เอกสารแนบหมายเลข 1

รายงานวิจัยฉบับสมบูรณ์

โครงการวัคซีนต่อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 ในการกระตุ้นแอนติบอดีจะเพาะต่อก้านของโปรตีน hemagglutinin ในคน

โดย

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สัญญาเลขที่ MRG5680078

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บทคัดย่อ

รหัสโครงการ: MRG5680078

ชื่อโครงการ: วัคซีนต่อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 ในการกระตุ้นแอนติบอดีจะเพาะต่อก้านของโปรตีน hemagglutinin ในคน

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Hemagglutinin (HA) โปรตีนของเชื้อไวรัสไข้หวัดใหญ่ประกอบไปด้วย 2 ส่วน คือ ส่วนหัว (globular head) และ ส่วนก้าน (stalk) โดย neutralizing epitopes จะอยู่บนส่วนหัวซึ่งเป็นบริเวณที่มีการเปลี่ยนแปลงได้ง่าย ในขณะที่ส่วนก้านจะมีการเปลี่ยนแปลงน้อยระหว่างไวรัสต่างสายพันธุ์ แต่การกระตุ้นแอนติบอดีต่อส่วนก้านนั้นเป็นไปได้ยากกว่าส่วนหัว การศึกษาที่ผ่านมาพบว่า วัคซีนไข้หวัดใหญ่ 2009 สามารถกระตุ้นแอนติบอดีต่อต้านก้านของ HA ได้ โครงการวิจัยนี้จึงต้องการศึกษาแอนติบอดีต่อต้านก้านของ HA ในอาสาสมัครคนไทยที่เคยได้รับวัคซีนไข้หวัดใหญ่ 2009 เปรียบเทียบกับอาสาสมัครที่ได้รับวัคซีนไข้หวัดใหญ่ก่อนปี 2009 โดยซีรั่มของอาสาสมัครจะถูกทดสอบด้วยวิธี ELISA โดยใช้โปรตีน HA cH6/1 และ cH9/1 ที่ถูกดัดแปลงให้ส่วนหัวเป็นของไวรัสสายพันธุ์ H9 และ H6 ที่ไม่ระบาดในคน แต่มียังคงส่วนก้านของ HA สายพันธุ์ H1 เพื่อดูปฏิกิริยาจำเพาะของซีรั่มแต่ละกลุ่มที่มีต่อก้านของโปรตีน HA ผลการทดลองพบว่า แอนติบอดีต่อต้านก้าน HA ในซีรั่มของอาสาสมัครที่เคยได้รับวัคซีนไข้หวัดใหญ่ 2009 ให้ผลการเข้าจับเป็นบวก ในขณะที่อาสาสมัครที่ได้รับวัคซีนก่อนปี 2009 ให้ผลการเข้าจับเป็นบวกน้อยกว่าอาสาสมัครที่เคยได้รับวัคซีนไข้หวัดใหญ่ 2009 กับทั้ง cH6/1 และ cH9/1 จากผลการทดลองแสดงให้เห็นว่าประชากรที่ได้รับวัคซีนหรือติดเชื้อจากไวรัสไข้หวัดใหญ่ 2009 H1N1 สามารถกระตุ้นแอนติบอดีต่อก้าน HA ได้ โดยซีรั่มบวกจะถูกนำไปทำ HAI (Hemagglutinin Inhibition assay) เพื่อยืนยันการไม่จับกับส่วนหัว H6 และ H9 แต่เป็นปฏิกิริยาต่อส่วนก้านเท่านั้น ก่อนจะถูกนำไปทำ neutralization assay เพื่อดูผลของการลบล้างฤทธิ์ของซีรั่มต่อ chimeric virus cH9/1N3 ผลการทดลองพบว่าซีรั่มที่ได้รับการฉีดวัคซีนไข้หวัดใหญ่ 2009 แสดงความสามารถในการลบล้างฤทธิ์จากการทดสอบกับไวรัสไข้หวัดใหญ่ลูกผสมเพียงเล็กน้อยเท่านั้น แม้ว่าความสามารถในการลบล้างฤทธิ์ที่ส่วนก้านจะไม่สามารถตรวจสอบได้ แต่ผลการทดลองแสดงให้เห็นว่าวัคซีนสามารถกระตุ้นแอนติบอดีต่อต้านก้าน HA ซึ่งอาจช่วยป้องกันการติดเชื้อไวรัสผ่านกลไกอื่นได้ยังคงต้องการการศึกษาต่อไป

Abstract

Project code: MRG5680078

Project Title: Pandemic H1N1 influenza vaccine and the induction of anti-hemagglutinin stalk antibodies in humans

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Project period: 3 June 2013 to 2 June 2015

