NEUTRALIZATION TITERS AGAINST INFLUENZA A (H3N2) AND INFLUENZA B VIRUSES AMONG A NON-VACCINATED POPULATION FROM THAILAND

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Abstract. Influenza A and B viruses are viral respiratory pathogens that can cause severe infections among birds and mammals. Neutralization assays using human sera are useful to evaluate the risk of circulating viruses to humans. In this study, 359 serum samples from healthy Thai volunteers, who had not been vaccinated against influenza for at least five years, were investigated by microneutralization (MN) assays against influenza A H3N2 and influenza B viruses in 2009. There was no significant difference in neutralization activities against 2006 and 2008 isolates of influenza A H3N2 viruses. However, neutralization titers to influenza B viruses among 2008 isolates were quite low. The results indicate the non-vaccinated study population had some neutralizing antibodies against influenza A H3N2 but not against influenza B viruses.

Keywords: influenza A virus, influenza B virus, neutralization titer, healthy Thais

INTRODUCTION

Influenza A and B viruses are the viral respiratory pathogens that can cause severe infections among birds and mammals. Since the first influenza A (H3N2) virus pandemic in 1968 which killed an estimated 750,000 people worldwide, the virus has had the tendency to predominate in prevalence over influenza A H1N1 and influenza B, especially over the past ten years (Finkelman *et al*, 2007; Colin *et al*, 2008). In Thailand, influenza (H3N2) virus predominated during 2005 and 2007 and type B viruses predominated in 2008 (Simmerman *et al*, 2009). Influenza viruses have both dynamic antigenic drifts

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and antigenic shifts. Antigenic drift is an accumulation of point mutations in the hemagglutinin (HA) gene, responsible for inhibiting receptor binding and preventing reinfection with the same strain, which allows the viruses to escape the immunologic pressure of the host (Hampson, 2002), whereas antigenic shift is the process by which two or more different strains of a virus combine to form a new subtype having a mixture of the surface antigens of the original strains (Carrat and Flahault, 2007). Molecular epidemiological studies have suggested antigenic changes occur in H3N2 virus more frequently than H1N1 virus (Rambaut *et al*, 2008). The World Health Organization (WHO) has been using antigenic, genetic and epidemiologic data from the current epidemic virus strains to identify antigenic variants with the potential to cause future epidemics and to develop vaccines (WHO, 2007). During 2007-2008 influenza season in the northern hemisphere there were changes in all vaccine strains: H1N1, H3N2 and influenza B viruses (WHO, 2008). This change in candidate vaccine strains suggests potential antigenic drift in 3 subtypes of influenza viruses. We reported significant antigenic drift in H1N1 viruses between 2006 and 2008 among healthy Thai volunteers in 2009, with remarkably lower neutralization activities against H1N1 isolates in 2008 than 2006 isolates (Kanai *et al*, 2010). Influenza A (H3N2) and influenza B viruses have been co-circulating worldwide with influenza A (H1N1) viruses. H1N1 and H3N2 viruses have been co-circulating worldwide since 1977 and have continued to do so even during the H1N1 2009 virus pandemic (WHO, 2011).

To better understand the epidemiology of influenza A (H3N2) and influenza B viruses in Thailand, we examined influenza virus neutralizing activities among non-vaccinated, healthy Thais against recent virus isolates to carry out risk analysis among Thais to current circulating viruses and the virus evolution driven by human antibodies.

MATERIALS AND METHODS

Healthy volunteer sera

In this study, we collected serum samples from 359 healthy volunteers in Prachuap Khiri Khan (*n*=119), Phetchabun (*n*=120) and Nakhon Sawan (*n*=120) Provinces, Thailand, during May to October 2009. None of the volunteers had vaccinations during the previous 5 years and signs of influenza infection during sampling. This study was approved by the ethics committee of the Department of Medical Sciences, Ministry of Public Health, Thailand. Written informed consent was obtained from each participant before inclusion in this study. Serum samples were treated with receptor destroying enzyme (RDE: Denka Seiken, Tokyo, Japan) at 37°C for 18 hours and followed by incubation at 56°C for 1 hour to remove non-specific neutralizing factors to influenza viruses.

Influenza viruses

The influenza A (H3N2) isolates in 2006 (TH/45/06; TH/46/06; TH/47/06; TH/49/06) and in 2008 (TH/743/08; TH/592/08) and influenza B isolates in 2008 (B/TAK/725/08; B/TRAT/433/08; TH/749/08, TH/533/08), which were clinical isolates in Thailand, were kindly provided by the Thai National Influenza Center (Thai NIC), National Institute of Health, Thailand. Viruses were cultured in Madin-Darby canine kidney (MDCK) cell culture and detected by the virus working dilution by focus-forming units (FFU) before use as previously described (Okuno *et al*, 1990).

