APJAI
Asian Pacific Journal of
Allergy and Immunology

ORIGINAL ARTICLE

Cross-reactive antibodies against H7N9 and H5N1 avian influenza viruses in Thai population

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Abstract

Background: Avian influenza H5N1 and H7N9 viruses have jumped across species from avian to humans and become a threat to public health. Not much is known about pre-existing cross-reactive antibodies against these avian viruses in human population.

Objective: To determine the prevalence of cross-reactive anti-HA and anti-NA antibodies to avian influenza H5N1 and H7N9 viruses in Thai population.

Method: Archival serum samples from 100 blood donors and 21 patients infected with 2009 pandemic influenza A (H1N1) (pdmH1N1) virus were investigated by hemagglutination-inhibition (HAI) and neuraminidase-inhibition (NAI) assays for anti-HA and anti-NA antibodies, respectively. The test antigens comprised 2 human viruses (pdmH1N1 and H3N2 viruses), and 6 reassortant viruses carrying *HA* and *NA* genes of avian H5N1 or H7N9 virus generated by reverse genetics.

Results: HAI antibody titers ≥ 10 were found in 58, 89, 0 and 15% of blood donors as tested against pdmH1N1, H3N2, H5N1 and H7N9 viruses, respectively. On the other hand, NAI antibodies were detected in 98, 94, 73 and 53% of blood donors when reverse genetic-derived viruses harboring NA gene from pdmH1N1, H3N2, H5N1 or H7N9 virus were used as the test antigens. Moreover, 66.7% of pdmH1N1 patients who had > 4 fold increase in HAI antibody titers in paired sera developed > 4 fold increase in NAI antibody titers.

Conclusions: Anti-NA antibody has broader reactivity than anti-HA antibody, therefore, it can be a supplement to anti-HA antibody in the prevention against novel influenza viruses.

Key words: Avian influenza H5N1 virus, Avian influenza H7N9 virus, Hemagglutination-inhibition assay, Neuraminidase-inhibition assay, reverse genetics

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Introduction

Influenza A virus harbors 2 surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) which play different roles in the virus infection.[1] At present, 18 HA and 11 NA subtypes have been identified in influenza virus type A;[2,3] while the circulating human influenza subtypes include 2009 pandemic influenza A(H1N1) (pdmH1N1), A(H3N2) and influenza B viruses. Occasionally, avian influenza viruses jump across species barrier to infect humans. The two most important avian viruses causing human threats are the highly pathogenic avian influenza (HPAI) A(H5N1) virus and low pathogenic avian influenza (LPAI) A(H7N9) virus.[4,5]

Since its re-emergence in People's Republic of China in 2003, HPAI H5N1 virus underwent genetic changes into several clades and subclades and spread worldwide. As of 4 April 2016, there were 850 human cases with 449 deaths (fatality rate of 52.8%) in 16 countries.[4] Thailand reported

DOI 10.12932/AP0788

the emergence of H5N1 virus in January 2004. With several strategic prevention and control plans, the virus disappeared from the Thai population in July 2006. In total, there were 25 human cases with 17 deaths (fatality rate of 68%).[4] Nevertheless, the outbreaks occurred in few poultry farms and lasted until 2008.[6] The LPAI H7N9 virus emerged in China in 2013.[7] As of 15 March 2016, laboratory confirmed human H7N9 cases reached 751 with 294 fatal outcomes (fatality rate of 39.1%).[5] However, the H7N9 virus is still confined to China, with the exception of a few cases who had history of travelling to China.[5] Subsequent studies suggested that H7N9 virus can bind both avian and human type receptors.[8] The ongoing circulation of these viruses continues to pose a pandemic threat due to their rapid geographical expansion and genetic diversity, and may eventually the adaptation to humans which may result in human-to-human transmission.[9]

