CHAPTER VII

SUMMARIZING DISCUSSION

Human pandemic influenza virus (pH1N1) is the predominant virus attack upper respiratory tract since its first pandemic in March, 2009 [97]. The outbreak of pH1N1 brings about public health concerns and loss of economics productivity. From Mexico city, the outbreak has been spread globally. Although the World Health Organization (WHO) declared the transformation of pH1N1 in becoming the seasonal flu, the molecular evolution of this virus needed to be monitored continuously. The more comprehension of virus characteristics, molecular dynamics and serological surveillance will lead to the more effective policies to control the outbreak and further pandemic such as vaccine and antiviral drug use and stockpiles. Because this virus has gradually evolved, the accurate, sensitive and specific rapid diagnosis of influenza virus infection required to be evaluated. Likewise, the study of seroprevalence is critical to monitor the dynamic of the outbreak and potential of species transmission of virus among population, but techniques use needs to be validated, especially the HI test which can be affected by many factors, such as erythrocyte species, serum samples and incubation time. This thesis aimed to display the current comprehensive study of pH1N1 virus in terms of genetic diversity and molecular evolution, detection and validation of antibody response to influenza A virus detection and evaluation of rapid diagnosis.

The concatenated genome of coding region of pH1N1 virus in Thailand showed the pattern of chronological distribution, but did not show a pattern of geographical distribution. Circulating in clade 5, 6, and 7, pH1N1 isolates from the 1st wave shared the

common node while those in from 2nd wave were mostly classified into clade 8 with many substitution sites before the phylogenic tree showed that isolates from the 3rd wave were classified into clade 11.1 and 11.2, suggested that the founder effect might occur during the 2nd and 3rd outbreak (during summer and rainy season of 2010). Any policies, such as school break period, the vaccine and drug use can account for this phenomenon. Although all pH1N1 genes were under purifying selection in Thailand, the evolutionary rate was comparatively higher compared to those in seasonal H1N1 and H3N2 virus [108]. It might assume that the high selective pressure was due to the virus just emerged in human population and needed to acclimatize to host factors by the change of amino acid, especially in genes coding for virus surface proteins, HA and NA. The virus would adapt for a while before it become more static like prior seasonal influenza. However, the niche displacement theory suggested differently. Another outcome from the virus mutation is the character displacement, suggested that the accumulated mutation will change the cell tropism or host species to extend the viral niche. Now, the genomic signature analysis suggested that there still had typical major signatures [86] and the pathogenicity of this virus was quite low, compared to H5N1 avian influenza virus. The pH1N1 virus circulated in clade11.1 and 11.2, resulted from the founder effect, expressed many interesting mutations that should be considered and continuously monitored. Some previous studies suggested the specific mutations that might change the viral virulence [83], but this recent study did not find any significant difference that could turn the pH1N1 isolates form mild to severe cases. The results in recent study did not show the non-synonymous mutation in HA gene, especially in antigenic sites Ca and vaccine was still effective to control the outbreak in Thailand, but the mutation still needed to be monitored and computer modeling was required for analysis the effect from other non-significant mutations that might alter the conformation of Ca site (Figure16).

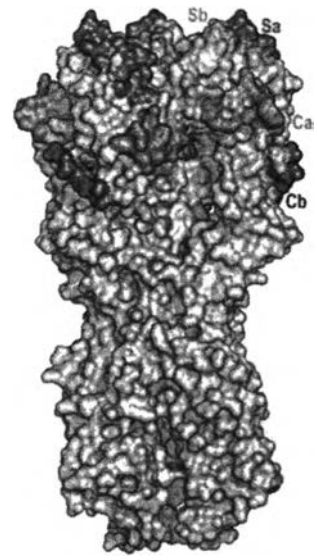

Figure 16. HA crystal structure showed antigenic sites of A/California/04/2009 (Xu, R, 2010)

Due to the gradual mutations of influenza virus, the molecular techniques used in diagnosis of influenza virus infection required to be regularly evaluated. The recent study analyzed 2 trademarks of RIDTs which were highly used in Thailand, SD and QuickVue which have different interpretation. Therefore, QuickVue test can interpret as negative, influenza A or B infection while SD can separate the pH1N1 infection from seasonal influenza virus. The results suggested that many factors influenced the accuracy, sensitivity and specificity of the test, therefore; age range of patients, period of influenza activity and influenza virus subtypes. Compared to other subtypes, H3N2 influenza virus seemed to be more false negatives. So, the results interpreted as H3N2 should be under careful consideration. The results obtained in adolescent and adult patients showed more false negative thanthose obtained in infants. This phenomenon might be resulted from the more viral shedding in children patients [122,123]. Although, the QuickVue test cannot specify pH1N1 infection from seasonal influenza A virus infection, the sensitivity and specificity of QuickVue test seemed to be higher than those obtained by SD test. Interestingly, the SD test yielded higher sensitivity than QuickVue during the high season of influenza activity. In this period, the clinicians should be more