Microneutralization assay

The microneutralization (MN) assay was carried out as described previously (Kanai *et al*, 2010). Pretreated sera were serially diluted (1:10 to 1:1,280) with Dulbecco's modified essential medium (DMEM) in a 96 well round-bottom microplate. Then, each serum dilution and then DMEM without serum as a control were each combined with an equal volume (25 l) of virus fluid adjusted to give a final control count of about 50-100 focus-forming units (FFU) per well and then incubated at 37ºC for 60 minutes in a 5% CO_2 incubator. After 16 hours, the cells were fixed with absolute ethanol and reacted with mouse monoclonal antibodies against influenza A virus NP protein (clone A3, 1:1,000 dilution) or influenza B virus NP protein (clone B2, 1:1,000 dilution), followed by horseradish peroxidase-conjugate goat anti-mouse IgG antibody (A2304, Sigma Aldrich, Steinheim, Germany. 1:400 dilution). Mouse monoclonal antibodies were kindly provided by Dr Yoshinobu Okuno (Kanonji Institute, the Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa, Japan). Virus-infected cells were visualized by 3,3-diaminobenzidine tetrahydrochloride hydrate (D5637; Sigma Aldrich, Steinheim, Germany). Human serum taken from vaccinated adults who had a positive antibody titer to influenza A H3N2 or influenza B virus was used as a positive control. The MN titers were expressed as a reciprocal of the maximum dilution giving a 50% reduction compared with the control.

Statistical analysis

The average MN titer among the virus isolates were compared by two-tailed Students *t*-test. The prevalences of positive samples were compared by chi-square test. A $p<0.05$ was considered statistically significant.

RESULTS

Seroprevalence and MN titer to subtype H3N2 viruses

Serum samples from 359 healthy volunteers collected during May to October 2009 were examined for their MN titer against influenza A (H3N2) (4 virus isolates in 2006 and 2 virus isolates in 2008). Mean MN titers against influenza A H3N2 and influenza B viruses are shown in Fig 1. The overall neutralization antibody levels against the H3N2 viruses in 2006 and 2008 isolates were comparable. Among H3N2 isolates during 2006 and 2008, a higher titer was detected in the 16-20 year old age group (average titer 272.8) and the 31-40 year old age group (average titer 239.3). The lowest titer was found among subjects in the older age (>41 years old) for virus isolates from 2006 and 2008 (average titer 134.0). Most individuals had similar MN titers against to each of the 4 isolates in 2006; only 8 of 119 subjects (6.7%) had a greater than 16-fold difference in MN titers among the 2006 isolates. Twenty-three of 119 subjects (19.3%) had a greater than 16-fold difference in the MN titer for 2 isolates in 2008; all 23 subjects had higher titers against TH/592/08 than TH/743/08 (Table 1).

A HI titer >40 is considered the minimum effective antibody level (Hancock *et al*, 2009). We examined the correlation between HI and MN titers against H1N1 viruses and found a MN titer >80-160 consistently corresponded to a HI titer >40 (Kanai *et al*, 2010). In this study, a MN titer >80 was considered as having an effective antibody titer. The prevalence of effective antibodies against H3N2 2006 isolates (63.7%) was significantly lower

Table 1
Distinct MN titers against 2 isolates of H3N2 viruses (TH/743/08 and TH/592/08) in 2008.

Sample ID	Age	MN titers to H3N2 viruses	
		TH/743/08	TH/592/08
PJ 46	16	40	640
PJ 48	16	20	320
PJ 44	18	10	160
PJ 49	18	40	>1,280
PJ 34	19	20	320
PJ 37	20	<10	160
PJ 39	21	40	640
PJ 3	21	10	160
PJ 19	22	10	160
PJ 9	28	20	320
PJ 33	28	40	>1,280
PJ 7	29	20	320
PJ 43	34	40	640
PJ 30	36	40	640
PJ 42	37	10	160
PJ 25	39	<10	160
PJ 11	41	20	320
PJ 2	42	<10	160
PJ 12	46	20	320
PJ 3	48	20	320
PJ 15	48	20	640
PJ 35	50	<10	80
PJ 6	52	<10	80

Subjects showing more than a 16-fold difference in MN titers are shown.

than against the H3N2 TH/592/08 isolate (94.1%) (p=0.004), but comparable to the H3N2 TH/743/08 isolate (69.4%) (p=0.435).

MN titers against influenza B virus

The MN titers against 4 isolates of influenza B virus in 2008 were examined. The MN titers against influenza B viruses in 2008 were lower than those against the influenza A H3N2 2008 isolates (p<0.001) (Fig 1). The MN titers in each age group varied by the virus isolate. There were no clear patterns among MN titers by age group found against H3N2 viruses. Low level of neutralizing antibodies were found among people of all age groups against influenza B isolates in 2008 with a prevalence of neutralizing antibodies of less than 50%. MN titers against influenza B viruses varied more than titers against H3N2 isolates. B/TRAT/433/08 has a remarkable ability to escape human antibodies, as was seen by the low prevalence of effective antibodies (MN titer >80). Interestingly, sero-prevalence rates of antibodies against B/TRAT/433/08 among 16-20 and 21-30 year olds were 2% (5/25) and 0% (0/35), respectively.

