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Determination of Antibody Response to the Human Pandemic Influenza H1N1 2009 among Patients with Influenza-like Illness and High Risk Groups

Jarika Makkoch[1], Sunchai Payungporn[2], Slinporn Prachayangprecha[1], Rachod Tantilertcharoen[3] and Yong Poovorawan[1]

SUMMARY The global population has been exposed to the novel pandemic H1N1 influenza virus since mid March 2009, causing the expansion of respiratory illness around the world, including Thailand. To evaluate the antibody titers against human pandemic influenza (H1N1) in Thai people with influenza-like illness (ILI), 45 paired serum samples (acute and convalescent) were subjected to hemagglutination inhibition (HI) test and real-time RT-PCR. Most serum samples of ILI patients positive by real-time RT-PCR displayed an at least four-fold antibody increase of HI titers against pandemic influenza (H1N1). In addition, to determine cross-reactivity with human seasonal H1N1 influenza, viral antigen from the seasonal H1N1 was used to detect antibody against seasonal H1N1 influenza and all sera showed negative results. We also studied the single sera samples from the high risk medical personals collected before and after the pandemic influenza (H1N1) outbreaks for antibodies against seasonal H3 influenza virus infection. The results showed lack of cross-reactivity to the human pandemic H1N1 influenza virus. HI antibody testing to pandemic influenza (H1N1) can be used for the diagnosis, preventive and control measures of potential outbreaks.

Since mid March 2009, the human pandemic influenza A virus (H1N1) has emerged continuously, causing mild to severe respiratory illness. From Mexico, the outbreak has spread to the United States and Canada and then expanded throughout the world. This novel pandemic virus was first discovered by the Centers of Disease Control and Prevention, United States (US-CDC), who declared the first case of pandemic influenza infection in the United States in southern California on April 21, 2009.[1] This pandemic virus strain expresses genetic reassortment among variable subtypes of influenza virus, including human seasonal influenza virus, avian influenza virus, Eurasian and North American (classical) lineages of swine influenza virus.[2,3] Furthermore, the accumulated evidence supports the theory that its hemagglutinin, one of its antigenic surface proteins, has originated from classical swine influenza, first appearing in swine populations in 1918 and that this triple-reassortant's antigenic epitope is similar to the influenza virus having circulated sporadically among humans for many decades.[3-5] Until now, there have been over 227,607 laboratory-confirmed cases of human pandemic influenza virus H1N1 infection

From the [1]Centre of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, [2]Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, [3] Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.
Correspondence: Yong Poovorawan
E-mail: Yong.P@chula.ac.th

with at least 12,220 deaths worldwide.[6]

As for the outbreak situation in Thailand, the Bureau of Emerging Infectious diseases, Department of Disease Control, Thai Ministry of Public Health documented the first two cases of infection on May 12, 2009.[7] However, both patients could recover and return home within a few days after infection. The first wave of outbreak occurred in several schools in the Bangkok Metropolitan area and some areas of the city of Pattaya. Both areas are overcrowded and due to close contact between people, the virus can be transmitted from person to person via droplet transmission.[8] Based on previous research, we have ascertained that the outbreak pattern of human pandemic influenza is established between mid June to late July of the previous year. The highest peak of the first outbreak in Thailand is reached in early July. In addition, this novel pandemic virus usually attacks children and adolescents between seven to twenty years old. This pattern of infection is different from that of seasonal influenza which covers all age groups.[9]

The second wave of the human pandemic influenza outbreak is supposed to be definite. Many health organizations of the entire world provided many strategies to prepare themselves for the second wave of pandemic. Vaccination is one of the effective strategies for preventing influenza virus infection. Initially, molecular characterization of the human pandemic virus has shown that the vaccine for human seasonal influenza virus (H1N1) cannot boost the specific antibody against the new strain of virus due to the distinct antigenic differences between the human pandemic influenza (H1N1) and human seasonal influenza (H1N1) while the hemagglutinin antigenic property of the human pandemic influenza is quite similar to the virus isolated from New York in 1976.[10,11] We may assume that some people have pre-existing antibody acquired by infection during the first wave of outbreak or due to circulation of influenza viruses with a similar epitope during the past decades. A study has suggested that people above the age of 60 years could have the antibody titer required to combat the novel pandemic strain. Hence, evaluating the antibody response to human pandemic influenza (H1N1) among Thai people will be essential for vaccine management which would be more effective once the vaccine against the human pandemic influenza (H1N1) is finally available in the next few months.