considerable in interpretation due to the more false positive results and aware of the more false negative that might occur during low season. For any underlying complications, the gold standard method, RT-PCR should be performed along with the rapid test for more accuracy. Applying a sensitive, specific and accurate rapid diagnostic test would facilitate the more efficient treatment.

During the pandemic, many strategies were used to control, including the vaccine policy. Before the vaccine would be applied, seroprevalence among the population should be obtained by using molecular techniques, such as MN and HI. In addition, there was query that whether pH1N1 virus or virus which had the same epitope had ever circulated among human population before the pandemic. Theoretically, the seroconversion ($\geq$4 folds) of antibody titers against influenza virus was showed between acute and convalescent serum [160]. The recent study performed the HI test in paired serum samples, separated as pH1N1-positive and pH1N1-negative sera by real-time RT-PCR. The results showed that almost all paired serum samples positive for pH1N1 infection revealed the $\geq$ 4 folds rising antibody while almost all serum sample with negative PCR result had not showed the sero-conversion, allowing to conclude that HI test was supported to use as the standard method for investigation the seroprevalence against pH1N1 virus among human population. Moreover, the recent study also showed that there was no cross-reactivity of seasonal H1N1 virus to pH1N1, which meant that individuals who already have antibody response to seasonal H1N1 virus were still possible to be infected by pH1N1 and seasonal H1N1 vaccine cannot protect population from pH1N1 pandemic. The limitation of this study was dependent to sampling period, that is to say, the acute serum samples should obtained from acute phase of the infection (< 7 days after symptoms onset or within 5-6 days after taking antiviral drug) and convalescent serum samples should obtained from convalescent

phase of the infection ($\geq$ 14 days after symptoms onset). In addition, MN test, as the direct method for seroprevalence study, should be performed together in further investigation. In conclusion, the strategies applied in this study may be used to determine the antibody response to human pandemic influenza H1N1 prior to and post vaccination with new vaccine which will be necessary for vaccine management and evaluation of vaccine efficiency.

The microneutralization (MN) test is the most direct method to investigate the antibody response to influenza virus. However, this method is quite time and labor-consuming. The hemagglutination inhibition (HI) assay is supplementary method by the less time and labor use, but this assay requires validation before use. In recent study investigated the erythrocyte binding affinity of pH1N1 virus and its consequence in the antibody titration in HI test. World Health Organization recommended using turkey erythrocyte in their HI protocol, but turkey erythrocyte is quite rare in Thailand. This study tried to fix the problem by finding the optional and more practical erythrocyte to perform HI assay. Many previous studies suggested the differences in erythrocyte binding efficiency depend on sialic acid receptor specificity which distributes in various ratios among host species of origin, such as type of sialic acid, type of linkage and oligosaccharide, and spatial arrangements on the sialic acid residue [19]. Typically, there are 2 major types of sialic acid receptor in targeted cells, N-glycolylneuraminic acid and N-acetyl neuraminic acid (Figure17).Mostly, horse and pig erythrocytes contain N-glycolylneuraminic acid receptor in different ratios while receptors on human erythrocytes mainly consist of N-acetyl neuraminic acid. The distribution of receptor types is in concordance of host range restriction of influenza virus. This recent study suggested that pH1N1 virus preferentially bound to goose erythrocyte while the efficiency in binding to turkey and human erythrocyte were comparable. Obviously, the

capability of pH1N1 virus in interaction with chick erythrocyte was low. Although turkey, goose and chicken are avian, but there were some distinctive difference of receptor distribution among these avian species. Another factor influenced the receptor binding preference was the glycosidic linkage on receptor molecule, the pH1N1 preferentially agglutinated the SA α-2,6- Gal, which was mostly in turkey and goose erythrocyte while SA molecule in chicken erythrocyte were α-2,3-glycosidic type (Figure18). The binding affinity of virus also had an effect on HI assay therefore; the HI titer obtained by using goose erythrocyte were highest, those obtained by human and turkey ranked second and third in comparable value while HI titers obtained from chicken erythrocyte were low and unstable.