Hemagglutinin (HA) protein of influenza virus is composed of two domains, globular head and stalk domain. Influenza neutralizing epitopes are mostly located on the highly variable globular head domain, while stalk domain is more conserved but less immunodominant than the globular head. Previous studies showed that 2009 H1N1 influenza virus vaccine can stimulate hemagglutinin stalk antibodies. This study investigated anti-HA stalk antibodies in Thai volunteers who received 2009 H1N1 influenza virus vaccine in comparison to those who received seasonal trivalent influenza vaccine before 2009. Volunteer sera were screen for stalk-reactive antibodies using ELISA against chimeric influenza HA proteins cH6/1 and cH9/1 which contain globular head from either H6 or H9 avian influenza viruses and the stalk from H1 virus. The result showed positive binding activity of 2009 influenza-vaccinated sera against to cH6/1 and cH9/1, while, pre- 2009 influenza vaccinated sera showed less binding activity. This result suggested that vaccination with or exposure to the pandemic 2009 H1N1 in this population could induce anti HA-stalk antibody. Positive sera were tested for absence of binding antibody to globular head of H6 and H9 by hemagglutination inhibition assay against H6 and H9 viruses before subjected to neutralization assay against cH9/1N3 chimeric virus. The 2009 influenza-vaccinated sera showed little neutralizing activity against the chimeric virus despite the presence of stalk-reactive antibodies. Although neutralizing activity against the stalk domain could not be detected, it can be seen that the vaccine can induce HA-stalk antibodies. These stalk-reactive antibodies may play roles in preventing infection via other mechanisms which remains to be investigated.

Keywords (3-5 words): influenza virus, H1N1, vaccine, hemagglutinin, stalk antibodies

Executive Summary

Influenza in an acute viral infection caused by influenza viruses that can be transmitted from person to person. Influenza viruses are important human respiratory pathogens which can cause seasonal infections as well as unpredictable pandemics. Every year, influenza infections cause a loss of productivity and a rise in health care costs. Worldwide, seasonal influenza causes severe illness in 3-5 million people and 200,000-500,000 deaths each year. Vaccination is currently the most effective way to prevent influenza infection. High avidity antibodies against the viral hemagglutinin (HA) protein can confer protection against influenza. These antibodies are usually induced after natural influenza infection or through vaccination.

Hemagglutinin (HA) protein of influenza virus is the main structure to attach to target cells for entry. HA protein is composed of two domains, globular head and stalk/stem domain. Globular head domain contains immunodominant antigenic sites for influenza virus but this domain is a highly variable. The globular head domain of the HA protein harbors the receptor binding site of the virus to sialic acid on the host cells surface. Protective antibodies typically display high affinity/avidity to the globular region of the HA and inhibit infection by blocking the attachment of virus to the host cells. However, these protective antibodies are very specific for a given strain or subtype of influenza virus. Influenza vaccines only rendered protection against viruses whose antigenicity is the same or very similar to the vaccine strains, which is why influenza vaccines needs to be updated each year to keep up with the circulating strains. The stalk domain is more conserved for each group of influenza subtypes but less immunodominant than the globular head. The level of broadly-reactive antibodies against the conserved HA stalk domain of influenza virus has been shown to be boosted followed the infection with pandemic 2009 H1N1 influenza A virus of which the globular head domain located on the hemagglutinin protein differs substantially from the circulating seasonal H1N1 strains. The concept of boosting these stalk-reactive antibodies which can elicit broad protection against diverse influenza viruses has quickly become the strategy for developing the universal influenza vaccine.

There is still very limited data on whether the pandemic 2009 H1N1 vaccine can also boost these antibodies. Therefore, it is essential to investigate whether the pandemic 2009 H1N1 vaccine could also confer the same boosting effect as of that occurs in the natural infection. In this study, sera samples from subjects who had received the pandemic 2009 H1N1 influenza A virus vaccine were assessed for the presence of anti-HA stalk antibodies in comparison to the control subjects that had been vaccinated with seasonal trivalent influenza vaccine before the occurrence of 2009 H1N1 influenza A virus pandemic. The sera were screened against recombinant chimeric HA proteins with HA stalk from the H1N1 influenza A virus, while the globular heads were modified to that of influenza subtypes (H6 and H9) not circulating in human population. Hemagglutination inhibition assay and immunofluorescence assay were used to further confirm the findings. The sera that were found to be reactive against the HA stalk domain were subjected to neutralization assay to test for the neutralizing activity against the virus. The information from this study would enrich our understanding on the mechanism of how these broadly cross-reactive antibodies can be naturally generated and the knowledge would be useful for the future development of influenza vaccines.