Various candidate assays can be used for detecting specific antibody titers in response to virus, such as microneutralization (MN) assay, and enzyme-linked immunosorbent assay (ELISA). One of these techniques is hemagglutination inhibition test (HI test). Among those techniques, microneutralization assay is the most laborious and requires expertise for infection of virus into cell culture and interpretation of the cytopathic effect (CPE), whereas ELISA is easier to process but more expensive and is occasionally prone to misinterpretation. HI test can be used for detection of specific antibody blocking the unique properties of HA to agglutinate the red blood cells. HI test represents a simple and inexpensive method which would be feasible and attractive for large-scale analysis.

In this study, we have aimed at evaluating the antibody titer specific to human pandemic influenza (H1N1) in high risk medical staffs and patients with influenza-like illness (ILI) during both acute and convalescent infection in Thailand confirmed by real-time RT-PCR to determine if antibody is produced from acute and convalescent infection in paired serum samples. Furthermore, we used single serum samples to assess cross reactivity between human pandemic and seasonal influenza virus (H1N1) in various groups of people. The data and strategies obtained from this study may be applied for determination of 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.

MATERIALS AND METHODS

Informed consent and ethical consideration

The project proposal had been approved by the ethics committee of the Faculty of Medicine, Chulalongkorn University. Patients were informed about the study objective and their written consent was obtained prior to specimen collection. Laboratory workers at the Center of Excellence in Clinical Virology also granted permission for their sera routinely stored in 2008 to be used in this project.

Population study

One hundred and sixty-two serum samples were divided into 5 groups, including:

Group I: Paired sera of ILI patients PCR positive and negative for human pandemic influenza (H1N1) infection

Forty-five sera (acute and convalescent paired samples) were available from patients diagnosed with acute respiratory tract illness with ILI (Influenza-like illness), which is defined by CDC as fever (100ºF or 37.8ºC) with sore throat or cough without other known causes,[12] attending the Out Patient Department, Chumpare Hospital, Khon Kaen province, Thailand. The acute sera were collected within the first 7 days after the onset of symptoms. The convalescent sera were collected at least 14 days after the onset of disease. In addition, nasopharyngeal swabs were collected from all 45 patients and screened for the human pandemic influenza (H1N1) by real-time RT-PCR.

Group II: Single sera of high risk medical staffs before outbreak of human pandemic influenza virus (H1N1)

Twenty single sera had been obtained from medical staffs at the Center of Excellence in Clinical Virology, Chulalongkorn University in the year 2008, before the pandemic influenza H1N1 2009 outbreak. All had been vaccinated against seasonal influenza virus for at least 2 years.

Group III: Single sera of healthy young adults before the outbreak of human pandemic influenza virus

Thirty-one stored sera were obtained from healthy young adults (18-20 years old) in the year 2008, before the outbreak of pandemic influenza (H1N1) 2009. None of them had ever received the seasonal influenza virus vaccine.

Group IV: Single sera of high risk medical staffs after the outbreak of human pandemic influenza (H1N1)

Forty-two single serum specimens were obtained from laboratory workers at the Center of Excellence in Clinical Virology, Chulalongkorn University in early December of 2009, after the first wave of pandemic influenza (H1N1) 2009 outbreak.

Group V: Single sera PCR positive for H3 human seasonal influenza virus

Five acute and one convalescent sera were obtained from 6 patients who attended the Out Patient Department, Chumpare Hospital, Khon Kaen province, Thailand. The nasopharyngeal swabs obtained from them were confirmed to be infected with H3 influenza A virus by real-time RT-PCR. The acute serum samples were collected within 7 days after the onset of symptoms. The convalescent serum sample was obtained on the 24th day after the onset of symptoms.