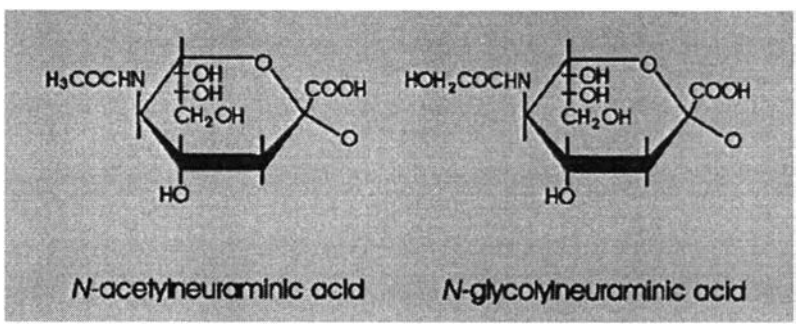

Figure 17. Structure of N-acetyl and N-glycolylneuraminic acids. These SA differ at position 5 of the pyranose ring. N-Acetylneuraminic acid is the precursor of N-glycolyneuraminic acid; enzymatic hydroxylation of the former results in the latter (Zusuki, Y, 2000).

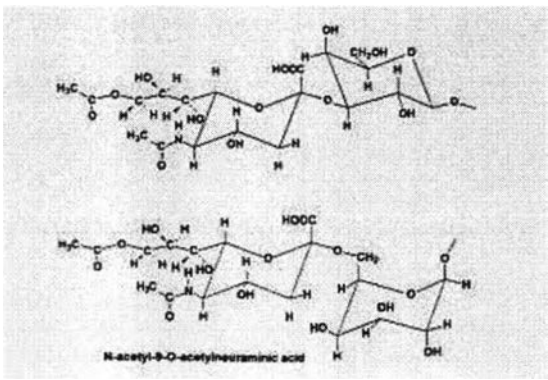

Figure 18. Structure of 2 types of glycosidic linkage of sialic receptor, α-2,3-glycosidic type (top) and sialic receptor, α-2,6-glycosidiclinkage (bottom)(Zimmer G, 1996)

Meanwhile, when the high HI titers by using goose erythrocyte were compared with MN titer, as the direct method to identify antibody response, many points had to be considered. The most sensitive HI titers in goose erythrocyte were not accurate as those in turkey when compared to MN titer. This suggested that the high HI titer obtained from goose erythrocyte might be an overestimation. Moreover, there was a contradiction in result of this study and previous study [154] which revealed that pH1N1 virus could not agglutinate goose erythrocyte at all. This might due to a species variation of geese which classified in 3 genera and their diversity might affect the distribution of surface molecule. This part needs further study by the sequencing and crystallization of sialic acid molecule in each genus. The study also reported the use of human erythrocyte to be comparable with turkey erythrocyte. However, using human red blood cells in HI assay required the preexistence of antibody test, especially when the outbreak ends and pH1N1 has become one of seasonal influenza virus, it would be difficult to find naïve human erythrocyte. This study provided the appropriate selection of erythrocyte species for HI assay that will allow construction of a more reliable database, which will be essential for further investigation and control of virus epidemics, such as herd immunity and vaccine policy.

From 1997-2004, many countries throughout Asia and Europe faced the outbreak of H5N1 influenza virus in poultry and avian-to-human transmission can be occurred. The avian influenza viruses had caused more than 500 laboratory-confirmed human cases and fatality rate over 60% [158], affecting the public health concerns and worldwide surveillance of influenza virus infection and antibody response is needed. This recent study identified the evidence of infection record of high risk people, which defined individuals who highly exposed to H5N1 avian influenza virus during its outbreak in poultry and felids. This study provided the information which would be useful

to assess the potential of H5N1 influenza virus in human-to-human or mammal-to-human transmission and determined the risk factor responsible for H5N1 transmission to human. The seroprevalence in high risk peoplewas obtained by using microneutralization assay and confirmed by hemagglutination inhibition assay. Approximately 4% of study population were sero-positive to H5N1 influenza virus. Most of infected people were chicken slaughters in outbreak area. Compared to previous studies, seroprevalence of H5N1 virus ranged from 0% to 12% [165], suggested many factors could be responsible for uncertain results in high risk population, such as the cut-off level used for sero-positive identification, sampling period, and virus isolates. Interestingly, the participants with sero-positive to H5N1 virus might be subclinical or asymptomatic infection. If so, the high fatality rate of H5N1 infection might be overestimated. However, the limitation of this study was the lack of acute serum samples which would provide the more accurate results.