Introduction

Seasonal influenza is an acute viral infection caused by influenza viruses that can spread easily from person to person. Each year, seasonal influenza causes severe illness in 3-5 million people and 200,000-500,000 deaths world-wide. High avidity antibodies against the viral hemagglutinin (HA) protein can confer protection against influenza. These antibodies are usually induced after natural influenza infection or through vaccination. Vaccination is currently the most effective way to prevent influenza infection. The immunodominant antigenic sites of influenza viruses are located on the globular head domain of the HA protein which harbor the receptor binding site of the virus to sialic acid on the host cells surface. Therefore, protective antibodies typically display high affinity/avidity to the globular region of the HA and inhibit infection by blocking the attachment of virus to the host cells. However, these protective antibodies are very specific for a given strain or subtype of influenza virus. The high mutation rate in the replication of influenza virus genome, especially around the receptor binding sites on the globular head domain, results in the selection of influenza virus to escape the existing protective antibodies. Thus, the influenza vaccine only rendered protection against viruses whose antigenicity is the same or very similar to the vaccine strains which is why influenza vaccines needs to be updated each year. The concept of a universal influenza vaccine which can elicit broad protection against diverse influenza viruses is particularly attractive for pandemic preparedness. Infection with pandemic 2009 H1N1 influenza A virus results in a boost in broadly-reactive antibodies to the conserved HA stalk domain. At present, there is still very limited data on whether the pandemic 2009 H1N1 vaccine can also boost these antibodies. Therefore, it is important to investigate whether the pandemic 2009 H1N1 vaccine could also rendered this boost.

Influenza viruses belong to the Orthomyxoviridae and are important human pathogens that cause a highly contagious respiratory disease. Influenza A viruses are responsible for the periodic widespread epidemics and several pandemics with high percentage of mortality rates. Influenza A virus is an enveloped virus containing eight segments of negative-sense, single-stranded RNA that encode for 11 proteins (1). They can be categorized into subtypes by their two surface antigens hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA and 9 NA subtypes have been reported. However, only H1, H2 and H3, with mainly N1 and N2, are circulated in the human population (2).

Hemagglutinin (HA) is a major homotrimeric transmembrane protein consists of a globular head domain and a membrane-proximal stalk/stem domain (3). HA primarily serves for the attachment of virus to cells sialic acid receptors and subsequently for the penetration of viral components into the cells. The globular head domain, which harbors the receptor binding site and immunodominant antigenic sites, mediates the attachment of the virus to the host cells and is the main target of neutralizing antibody, while the stalk region, where the fusion peptide is located, mediates fusion and entry of the viral membrane into the host cell (3-4).

Periodic changes on the globular head of hemagglutinin (HA) caused by the selective pressure of anti-hemagglutinin antibody and the high mutation rate in influenza virus replication can result in antigenic drift which contribute to a substantial part of diversity seen across influenza A virus subtypes. Occasionally, antigenic shift, which confers a more drastic change in the antigenicity of HA, can also occur and can result in an influenza pandemic (5). In contrast to the globular head domain, the stalk

domain of HA has a function which requires a more conserved conformational structure, therefore mutations in the stalk are not well-tolerated and thus the region remains conserved (6).

Currently, many groups of researchers are interested in the concept of universal vaccine for influenza A viruses and many recent works have been focusing on the characterization of the anti-HA stalk domain antibodies (7-16). These antibodies promise much broader activity than those of the anti-globular head domain, as the stalk domain of the HA is highly conserved across influenza A virus subtypes and thus can confer reactivity to a variety of influenza A virus strains (7-16). It is believed that the anti-HA stalk domain antibodies can be efficiently boosted when individuals are exposed to HA antigens which the globular head domain differ substantially from previous exposures while the stalk domains remain conserved (17).

In 2009, an influenza pandemic occurred when a novel swine influenza virus, now classified as the 2009 pandemic H1N1 influenza A virus, emerges against which there is little or no immunity in the general population. It is interesting to note that the 2009 pandemic H1N1 influenza A virus subsequently replaced the normally circulating seasonal influenza A viruses. It was speculated that the elimination of the seasonal H1N1 viruses is the result of the induction of HA stalk domain specific antibodies. Studies in both naturally infected human (18) and a mouse model (19) have shown that the 2009 pandemic H1N1 influenza A virus strain, which is immunologically distinct from other seasonal influenza A viruses in circulation, can elicit the virus-neutralizing antibodies specific to the HA stalk domain. Moreover, recent studies have reported that HA stalk domain specific monoclonal antibodies and antibody-producing cells can be isolated from the pandemic 2009 H1N1 vaccinees (20-21).

The observations that the broadly cross-reactive antibodies that bind to the HA stalk domain can be boosted by infection with pandemic H1N1 influenza A viruses which contain hemagglutinin proteins with globular heads that differ substantially from seasonal strains has become a promising strategy for creating a universal vaccine for influenza A viruses. Therefore, it would be valuable to investigate whether the pandemic 2009 H1N1 vaccine, which the virus globular head domain differ substantially in comparison to seasonal strains, could also boost the stalk-reactive antibodies.