These single sera in groups II, III, IV and V were used as control sera to test the cross-reactive antibody response between seasonal H1N1 and pandemic influenza H1N1 2009 virus infection. All samples were kept at -70°C until tested.

Detection of human pandemic influenza (H1N1) by real-time RT-PCR

The nasopharyngeal or throat swab samples of patients with ILI were collected in 2 ml of viral transport medium and transported in a biohazard ice box. The specimens were sent within 48 hours to the Center of Excellence in Clinical Virology for diagnosis of the human pandemic influenza (H1N1). Then, 200 µl of nasopharyngeal swab medium was used for RNA extraction using the Viral Nucleic Acid Extraction Kit (RBC Bioscience Co, Taiwan) following the manufacturer's recommendation. RNA obtained from each sample was used as a template for detection of the human pandemic influenza virus (H1N1) by real-time RT-PCR with primers and probes as previously described.[13] Real-time RT-PCR was carried out using the SuperScript III Platinum One-Step RT-PCR system (Invitrogen, California, USA) and performed on Rotor-Gene 3000 (Corbett Research, New South Wales, Australia) applying conditions described previously.[9]

Virus propagation

The human pandemic influenza (H1N1) and the human seasonal influenza (H1N1) used as virus antigen in this study are A/Thailand/CU-H88/09 (CU-H88) and A/Thailand/CU-41/06 (CU-41), re-

spectively. The viruses were successfully isolated from the previous outbreaks of influenza virus in Thailand.[9,14] Then virus propagation for use as viral antigen was performed by inoculation of the virus stocks into the allantoic cavity of 10-day-old chick embryonated eggs. After 48 hours of incubation, the allantoic fluid was harvested and clarified by centrifugation at 1,200 x *g* for 10 minutes. The allantoic supernatants were used for determination of the viral titers by hemagglutination assay (HA) as previously described.[15] All virus propagation procedures were conducted in a bio-safety level 2+ (BSL2+) laboratory.

Hemagglutination inhibition assay

Serum samples were treated with receptor-destroying enzyme (RDE) produced by *Vibrio cholerae* Ogawa type 558 (Denka Seiken, Co. Ltd., Tokyo, Japan) in 1:3 ratios of serum:RDE. The serum/ RDE mixtures were incubated at 37°C for 16 hours. Subsequently, they were heat inactivated at 56°C for 30 minutes followed by addition of 0.85% vol physiological saline to arrive at a final serum dilution of 1/10. The HI assays were performed with 0.5% turkey erythrocytes in V-shaped 96-well microtiter plates (Greiner Bio-One GmbH, Kremsmuenster, Austria), as previously described. [15,16]

Data analysis

Data analysis of the real-time PCR assay was performed using the Rotor-Gene data analysis software, Version 6.0 (Corbett research supporting program). All results obtained from HI test were photographed immediately after the end of the process and then analyzed without bias by the same investigator. Statistical data were analyzed using SPSS for Windows version 17.0 software package.

Table 1 Paired sera with real-time RT-PCR positive for human pandemic influenza (H1N1)

No.	Age (years)	Gender	Day after symptom onset	Days	HI against pandemic H1N1		HI against seasonal H1N1	
					Acute	Convalescent	Acute	Convalescent
1	18	F	3	16	320	640	40	40
2	11	F	2	16	40	40	80	20
3	65	F	2	16	10	40	20	10
4	9	F	3	16	20	40	40	40
5	38	M	3	16	20	320	20	10
6	15	F	5	16	20	320	160	20
7	12	F	4	16	40	640	40	40
8	12	F	5	16	10	320	80	10
9	10	F	5	14	20	320	20	10
10	10	M	4	14	20	320	20	20
11	10	F	4	14	20	20	40	20
12	4	M	2	14	20	640	40	40
13	10	M	2	14	20	320	80	80
14	16	F	3	14	20	320	160	80
15	14	F	3	14	20	1,280	80	20
16	8	F	8	14	20	320	160	20
17	15	M	5	14	10	160	20	40
18	5	M	4	16	20	80	80	10
19	6	M	2	16	10	1,280	20	10
20	10	F	3	16	20	320	80	10
21	14	M	3	16	10	80	40	20
22	7	M	2	16	<10	<10	20	<10