Low level of immunity is one of key elements to determine the in taking of the new influenza strain by human population.[10] Anti-HA antibody confers complete protection, while anti-NA antibody confers partial protection.[11] Hemagglutination-inhibition (HAI) and neutralization (NT) assays are employed to measure anti-HA antibodies in laboratories worldwide,[12] while the method to measure anti-NA antibody is not well standardized. Determination for anti-NA antibody in human sera using native virus as the test antigen normally result in the background interference from anti-HA antibody generated by natural influenza virus infection or vaccination. HA is immunodominant; and in a virion, it is present in higher amount than NA (HA:NA ratio of 4-5:1).[13] The binding between anti-HA antibodies and HA molecules will interfere with anti-NA antibodies to gain access to NA molecules or with the access of NA to substrate. Recently, the method for measuring anti-NA antibodies against pdmH1N1 and H3N2 viruses by neuraminidase-inhibition (NAI) assay has been established using pseudotyped viruses or reassortant viruses carrying HA subtype of non-human origin as the test viruses.[14,15] With this approach, interference from binding between anti-HA antibody and HA antigen of non-human virus origin can be avoided. This study aimed to determine the prevalence of anti-HA and anti-NA antibodies against pdmH1N1, H3N2, H5N1 and H7N9 viruses in Thai blood donors, and also the cross-reactive anti-HA and anti-NA antibodies against viruses of heterologous HA and NA subtypes in paired sera of pdmH1N1 patients. To accomplish these objectives, we generated 4 reassortant influenza viruses carrying H4 *HA* gene of an avian virus in combination with *NA* gene of distinct viruses of avian or human origin for determining anti-NA antibody by NAI assay. In parallel, we also determined anti-HA antibodies by HAI assay using both wild-type and reassortant viruses as the test antigens.

Methods

Ethical issues

An ethical approval for the study was granted by the Institutional Review Boards of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand

Sample size estimation

The equation $n = [P(1-P)(Z_{1-\alpha/2})^2]/e^2$ was used for sample size estimation. As the prevalence of anti-N9 antibody has never been reported in human population, a 50% positive proportion [P] was set. This setting produces a conservative estimation of variance. "e" was set as 0.1 to represent the difference between the estimated prevalence and one side of the confidence limit. The "α" value of 0.05 is considered as the significance level; and the critical standard score "Z" is 1.96. Thus, the estimated sample size (n) was 96.

Human serum samples

Archival serum samples from 2 groups of subjects were employed in this study. The first group comprised 100 blood donors from the Thai Red Cross Society in 2013; and the second group comprised 21 pdmH1N1 patients, whose paired blood samples were collected by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand during an outbreak investigation in 2010.

Wild-type influenza viruses

Influenza viruses used in this study included two human viruses: 2009 pandemic virus, A/California/07/2009 (CA-07), from the National Institute for Biological Standards and Control, UK; and A/Thailand/NMA-1/2011 (H3N2) [A/Texas/50/2012 (H3N2)-like virus]; one HPAI virus, A/Laos/Nong Khai 1/2007 (H5N1) clade 2.3.4 (Nong Khai 1 virus); and one LPAI virus, A/Duck/Shan Tou/461/2000 (H4N9), kindly provided by Prof. Robert G. Webster, St.Jude Children Research Hospital, TN, USA. These viruses were grown in Madin-Darby canine kidney (MDCK) cells in Eagle's Minimum Essential Medium (EMEM) (Gibco, Grand Island, NY, USA), except the LPAI A(H4N9) virus, which was grown in chicken embryonated eggs. Only the virus growth media for human viruses were supplemented with trypsin-tosyl phenylalanyl chloromethyl ketone (trypsin-TPCK). Cell culture supernatants containing viruses and allantoic fluids from virus-inoculated eggs were harvested, centrifuged, aliquoted and stored at -70 C.

Generation of reassortant viruses by reverse genetics

Reverse genetics technique[16] was employed to construct reassortant influenza viruses using the pHW2000 recombinant plasmids kindly provided by Prof. Robert G. Webster. The technique comprised 8 recombinant plasmids carrying each gene of A/Puerto Rico/8/1934 (H1N1) virus (PR8 virus). The recombinant plasmids carrying H7 *HA* or N9 *NA* gene derived from A/Anhui/1/2013 (H7N9) virus were kindly provided by Dr. Yuelong Shu, Chinese National Influenza Center. The multiple basic amino acids (RERRRK/R) at the HA cleavage site of Nong Khai 1 virus was altered to the monobasic amino acid sequence IET/R of an avirulent avian virus subtype H6.