Objectives

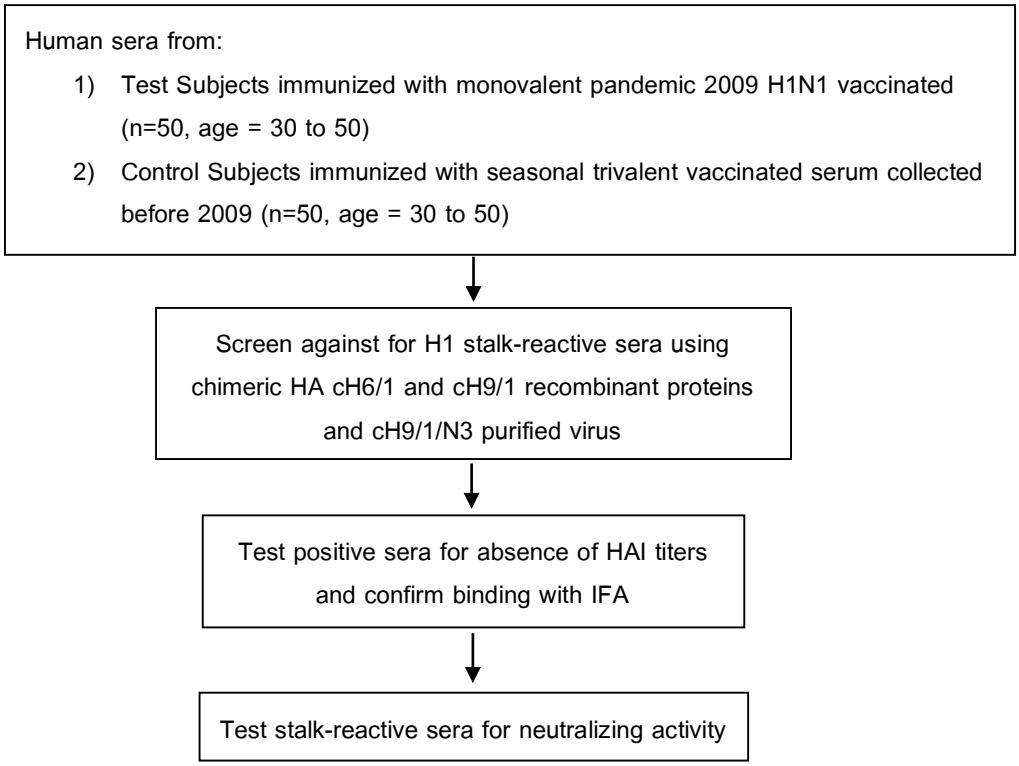
The objectives of this study are to:

1. investigate whether sera samples from subjects who had received the pandemic 2009 H1N1 influenza A virus vaccine display elevated presence of anti-HA stalk antibodies in comparison to the age-matched control group that had been vaccinated with seasonal trivalent influenza vaccine before the occurrence of 2009 H1N1 influenza A virus pandemic.
2. investigate presence of possible stalk-reactive neutralizing antibodies in the sera by hemagglutination inhibition (HI) assay using sera that show ELISA positive binding to the chimeric hemagglutinins.
3. investigate the neutralizing activity of stalk-reactive sera against the virus with recombinant chimeric HA.

Methodology

Scope of research

This study involved the assessment of sera samples from subjects who had received the pandemic 2009 H1N1 influenza A virus vaccine for the elevated presence of anti-HA stalk antibodies in comparison to the control group that had been vaccinated with seasonal trivalent influenza vaccine before the occurrence of 2009 H1N1 influenza A virus pandemic. The study was carried out by first screening the samples against recombinant chimeric HA proteins with HA stalk from the H1N1 influenza A virus, while the globular heads were modified to that of influenza subtypes (H6 and H9) not circulating in human population. Hemagglutination inhibition assay and immunofluorescence assay was used to further assist with the screening. The sera that were found to be reactive against the HA stalk domain were subjected to neutralization assay to test for the neutralizing activity against the virus.



Human serum samples

Human sera will be taken from the archival sera collected during the clinical trial by the Ministry of Public Health in collaboration with Professor Pilaipan Puthavathana. The sera were collected from subjects who had received the pandemic H1N1 vaccine from the end of 2009 to early 2010. The number of the test subject sera will be $n \geq 50$ and age ranging from 30-50 years. The control subjects will be taken from the archival sera collected from healthcare personnel working at Siriraj Hospital who had received seasonal trivalent vaccine before year 2009. The number of the control subject sera will be $n \geq 50$ and age ranging from 30-50 years. The test subject sera will be provided by Professor Pilaipan Puthavathana and the control sera will be obtained from Professor Prasert Auewarakul.

Recombinant influenza virus proteins

Recombinant Influenza virus proteins use in this study

cH6/1 HA	Globular head from A/mallard/Sweden/81/02 Stalk domain of A/Puerto Rico/8/1934
cH9/1 HA	Globular head from A/guinea fowl/Hong Kong/WF10/99 Stalk domain of A/Puerto Rico/8/1934

The recombinant influenza virus HA proteins cH6/1 and cH9/1 used for the screening of sera were provided by Professor Peter Palese. cH6/1 HA contains a globular head from A/mallard/Sweden/81/02, while cH9/1 HA contains a globular head from A/guinea fowl/Hong Kong/WF10/99. The stalk domain for both proteins is from A/Puerto Rico/8/1934. The recombinant proteins were expressed in the baculovirus system. Protein purity and identity was tested by SDS-PAGE, Coomassie staining and Western blot. Protein concentration was determined with Bradford reagent.