RESULTS

Comparison of HI titer against human pandemic influenza (H1N1) and seasonal influenza (H1N1) in ILI patients with paired sera

Detection of human pandemic influenza (H1N1) from ILI patients

Based on the real-time RT-PCR diagnosis performed on nasopharyngeal or throat swab samples from 45 patients with influenza-like illness, 22 samples were positive for the human pandemic influenza (H1N1) whereas another 23 individuals yielded negative results. Based on these results, patients with ILI were divided into 2 groups, i.e., those positive and negative for human pandemic influenza (H1N1).

HI titers in ILI patients positive for the human pandemic influenza (H1N1)

Of 45 ILI patients with paired sera, 22 individuals were positive for the human pandemic influenza infection. In this group, almost all of the antibody titers against the human pandemic influenza virus in acute sera (21 in 22 individuals; 95.45%) ranged from <10 to 40. The geometric mean titer in this group amounted to 16.82 with a median at 20 while the antibody titer of convalescent sera ranged from <10 to 1,280. The median was 340. Most convalescent sera (20 in 22 samples; 90.90%) exhibited HI titers ≥ 40 and were considered as evidence of antibody response to the human pandemic influenza (H1N1). Seventeen patients had significant seroconversion or increase in antibody titers (≥ 4 fold)

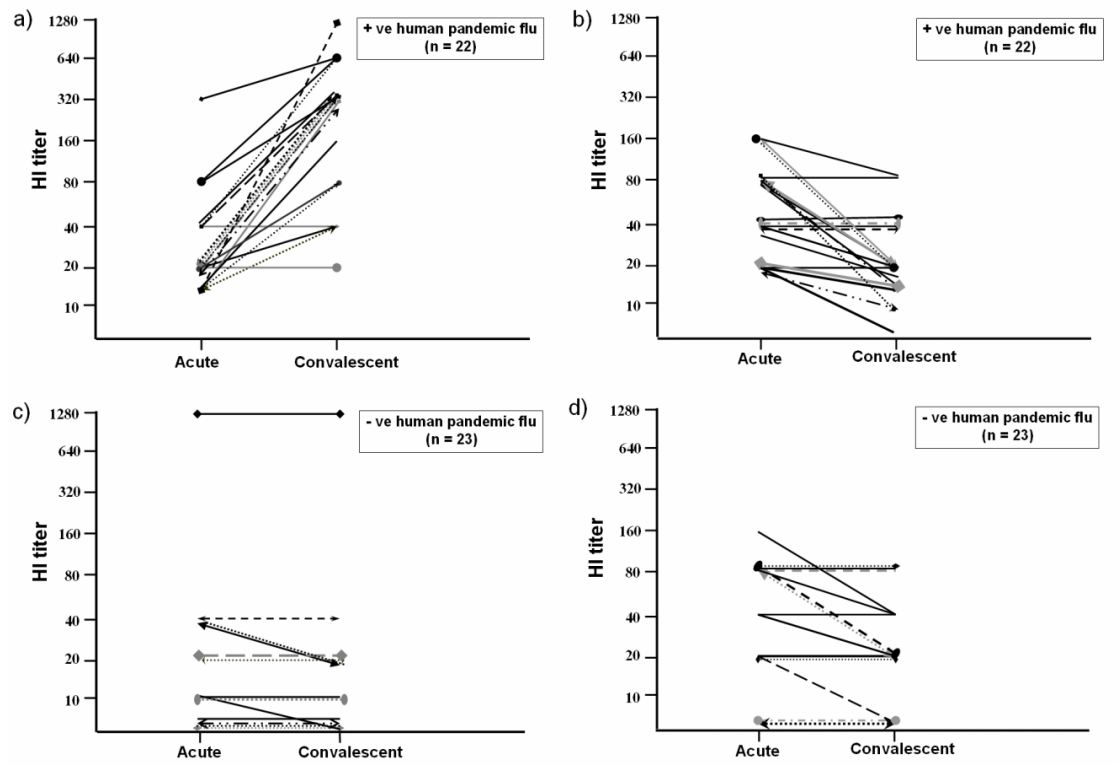

Fig. 1 Sero-conversion of HI titer in patients with influenza-like illness (a) Increase in antibody against the human pandemic flu (H1N1) in patients with the pandemic H1N1 positive PCR result. (b) Antibody titers against human seasonal flu (H1N1) in patients with the pandemic H1N1 positive PCR result. (c) Antibody titers against the human pandemic flu (H1N1) virus in patients with the pandemic H1N1 negative PCR result and (d) Antibody titers against human seasonal flu (H1N1) virus in patients with the pandemic H1N1 negative PCR result.

while 5 patients had 2-fold or stable HI titers. The results are summarized in Table 1 and the increase in antibody titers against human pandemic H1N1 in this group is illustrated in Fig. 1a.