To this end, the HA or NA full genomic segments of test viruses were amplified by PCR using the universal primers designed by Hoffmann, et al,[17] and the amplified products were cloned into the pHW2000 plasmid vector. Subsequently, the recombinant plasmids in combination with the other 6 recombinant plasmids carrying the internal genes of PR8 virus were used to transfect the co-cultures of MDCK cells and

human embryonic kidney 293 T (HEK-293T) cells by *Trans*IT-LT1 solution (MirusBio, Madison, WI). The inoculated cell monolayers were incubated at 37 C in a CO_2 incubator and observed daily for cytopathic effects. Following virus collection, the reassorted *HA* and *NA* genes were verified by nucleotide sequencing.

HA and NA identity identification

HA and NA amino acid sequences of the study viruses were aligned by the Influenza Research Database tool (http://www.fludb.org/), and the percent identities of those amino acid sequences were calculated using Standard Protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Hemagglutination-inhibition assay

The HAI assay was conducted in 96-well "V" shaped microtiter plates in duplicate according to the protocol previously described.[18] The test sera were mixed with receptor-destroying enzyme (RDE) (Denka Seiken, Tokoyo, Japan) at the ratio of 1:3 for 18 hours at 37 C, followed by heat inactivation for 45 minutes at 56 C and absorption with goose erythrocyte suspension for 60 minutes at 4 C to remove nonspecific agglutininator. The treated sera were diluted serially in a 2-fold dilution manner, starting from 1:10 to 1:1280. In a reaction well, 25 µl of the diluted serum was incubated with a 25 µl volume containing 4 HA units of the test virus for 30 minutes at room temperature, followed by adding of 50 µl 0.5% goose erythrocyte suspension. Virus back titration was performed in parallel. The end result was read after the reaction plate was incubated for 30 minutes at 4 C. Antibody titer is defined as the reciprocal of the highest serum dilution that completely inhibits hemagglutination reaction.

Neuraminidase-inhibition assay

Enzyme-linked lectin based NAI assay was performed to determine anti-NA antibody that could inhibit NA enzymatic activity of the test viruses with distinct NA subtypes. The procedure was according to that described previously.[19] Prior to each NAI assay, each test virus was titrated for its NA activity by NA assay as follow. The virus suspension was 2-fold serially diluted starting from the dilution 1:2 to 1:2048 with the sample diluent containing 1% bovine serum albumin and 0.5% Tween 20 in Dulbecco's phosphate buffer saline (Sigma-Aldrich, St. Louis, MO, USA). Then, 50 µl of each virus dilution and 50 µl of sample diluent were transferred into a well of the 96-well plate pre-coated with fetuin (Sigma-Aldrich) and incubated for 17 hours at 37 C. Subsequently, peanut agglutinin conjugated-horseradish peroxidase (PNA-HRP) (Sigma-Aldrich) was added, and the plate was further incubated at room temperature for 2 hours in the dark. *O*-phenylenediamine dihydrochloride (OPD) was employed as the chromogenic substrate. The optical density (OD) at 490 nm was determined using an automatic plate reader. The OD values indicating NA enzyme activity were plotted against the virus dilutions as a sigmoidal regression curves. The virus dilution that yielded 90–95% maximum signal at the OD value of about 2.0 was chosen for further use in NAI assay.

To measure the NAI antibody titers, each serum sample was pretreated with RDE followed by heat inactivation similar to that mentioned in HAI assay. The treated sera were 2-fold serially diluted with sample diluent starting from 1:10 to 1:1280. A 50 µl volume of each diluted serum was added into a fetuin pre-coated well in duplicate, followed by 50 µl of the virus at working dilution. Further steps of the reaction were as described above for NA assay. Each experiment included at least 4 wells containing the virus at working concentration as the virus control, and at least 4 wells containing only sample diluent as the background control. The end result was calculated by subtracting the mean O.D. value of the test wells with the mean O.D. value of the background wells. The NAI antibody titer was obtained from the reciprocal of the highest serum dilution that yielded 50% inhibition of the virus control.