Cells

MDCK cells (Madin-Darby Canine Kidney cell line) will be maintained in Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, USA) in the presence of penicillin, gentamycin and fungizone at 37°C under a 5% CO_2 and passages twice weekly.

Viruses

Influenza viruses use in this study

Strain of viruses	Source of viruses
Pandemic H1N1 (2009) A/Nonthaburi/104/2009(H1N1)	Siriraj ICRC [a]
Avian influenza H6N8 viruses: A/Heron/Hong Kong/LC10/2002 H9N2 viruses: A/Chicken/Hong Kong/G9/97	Siriraj ICRC
ChimericH9/1: H9: B A/guinea fowl/Hong Kong/WF10/99 H1 A/Puerto Rico/8/34(PR8) and N3 NA from A/Swine/Missouri/4296424/06 virus	Professor Peter Palese

Virus was propagated on MDCK cells in DMEM containing 1 µg/ml l-1-tosylamide-2-phenylethyl chloromethyl ketone-treated (TPCK)-trypsin (Sigma-Aldrich) and 10-day old embryonated chicken eggs. The chimeric HA cH9/1 N3 reverse-genetic virus was obtained from Professor Peter Palese. The virus was generated using reverse genetics plasmids that encode vRNA and mRNA include the six wild-type viral segments from A/Puerto Rico/8/34 (PR8) for the internal genes, plasmids encoding cH9/1 HA containing a globular head from A/guinea fowl/Hong Kong/WF10/99 and a stalk domain from A/Puerto Rico/8/1934, and N3 NA from A/Swine/Missouri/4296424/06 virus.

Purified preparations of whole virus

Purified viral particles used for the screening of sera were prepared by harvesting allantoic fluid or tissue culture media and spinning at 80,000 x g for two hours over a 20% sucrose cushion. Pelleted viruses were then washed once with 1X phosphate buffered saline (PBS) and spun at 80,000 x g for an hour and reconstituted with 1X PBS and stored at -80ºC until further use.

Enzyme-linked immunosorbent assay (ELISA)

Titers of anti-HA stalk domain antibodies in the sera were determined by enzyme-linked immunosorbent assay (ELISA). 50 µl of purified recombinant chimeric hemagglutinins (cH6/1 or cH9/1 HA) at 2 µg/ml or 5 µg/ml of purified virus were coated overnight onto 96-well plate (Immulon 2; Nunc) at 4ºC. Then plate was washed twice with 0.025% tween/1X PBS and blocked with 5% non-fat milk in 1X PBS for 30 minutes at RT. Serum was diluted serially in 5% non-fat milk and incubated for 1 hour at RT, then washed with PBS-T. Goat anti-human IgG-horseradish peroxidase (HRP) (Meridian Life Science Inc.) secondary antibody was diluted 1:5000 in 5% non-fat milk, 50 µl was added to the wells and incubated for 1 hour at RT, then washed. TMB peroxidase substrate system (KPL, USA) was added into each well and incubated in the dark for 10 min at room temperature. 1 M H2SO4 was added to stop the reaction and the plate was read at OD 450/630 by ELISA plate reader.

Treatment of sera

Before use, sera were treated with 3 volumes of RDE at 37°C overnight and inactivated at 56 °C for 30 min. Samples were allowed to cooled to RT before being adsorbed with red blood cells or further treated with three volumes of 11 mM potassium periodate solution and incubated for 15 min at RT, then three volumes of 1% glycerol saline solution were added to samples and incubate for another 15 min at RT. The treated serum was adsorbed with red blood cells (GRBCs) for 1 hour, mix by inverting every 15 min, before centrifugation and sera collection.

Virus infection

Infection was carried out by inoculating the virus diluted in Minimum Essential Medium (MEM) in the presence of penicillin, gentamycin and fungizone with 2µg/ml TPCK-trypsin onto MDCK cell monolayers and incubated for 1 hour at 37°C under a 5% CO_2 before removing the inoculums and replaced with MEM with 2µg/ml TPCK-trypsin in the presence or absence of additional sialidase/neuraminidase (Sigma).

Viral TCID50 titration

50% tissue culture infectious doses (TCID50) titer was determined using viral nucleoprotein (NP) based ELISA. Virus infection for titration was carried out as previously describes by infecting MDCK cells monolayer with serially diluted virus. After infection, cells will be incubated for 20 h at 37°C, 5% CO_2. The virus titer will be determined using NP ELISA as the end point measurement for virus infection. The viral titer was calculated using Reed and Muench method and presented as 50% tissue culture infectious dose.

NP ELISA was used to measure the level of viral nucleoprotein (NP) expression within the infected cells to indicate the level of infectivity. NP ELISA was carried out by fixing the infected cells with 80% cold acetone for 1 h at 4°C. After washing, the plate was blocked with 3% hydrogen peroxide for 30 min at room temperature, then mouse monoclonal antibody to influenza A nucleoprotein (Chemicon, USA), diluted 1:1000 in blocking buffer (PBS pH 7.2 with 1% BSA and 0.1% Tween-20), was added to each well and allowed to incubate for an hour at 37°C. After the incubation, the plate was washed and the goat anti-mouse antibody conjugated to horseradish peroxidase (Southern Biotech Associates, USA), diluted 1:2000 in blocking buffer, was added and allowed to incubate at 37°C for an hour, then washed. TMB peroxidase substrate system (KPL, USA) was added into each well and incubated in the dark for 10 min at room temperature. 1 M H2SO4 was added to stop the reaction and the plate will be read at OD 450/630 by ELISA plate reader.