As for the HI titer against seasonal H1N1 influenza virus in ILI patients positive for the human pandemic influenza (H1N1) by real-time RT-PCR, the HI titer of acute serum samples ranged from 20 to 160 with median values of 46.82 and 40, respectively while the HI titer of convalescent serum samples ranged from < 10 to 80 with of 21.36 and 20, respectively. The results are summarized in Table 1 and the antibody titers against human seasonal influenza H1N1 in this group are illustrated in Fig. 1b.

HI titers in ILI patients negative for the human pandemic influenza (H1N1)

From 45 ILI patients with paired sera, 23 samples were negative for the human pandemic influenza infection. Most of the antibody titers against the human pandemic influenza virus in both acute and convalescent sera (21 in 23 patients; 91.30%) ranged from < 10 to 40 with median values of 10 and 20, respectively. The sero-conversion against human pandemic influenza H1N1 2009 virus infection in this group showed no significant differences in HI titers (< 4 fold) between acute and convalescent sera, which signified negative HI titers. The antibody titers against human pandemic influenza (H1N1) in this group are shown in Table 2 and Fig. 1c.

As for the HI titer against seasonal H1N1 influenza virus in ILI patients negative for the human pandemic influenza (H1N1) strain, almost every patient in this group (19 in 23 patients; 82.61%) already had antibody response to seasonal H1N1 influenza virus. The HI titers against seasonal H1N1

Table 2 Paired sera with real-time RT-PCR negative for human pandemic influenza (H1N1)

No.	Age (years)	Gender (M/F)	Day after symptom onset	Days	HI against pandemic H1N1		HI against seasonal H1N1	
					Acute	Convalescent	Acute	Convalescent
23	5	M	2	16	<10	<10	40	20
24	15	F	3	16	<10	<10	160	40
25	5	M	3	16	40	40	20	20
26	13	M	2	16	<10	<10	80	20
27	24	F	4	14	320	320	80	20
28	1	F	5	14	20	20	80	80
29	4	F	4	14	40	20	80	80
30	46	F	8	14	<10	<10	<10	<10
31	11	M	2	14	10	10	20	20
32	34	F	5	34	<10	<10	80	80
33	13	F	4	15	<10	<10	40	40
34	9	M	1	13	40	20	80	40
35	57	F	18	12	1,280	1,280	40	40
36	5	F	4	12	10	10	40	40
37	10	M	1	15	10	10	80	20
38	8	M	6	12	10	<10	40	20
39	1	M	5	14	10	10	40	160
40	10	M	5	14	10	<10	20	40
41	10	M	5	14	10	<10	40	10
42	0.75	F	7	14	10	10	40	20
43	0.08	F	5	14	10	10	10	10
44	7	M	3	14	10	<10	80	80
45	15	F	5	14	20	10	80	80

ranged from <10 to 160 both in acute and convalescent sera with median values for both at 40. The antibody titers against human pandemic influenza H1N1 2009 in this group are shown in Table 2 and Fig. 1d.