Statistical Analysis

All statistical analyses were done using the R statistical package (R 3.2.1, http://www.r-project.org/). The test sera that yielded negative result (titer < 10) in HAI or NAI assays were assigned to contain the antibody titer of 5; and the titers greater than the final serum dilution were multiplied by 2 for geometric mean titer (GMT) and 95% confidence interval (CI) calculation. The correlation coefficients (r) between NAI antibody titers against each NA antigen was determined by Pearson test.

Results

Construction of reverse genetics-derived viruses

A total of 6 reverse genetics-derived viruses (rg-viruses) were constructed as shown in Table 1. These reassortant viruses carried *HA* and *NA* genes from various donors and the other 6 internal genes from the PR8 parental backbone.

Identity of amino acid sequences in HA and NA of distinct virus subtypes

Amino acid sequences of HA and NA proteins of the study viruses were aligned (data not shown), and the percent identity of the protein sequences are summarized in Table 2. The result showed the highest identity of 63% between HA of H5N1 and HA of pdmH1N1 viruses, and the lowest identity of 39% between HA of H5N1 and HA of H3N2 viruses. On the other hand, the identity of NA proteins among the test viruses is around 40%, but it reaches 83% between NA of pdmH1N1 and NA of H5N1 viruses. Similar to the other viruses deposited in GenBank database, the NA proteins of our study viruses contained a conserved sequence "ILRTQESEC" at the enzyme active site.

Determination for HAI antibody in blood donors

Single serum samples from 100 blood donors were assayed for HAI antibody to 4 viruses, including pdmH1N1 (CA-07), H3N2 (Texas-like), rgH5N1 and rgH7N9 influenza viruses. The result showed that the antibody titers of ≥ 10 were found in 58, 89, 0 and 15% of the blood donors as tested against pdmH1, H3, H5 and H7 viruses, respectively. In details, 19% of them had HAI titers ≥ 40 with GMT of 12.48 (95% CI, 10.14-15.36) against pdmH1N1 virus, and 21% had HAI titers ≥ 40 with GMT of 16.47 (95% CI, 14.38-18.86) against H3N2 virus. None had HAI

DOI 10.12932/AP0788

Table 1. *HA* and *NA* gene origins in reverse genetics-derived viruses

Reassortant virus	*HA* gene donor	*NA* gene donor
rgH5N1	A/Laos/Nong Khai 1/2007(H5N1)	A/Laos/Nong Khai 1/2007(H5N1)
rgH7N9	A/Anhui/1/2013(H7N9)	A/Anhui/1/2013(H7N9)
rgH4(pdmH1N1) NA	A/Duck/Shan Tou/461/2000(H4N9)	A/California/07/2009 (pdmH1N1)
rgH4(H3N2)NA	A/Duck/Shan Tou /461/2000(H4N9)	A/Thailand/NMA-1/2011 (H3N2)
rgH4(H5N1)NA	A/Duck/Shan Tou /461/2000(H4N9)	A/Laos/Nong Khai 1/2007(H5N1)
rgH4(H7N9)NA	A/Duck/Shan Tou /461/2000(H4N9)	A/Anhui/1/2013(H7N9)

titers ≥ 40 against H5N1 (GMT of 5: 95% CI, 5-5) and H7N9 viruses (GMT of 5.55: 95% CI, 5.28-5.83) (Figure 1), suggesting poor reactivity of HAI antibody across distinct HA subtypes.

Determination for NAI antibody in blood donors

All 100 serum samples were also assayed for anti-NA antibody against N1, N2 and N9 NA by NAI assay using 4 reassortant viruses i.e., rgH4(pdmH1N1)NA, rgH4(H3N2)NA, rgH4(H5N1)NA and rgH4(H7N9)NA viruses as test antigens. All of these viruses belonged to H4, the subtype that has never been found to cause human infection. As such, the binding between pre-existing anti-HA antibodies in human sera and the H4 HA expressing on the surface of the test virions did not occur. Therefore, anti-NA antibodies can gain access to NA protein on the virion surface without interference from the pre-existing anti-HA antibodies.