Hemagglutination Assay

The hemagglutination assay was used to determine the hemagglutination titers of viruses. Briefly, 50 µl of PBS (pH 7.2) was added in each well of 96 well microplate except the first column. Each culture supernatant was added to the first column 50 µl. Goose red blood cells (GRBCs) control was prepared by adding 50 µl of PBS instead of culture supernatant. Serial two-fold dilutions were made by transferring 50 µl from the first well. Final 50 µl was discarded. GRBCs suspension was added 50 µl to each well on the plate. The plate was incubated at 4°C for 30 min. The GRBCs control was checked for complete settling of RBCs. The hemagglutination titer was read at the highest virus dilution that caused complete hemagglutination.

Hemagglutination inhibition (HAI) assays

HAI assay was used to re-screen the sera after the initial screening with ELISA to confirm that there is no HAI titer, ie. do not bind to the globular head of the virus. 25 µl of 8 HAU/50µl of virus was pre-incubated with 25 µl of serially diluted sera for 1 hour on ice. 0.5% of GRBCs was then added to the virus and sera mixture, gently shaken and incubated on ice for an additional one hour before reading. PBS with virus and no sera was used as a negative control while PBS with no virus and no sera will serve as a background control.

Immunofluorescence

Immunofluorescence was used to visually confirm the binding of antibodies to the stalk of the chimeric HA of the reverse-genetic virus. MDCKs was infected with virus at an MOI of 5 for 12 to 16 hours without the presence of trypsin. Cells were fixed with 80% ice-cold acetone and blocked with 5% non-fat (NF) milk/1X PBS for 30 minutes at RT. Sera were diluted optimally in 5% NF-milk and

incubated onto the cells at 37 °C for 1 hour. The cells were washed three times with 1X PBS and then incubated with anti-human IgG FITC conjugate at a dilution of 1:1000 for 1 hour at RT. Fluorescence reactivity was visualized using fluorescence microscope.

Microneutralization assay

Microneutralization assay was used to test whether the sera which are reactive to the HA stalk domain also have the ability to neutralize the virus. Virus was diluted to 200 TCID50/100µL with 1X minimum essential media (MEM) and then incubated with a serially diluted serum for 1 hour at 37ºC, 5% CO_2. MDCKs in a 96-well plate format was washed with 1X PBS. Serum/virus mixtures were transferred to wells and incubate for 1 hour at 37ºC, 5% CO_2. Cells were washed once with 1X MEM and reincubate with 1X MEM supplemented with trypsin containing equivalent concentrations of diluted serum. At 18 to 22 hpi, cells were fixed and antibody treatments were identical to those used during $TCID_{50}$ determination using nucleoprotein (NP) based ELISA method as previously described. Neutralization titers were defined as the dilution of serum which resulted in at least 50 % inhibition of infectivity.

Plaque reduction neutralization (PRNT) assay

Plaque assay is a quantitative assay used to visualize infection of influenza viruses by plaque apparent. PRNT assay was used to test whether the stalk-reaction sera have the ability to neutralize the virus. Virus was diluted to 200 TCID50/100µL with 1X minimum essential media (MEM) and then incubated with a serially diluted serum for 1 hour at 37ºC, 5% CO_2. MDCKs in a 12-well plate format was washed with 1X PBS. Serum/virus mixtures were transferred to wells and incubate for 1 hour at 37ºC, 5% CO_2; gently shaking plate every 10 minutes to allow even virus adsorption. 2X viral growth media and 1.6% low melting temperature agarose (LMP) were mixed together in a ratio 1:1, then 2 ml of the mixture was overlay onto each well. The plate was incubated for 72 hours at 37°c with 5% CO2. After incubation, plat was fixed with 10% buffered formalin for 1.30 hours. The overlay solution was discarded. The plate was rinsed and cellse were stained 1% crystal violet for 30 minutes at room temperature before rinsing with with tap water and dried. The number of plaques in each culture well were counted and the number of plaques forming unit per ml (PFU/ml) was calculated.

Results

Comparison of the anti HA-stalk activity between 2009 and pre-2009 vaccinated sera against chimeric influenza HA proteins (cH6/1 and cH9/1)

To study the binding activity of HA stalk-reactive antibodies of influenza A virus in sera samples, the ELISA was performed. For the influenza protein cH6/1, 49 monovalent (Figure 1A) and 50 trivalent vaccinated serum (Figure 1B) were positive at dilution 1:100. At dilution 1:200, 42 monovalent (Figure 2A) and 19 trivalent vaccinated serum (Figure 2B) showed positive result. The result of the dilution 1:400 showed 29 positive monovalent (Figure 3A) and 8 weak positive trivalent vaccinated serum (Figure 3B). For the influenza protein cH9/1, 50 monovalent and trivalent were positive at dilution 1:100 (Figure 4A and 4B). At the dilution 1:200, 48 monovalent (Figure 5A) and 20 trivalent vaccinated sera (Figure 5B) showed positive result. The result of the dilution 1:400 showed 32 positive monovalent (Figure 6A) and 4 weak positive trivalent vaccinated sera (Figure 6B).