Comparison of HI titers against human pandemic influenza (H1N1) among single sera and antigenic cross reactivity to human H1N1 influenza virus

In order to test for cross reactive antibodies between the human pandemic influenza (H1N1) and seasonal influenza (H1N1), 78 single cross sectional sera were collected and divided into 4 groups (groups II-IV as described above).

The HI test results for human pandemic influenza (H1N1) revealed that all samples from high risk medical staffs (group II, n = 20) and healthy young adults (group III, n = 31) collected before the outbreak of human pandemic influenza (H1N1) had antibody titers below 40 (ranging from < 10 to 20). Comparison of the HI titers against seasonal influenza between group II with all individuals vaccinated against seasonal influenza virus for at least 2 years and group III with no-one ever vaccinated against seasonal influenza, showed the geometric mean titers of these 2 groups at 94.17 and 34.82, respectively (data not shown). Most samples (32 in 42 individuals; 76.20%) from high risk medical staffs collected after the outbreaks of human pandemic influenza (H1N1) showed positive HI titers (≥ 40) against the H1N1 human pandemic influenza (Fig. 2). Likewise, none of six serum samples with their respective nasopharyngeal swabs positive for H3, displayed any HI titers (HI titer < 10; Fig. 2) against either human pandemic influenza or seasonal influenza (H1N1). Based on the HI assays, we concluded that there is no cross reactivity between human pandemic influenza virus (H1N1) and seasonal H1N1 or H3N2 influenza viruses.

