)	30 µl WS NI + 720 µl AB	750	20,000 nM	6,600 nM
2(1:5)	100 µl Dil 1 + 400 µl AB	400	4,000 nM	1,320 nM
3(1:5)	100 µl Dil 2 + 400 µl AB	400	800 nM	264 nM
4(1:5)	100 µl Dil 3 + 400 µl AB	400	160 nM	52.8 nM
5(1:5)	100 µl Dil 4 + 400 µl AB	400	32 nM	10.56 nM
6(1:5)	100 µl Dil 5 + 400 µl AB	400	6.4 nM	2.11 nM
7(1:5)	100 µl Dil 6 + 400 µl AB	400	1.28 nM	0.422 nM
8(1:5)	100 µl Dil 7 + 400 µl AB	400	0.256 nM	0.084 nM

NA-XTD™ Substrate

For one 96-well plate, NA-XTD™ Substrate is diluted 1:1000 in NA-XTD™ Assay Buffer at room temperature.

NA-XTD™ Substrate	4	µl
NA-XTD™ Assay Buffer	4	ml

APPENDIX B

Appendix B-1: Database of samples in this study

No	Specimen No	Strain	Threshold cycle value (Ct)
1	FLU-PMKA3098	A pdm H1N1	17
2	FLU-PMKD059	A pdm H1N1	21
3	FLU-PMKB0237	A pdm H1N1	16
4	FLU-PMKD0633	A pdm H1N1	21
5	FLU-PMKD0531	A pdm H1N1	29
6	FLU-PMKD0568	A pdm H1N1	30
7	FLU-PMKD0574	A pdm H1N1	28
8	FLU-PMKA3020	A pdm H1N1	24
9	FLU-PMKD0588	A pdm H1N1	25
10	FLU-PMKA3058	A pdm H1N1	19
11	FLU-PMKD0590	A pdm H1N1	22
12	FLU-PMKD0596	A pdm H1N1	23
13	FLU-PMKA3117	A pdm H1N1	23
14	FLU-PMKA3153	A pdm H1N1	21
15	FLU-PMKA3177	A pdm H1N1	23
16	FLU-PMKA3200	A pdm H1N1	24
17	FLU-PMKA3201	A pdm H1N1	25
18	FLU-PMKD0612	A pdm H1N1	25
19	FLU-PMKD0615	A pdm H1N1	24
20	FLU-PMKA3244	A pdm H1N1	25
21	FLU-PMKD0670	A pdm H1N1	25
22	FLU-PMKD0717	A pdm H1N1	27
23	FLU-PMKA2466	A H3N2	24
24	FLU-PMKA2485	A H3N2	22

No	Specimen No	Strain	Threshold cycle value (Ct)
25	FLU-PMKD0526	A H3N2	23
26	FLU-PMKA2593	A H3N2	20
27	FLU-PMKD0545	A H3N2	22
28	FLU-PMKA2731	A H3N2	23
29	FLU-PMKA2798	A H3N2	14
30	FLU-PMKA2810	A H3N2	24
31	FLU-PMKA2834	A H3N2	25
32	FLU-PMKA2840	A H3N2	23
33	FLU-PMKD0564	A H3N2	21
34	FLU-PMKA3039	A H3N2	27
35	FLU-PMKA3473	A H3N2	24
36	FLU-PMKA3707	A H3N2	27
37	FLU-PMKB0284	A H3N2	23
38	FLU-PMKD0693	A H3N2	23
39	FLU-PMKD0708	A H3N2	22
40	FLU-PMKD0722	A H3N2	23
41	FLU-PMKA2994	B	19
42	FLU-PMKA3335	B	19
43	FLU-PMKA3209	B	16
44	FLU-PMKA2493	B	27
45	FLU-PMKA2928	B	22
46	FLU-PMKA3008	B	24
47	FLU-PMKA3012	B	23
48	FLU-PMKA3014	B	25
49	FLU-PMKA3044	B	22
50	FLU-PMKA3062	B	23

No	Specimen No	Strain	Threshold cycle value (Ct)
51	FLU-PMKA3093	B	25
52	FLU-PMKA3103	B	24
53	FLU-PMKD0600	B	24
54	FLU-PMKA3125	B	25
55	FLU-PMKA3131	B	17
56	FLU-PMKD0603	B	23
57	FLU-PMKD0604	B	23
58	FLU-PMKA3143	B	24
59	FLU-PMKA3189	B	22
60	FLU-PMKA3219	B	23
61	FLU-PMKA3230	B	19
62	FLU-PMKD0617	B	25
63	FLU-PMKD0618	B	23
64	FLU-PMKA3236	B	23
65	FLU-PMKA3312	B	23
66	FLU-PMKA3313	B	23
67	FLU-PMKA3317	B	23
68	FLU-PMKA3327	B	24
69	FLU-PMKD0648	B	23
70	FLU-PMKA3400	B	32
71	FLU-PMKA3705	B	25
72	FLU-PMKD0702	B	21
73	FLU-PMKD0726	B	19

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