The data clearly showed that the monovalent vaccinated group had higher frequency and level of anti H1 stalk antibodies as measured by the ELISA. This is in agreement with previously published data and supports the hypothesis that exposure to pandemic H1N1 virus induced antibody to the HA stalk. It was previously shown that antibody to HA stalk was not common in people exposed to seasonal influenza viruses before 2009. We therefore made an assumption that the OD in the trivalent vaccinated group represented background level. With this assumption, the OD at dilution 1:100 showed too high background since most samples in both groups showed positive result, whereas the dilution 1:400 showed much lower positive rate in the monovalent vaccinated group as compared to the OD at dilution 1:200. It was therefore concluded that the dilution 1:200 was suitable for a screening for antibody to HA stalk using this ELISA.

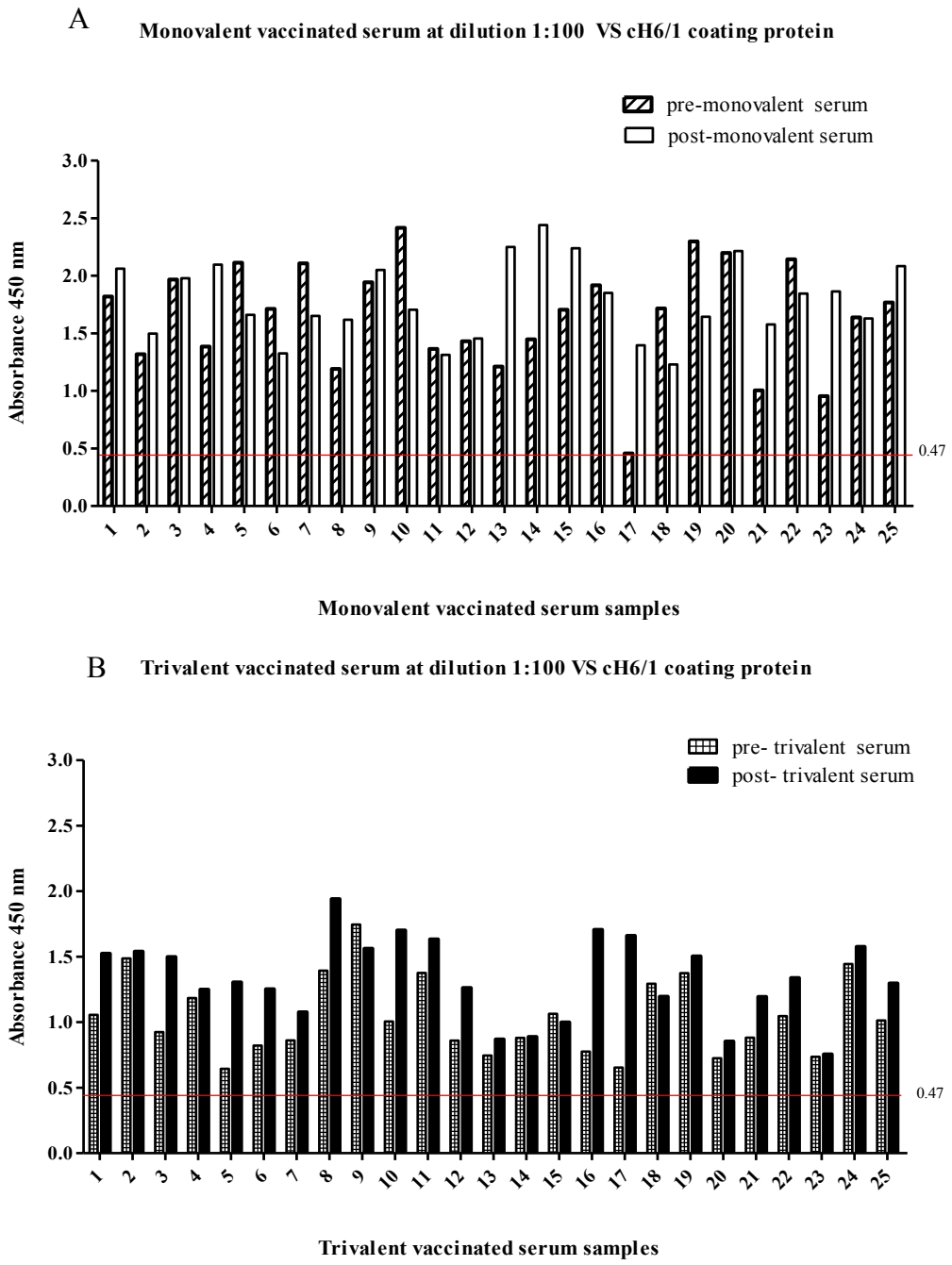


Figure 1 Reactivity of HA-stalk antibody in human sera against chimeric influenza protein (cH6/1) at sera dilution 1:100. Y axis showed number of pair sera (2 bars); two-tone pattern bars were pre-vaccinated sera (D0), light bar and dark bars were Post-vaccinated sera (D30). The cross line represents the cut off; 0.47. Upper graph (A) showed the OD of the monovalent vaccinated serum. Lower graph (B) showed the OD of the trivalent vaccinated serum.