The result showed that most of the subjects i.e., 98, 94, 73 and 53% had antibodies against NA of pdmH1N1, H3N2, H5N1 and H7N9 viruses, respectively. The GMTs of NAI antibodies were 164.50 (95% CI, 128.37-210.79) for N1 derived from pdmH1N1, 111.6 (95% CI, 84.48-147.36) for N2 derived from H3N2, 20.85 (95% CI, 16.43-26.46) for N1 derived from H5N1 and 10.94 (95% CI, 8.98-13.34) for N9 derived from H7N9 virus (Figure 2).

Table 2. Percent amino acid identity among HA and NA proteins of distinct viruses

	HA					NA			
	H1N1	H3N2	H5N1	H7N9		H1N1	H3N2	H5N1	H7N9
H1N1	100				H1N1	100			
H3N2	43	100			H3N2	43	100		
H5N1	63	39	100		H5N1	83	42	100	
H7N9	41	47	42	100	H7N9	45	45	44	100

H1N1, A/California/07/2009; H3N2, A/Thailand/NMA-1/2011; H5N1, A/Laos/Nong Khai 1/2007; H7N9, A/Anhui/1/2013

Correlation between NAI antibody titers against distinct NA subtypes derived from human and avian influenza viruses in blood donors

Our data suggested that the majority of our subjects were previously infected with human influenza viruses and resulted in the induction of cross-reactive antibodies across distinct NA subtypes. We then further determined the correlation between levels of NAI antibodies against NA of human viruses and those against NA of avian viruses (Figure 3). It was found that the NAI antibody titers against NA originated from pdmH1N1 and H5N1 viruses were well correlated (r>0.75) as determined by Pearson correlation test. This result was in line with the highest degree of identity between amino acid sequences of NA derived from pdmH1N1 and H5N1 viruses. On the other hand, levels of NAI antibody to human N1 or N2 subtypes poorly correlated with those to N9 subtype (r<0.45).

Cross-reactivity of anti-N1 antibody from pdmH1N1 patients with distinct NA from human and avian viruses

To explore whether natural infection by human influenza virus could induce anti-NA antibody that cross-reacted against distinct NA of human and avian viruses, paired sera from 21 H1N1pdm patients who developed ≥ 4-fold rise in HAI antibody titers to pdmH1N1 virus were determined for cross-reactive anti-NAI antibodies using rg-viruses expressing distinct NA proteins as test antigens (Table 3). The result demonstrated a 4-fold or greater rise in NAI antibody titer to pdmN1 in 14 patients (66.7%); whereas an increase in NAI antibody titer was detected in 1 patient (4.8%) for N2 derived from human H3N2, 2 patients (9.5%) for N9 derived from avian H7N9, and 10 patients (47.6%) for N1 derived from

Figure 1. HAI antibody titers against distinct HA subtypes derived from human and avian influenza viruses in 100 blood donors. (GMT, geometric mean titer; 95% CI, confidence interval)

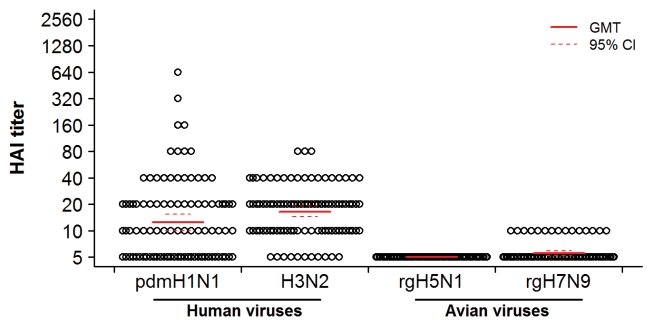


Figure 2. NAI antibody titers against distinct NA subtypes derived from human and avian influenza viruses in 100 blood donors. (GMT, geometric mean titer; 95% CI, confidence interval)