DISCUSSION

In this study, 359 samples collected from non-vaccinated, healthy volunteers were examined for neutralizing activity against influenza A (H3N2) and influenza B viruses. The study demonstrated:1) no antigenic changes between 2006 and 2008 in H3N2 viruses and 2) low neutralizing activity against influenza B viruses in 2008. The WHO changed recommendations for the influenza A strains of H3N2 used in the vaccine for the 2007-2008 influenza season. Significant differences in neutralizing activity against H3N2 isolates would be expected between 2006 and 2008 and would be expected against H1N1 viruses (Kanai *et al*, 2010). The samples in this study were tested against H1N1 virus. The MN titer among the younger population against H1N1 2008 isolates was found by another study (Kanai *et al*, 2010) to be nearly 900.

Since normal human serum is known to have non-specific neutralizing factors against influenza viruses (Ananthanarayan and Paniker, 1960), the samples were

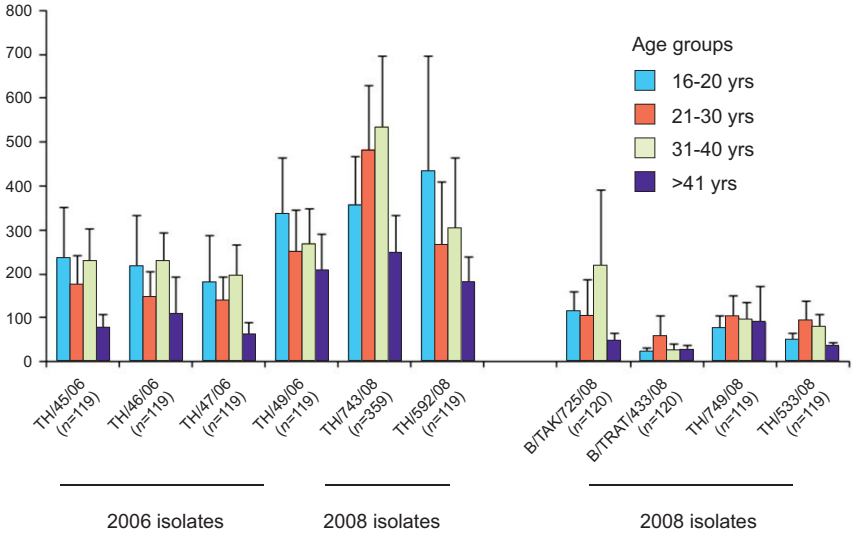

Fig 1–MN antibody titers among 359 healthy volunteers from Thailand against A (H3N2) and influenza B viruses.

treated with respector destroying emzyme (RDE) before testing to remove non-specific inhibitors. Although a negative control using human serum, which could assure the specificity of neutralizing reaction, was not used in this study, a positive reaction can be considered specific for neutralizing antibodies, since many samples were negative against influenza A (H3N2) and influenza B viruses. This study found the population had neutralizing antibodies against H3N2 isolates in 2006 and 2008 with more than a 50% prevalence of effective antibodies. Interestingly, the MN titer against H3N2 virus isolates in 2008 was slightly higher than against H3N2 isolates in 2006, in contrast to the general theory that virus antigenicity changes toward escape from natural antibodies. The effect of antigenic change due to human herd immunity was low, even if changes had occurred in circulating H3N2 viruses in 2008. Neutralization titers against H3N2 viruses in 2006 and 2008 were characterized by distinct age patterns, with higher activity among 16-20 and 31-40-year olds. However, there were no significant differences in neutralization activities between the 2006 and 2008 isolates. Subjects showed distinct MN titers for the 2 isolates of H3N2 2008 viruses. The MN titer against TH/743/08, highest among the 31-40 year old age group and lower among the 21-30 year old age group was distinct for each of the 5 H3H5 isolates examined. Although the sequence data are not available for the isolates used in this study, the current data suggest TH/743/08 is an antigenically evolved virus.

There were low effective neutralization antibodies against influenza B virus isolates in 2008. The lowest titer was seen against B/TRAT/433/08 (mean MN titer of 33) among all age groups. This indicates the human herd immunity was vulnerable to influenza B viruses in 2008. Due to the limited availability of virus isolates, it was not possible to examine influenza B viruses before 2008. The 2008 influenza B virus isolates might be escaped mutations from the herd immunity. During 2008, MN titers against influenza A (H3N2) viruses were higher than those against influenza B viruses. The remarkably low titers against influenza B viruses suggests influenza B virus will predominate during the following season. However, in 2009 after collecting serum samples, pandemic H1N1 2009 predominated in Thailand and only sporadic cases of influenza B virus were reported in Southeast Asian countries (WHO, 2009). This fact indicates

neutralization activity among humans is not the only factor that determines circulation of influenza viruses. Routine investigations for neutralization activity is still important to understand the epidemiology of influenza virus and to predict the epidemiological status during the following season. This type of study should be performed periodically.

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