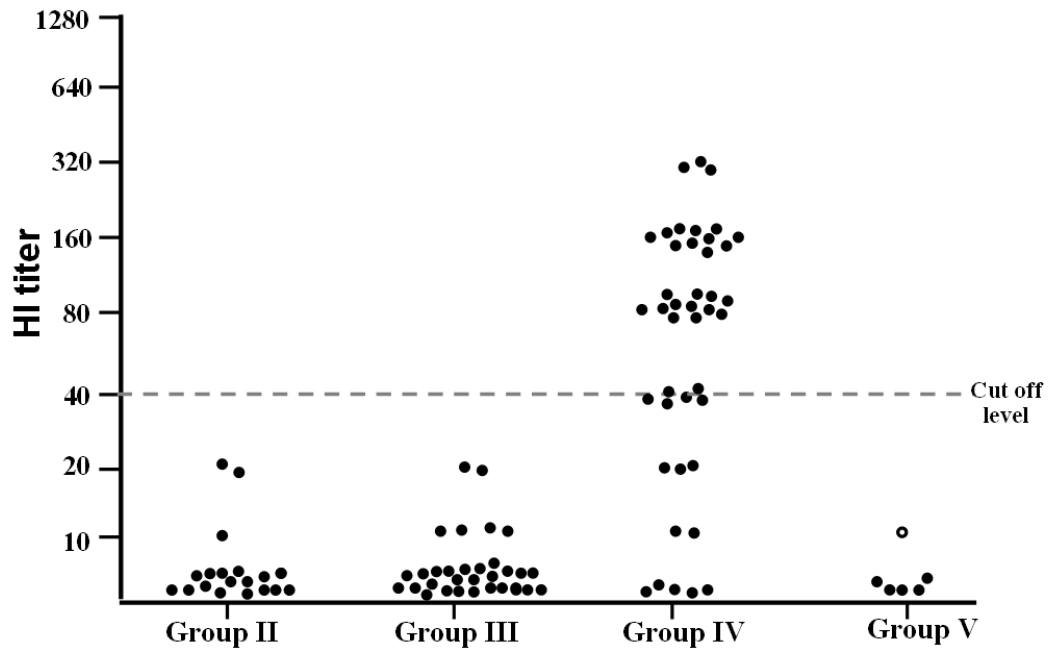

Fig. 2 Antibody titers against the human pandemic influenza (H1N1) in single sera obtained from various groups. Group II: Single sera of high risk medical personals before outbreak of human pandemic influenza virus (n = 20); Group III: Single sera of healthy young adults before the outbreak of human pandemic influenza (n = 31); Group IV: Single sera of high risk medical personals after the outbreak of human pandemic influenza (n = 42); Group V: Single sera PCR positive for H3 human seasonal influenza virus (n = 6). Black circles and a hallow circle represent samples from acute and convalescent sera, respectively.

DISCUSSION

In this study, we performed the HI test in paired serum samples of ILI patients to detect the different titers against human pandemic H1N1 influenza virus. All paired serum samples were separated into 2 groups according to real-time RT-PCR results, i.e., sera positive and sera negative for human pandemic influenza H1N1 2009 virus infection. Based on HI assays for pandemic influenza (A/Thailand/CU-H88/2009), most serum samples positive for pandemic human influenza H1N1 2009 displayed significant (≥ 4-fold) sero-conversion. Most HI titers in acute sera were defined as negative (< 40) whereas the convalescent sera produced positive HI titers (≥ 40). In contrast, all serum samples negative for human pandemic influenza H1N1 2009 by real-time RT-PCR demonstrated stationary or decreased HI titers against human pandemic influenza H1N12009 virus. The HI titers against seasonal H1N1 virus (A/Thailand/CU-41/2006) in 2 groups of serum were not significantly different and presented low titers. These results allowed for the conclusion that the HI test, which is the standard test and recommended by WHO, is reliable for serological diagnosis in people infected with human pandemic influenza virus (H1N1) and HI titers in acute and convalescent sera would show at least a 4-fold increase.

There were 2 cases in the ILI group negative for human pandemic influenza H1N1 2009 by real-time RT-PCR which should be considered. These cases showed high but stationary HI titers in paired serum samples (320-320 and 1280-1280). The 2 patients were supposed to be infected with the human pandemic H1N1 influenza virus prior to serum sampling and the influenza-like illness was caused by infections with other viruses, such as parainfluenza virus, adenovirus etc. On the other hand, this patient, who was a teenager, may have been admitted and the nasopharyngeal swab for real-time RT-PCR detection and serum for HI test may have been obtained after he had already been infected for several days. Applying real-time RT-PCR, viral load can be detected within 15 days after onset of symptoms [17], or within 5-6 days if the patient was treated with the antiviral drug Oseltamivir [18] while the antibody titer would normally rise between 14-28 days after infection. [19] The finding also found in perinatal infection with the antibody rising in the third to forth week. [20]

To ensure the HI test's specificity for human pandemic influenza H1N1 2009, we tested for cross-reactivity against pandemic influenza. The single sera collected before the outbreak, were subjected to HI test for either human pandemic influenza (H1N1) or seasonal influenza (H1N1). The results shown in Fig. 2 suggest that there was no cross-reactivity between the two virus strains. As for high risk healthcare staff, there were 10 pairs of sera collected from the same individuals in 2008 and 2009. According to the HI test, the HI titers increased significantly between 2008 and 2009. Many of the sera collected in 2009 already had the antibody against human pandemic influenza which may have been caused by close contact with patients. These members of the medical staff may also have applied various strategies protecting themselves from symptomatic virus infection. Due to lack of cross-reactivity against pandemic H1N1, the seasonal influenza vaccine could not protect anyone from the human pandemic H1N1.

In conclusion, our results have shown lack of cross reactivity in antibody titers obtained from HI test between the human pandemic influenza (H1N1) and seasonal influenza (H1N1). Moreover, comparison of the HI results between acute and convalescent sera have shown a significant increase in the specific antibody titers against human pandemic influenza (H1N1). Therefore, 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.

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