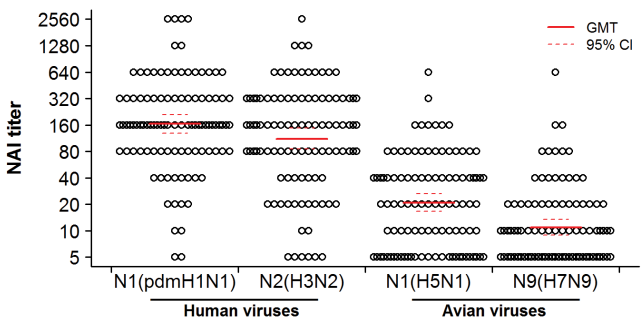


Table 3. Increase in NAI antibody titers in paired sera from the pdmH1N1 patients as tested against various NA subtypes by fetuin-based NAI assay (n=21)

NA subtype	Number of patients with NAI antibody titer increase by fold (%)		
	0	2	≥4
N1 (pdmH1N1)	2 (9.5)	5 (23.8)	14 (66.7)
N2 (H3N2)	18 (85.7)	2 (9.5)	1 (4.8)
N1(H5N1)	5 (23.8)	6 (28.6)	10 (47.6)
N9 (H7N9)	14 (66.7)	5 (23.8)	2 (9.5)

avian H5N1 virus. The finding suggested that the cross-reactive NAI antibodies against distinct NA of human and avian virus origins are commonly induced by natural infection. The cross-reactivity was high for the virus carrying homosubtypic NA.

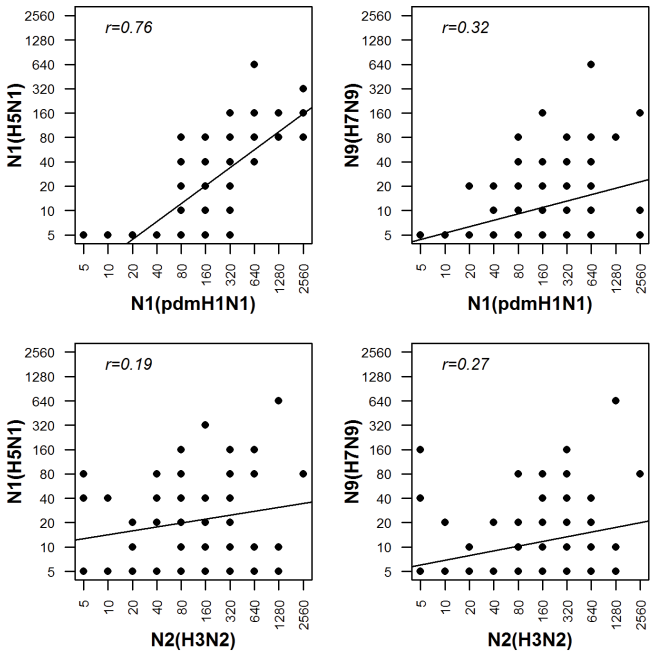
Discussion

Serological surveillance by HAI assay is mostly carried out in laboratories worldwide to assess the previous exposure, prevalence of infection and protective immunity to contemporary influenza strains. Less is known about anti-NA antibody. Nevertheless, epidemiological and experimental data suggested that anti-NA antibody could modify the disease severity[11] and protect mice against the virus lethal challenge.[20]

In this study, HAI antibodies at titer ≥ 10 against pdmH1N1, H3N2, H5N1 and H7N9 viruses were detected in 58, 89, 0 and 15% of blood donors, respectively. It is generally known that HAI antibodies are highly strain-specific. No cross-reactivity between the pdmH1N1 and H3N2 influenza viruses has been demonstrated.[21] Therefore, our result demonstrated that most of the Thai people have been infected with human influenza viruses. None of blood donors in this study contained HAI antibody to H5N1 virus. Using micro-neutralization assay, we previously reported very rare H5N1 seropositive persons among poultry farmers and villagers resided in areas of poultry die-off in 2004.[22,23] Similar finding was reported in Cambodia where none of 351 subjects from 93 households had neutralizing antibody to H5N1 virus in 2005.[24]

Even though 15% of the Thai blood donors had HAI antibody to H7N9 virus, the GMT of 5.55 was considered very low. Thailand is outside of the H7N9 outbreak area; therefore, presence of low HAI antibody titers to H7N9 in blood donors might be due to cross-reactivity with antigens of other human influenza viruses exposed previously. Nevertheless, we could not exclude that this cross-reactivity might be elicited by influenza vaccination. There were a number of studies on the prevalence of antibody to H7N9 virus in the general population.[25-29] None, except those from China, demonstrated high prevalence of anti-H7N9 antibody. The prevalence of H7N9 HAI antibody titers of ≥ 40 was 0.8%,[25] while that of neutralizing antibody titers ranged from 0 to 14% in the general population from different areas across China.[26] The H7N9 HAI antibody titers of ≥ 40 were detected in 7.6% of poultry workers living in the outbreak area.[25]

Figure 3. Scatter plot and correlation coefficient of NAI antibody titers against distinct NA subtypes (*r*: correlation coefficient)



Our study showed that most of Thai blood donors had NAI antibodies to various NA strains with the prevalence of 98, 94, 73 and 53% against NA of pdmH1N1, H3N2, H5N1, and H7N9 viruses, respectively. We also investigated 21 pdmH1N1 patients who had > 4-fold rise in HAI antibody titers in paired sera and found that 66.7% of them developed > 4-fold rise in NAI antibody titer. An increase in NAI antibody titers against NA of H5N1, H3N2 and H7N9 viruses was also detected in 47.6, 4.8 and 9.5% of this group of patients, respectively. Our result suggested that NAI antibody broadly reacted across distinct NA of various test viruses, and was supported by several other studies.[30,31] Mice immunized with recombinant NA proteins derived from A/Vietnam/1203/2004 (H5N1) developed high titers of NA-specific IgG and NAI antibodies against H5N1, pdmH1N1, H3N2, and H7N9 viruses.[30] Moreover, the universally conserved peptide "ILRTQESEC", which induced broadly reactive NAI antibodies across all influenza NA subtypes,[31] was also found in all of our study viruses (data not shown). The result from a clinical trial found that individuals who received the inactivated A/New Jersey/76 (H1N1) seasonal influenza monovalent vaccine in 1976 developed high anti-NA antibody titers against both the vaccine strain and the pdmH1N1 virus. When passively transferred to mice, these human sera protected mice against a lethal challenge with a reassortant virus containing NA of the pdmH1N1 origin, but a mismatched HA.[32]

The NA amino acid identity of 83% between the pdmH1N1 and H5N1 viruses might explain the strong correlation (*r*=0.76) between NAI antibody levels against these two strains. The close relatedness in antigenic characteristics between NA belonging to H5N1 and pdmH1N1 viruses was also observed in previous study.[33] NAI antibody induced by pdmH1N1 virus provided cross-protection against lethal H5N1 virus challenge in mice.[34]

Even though anti-HA antibody against H5N1 and H7N9 viruses was rarely detected, the NAI antibodies against the two avian viruses were much more common in the Thai population. Compared to HA, NA suffers less selective pressure, resulting in slower rate of NA evolution. Thus, diversity of NA protein is less than that of HA protein.[35] Collectively, these data suggest that to develop broadly reactive and long-lasting immunity, the induction of anti-NA antibody as a supplement to anti-HA antibody is desirable.

Acknowledgments

This study was partially supported by the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative, and also Siriraj Graduate Thesis Scholarship Program for International Students from Neighboring Countries. We thank Dr. Yuelong Shu at China CDC for providing the H7N9 virus HA and NA gene segments; Prof. Robert G. Webster, St. Jude Children's Research Hospital, Memphis, USA, for the recombinant pHW2000 reverse genetic plasmids and Dr Sutha Sangiambut, National Science and Technology Development Agency, Thailand, for preparation of the manuscript.

Conflict of interest statements

We have no competing interests

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