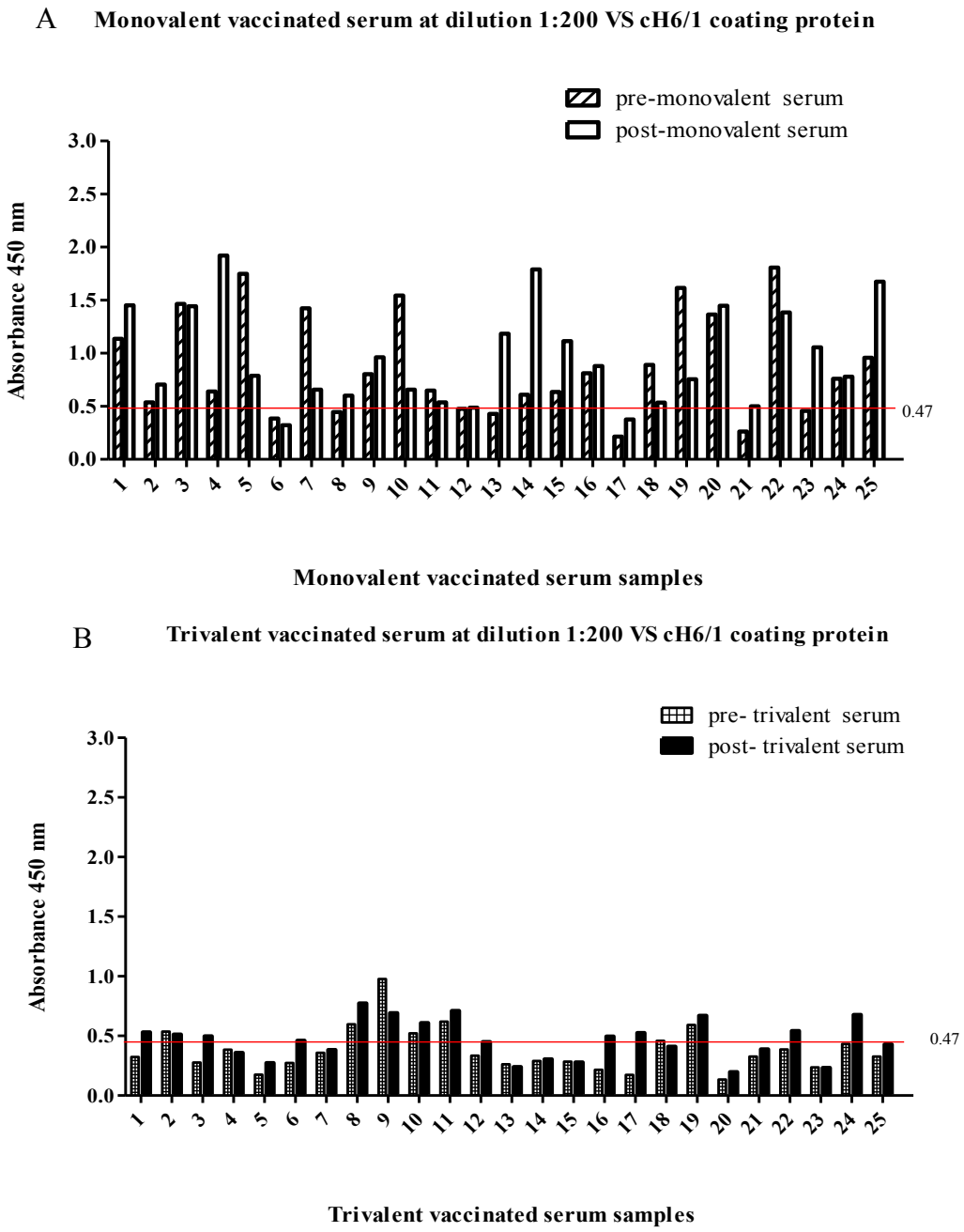


Figure 2 Reactivity of HA-stalk antibody in human sera against chimeric influenza protein (cH6/1) at sera dilution 1:200. Y axis showed number of pair sera (2 bars); two-tone pattern bars were Pre-vaccinated sera (D0), light bar and dark bars were Post-vaccinated sera (D30). The cross line represents the cut off; 0.47. Upper graph (A) showed the OD of the monovalent vaccinated serum. Lower graph (B) showed the OD of the trivalent vaccinated serum.

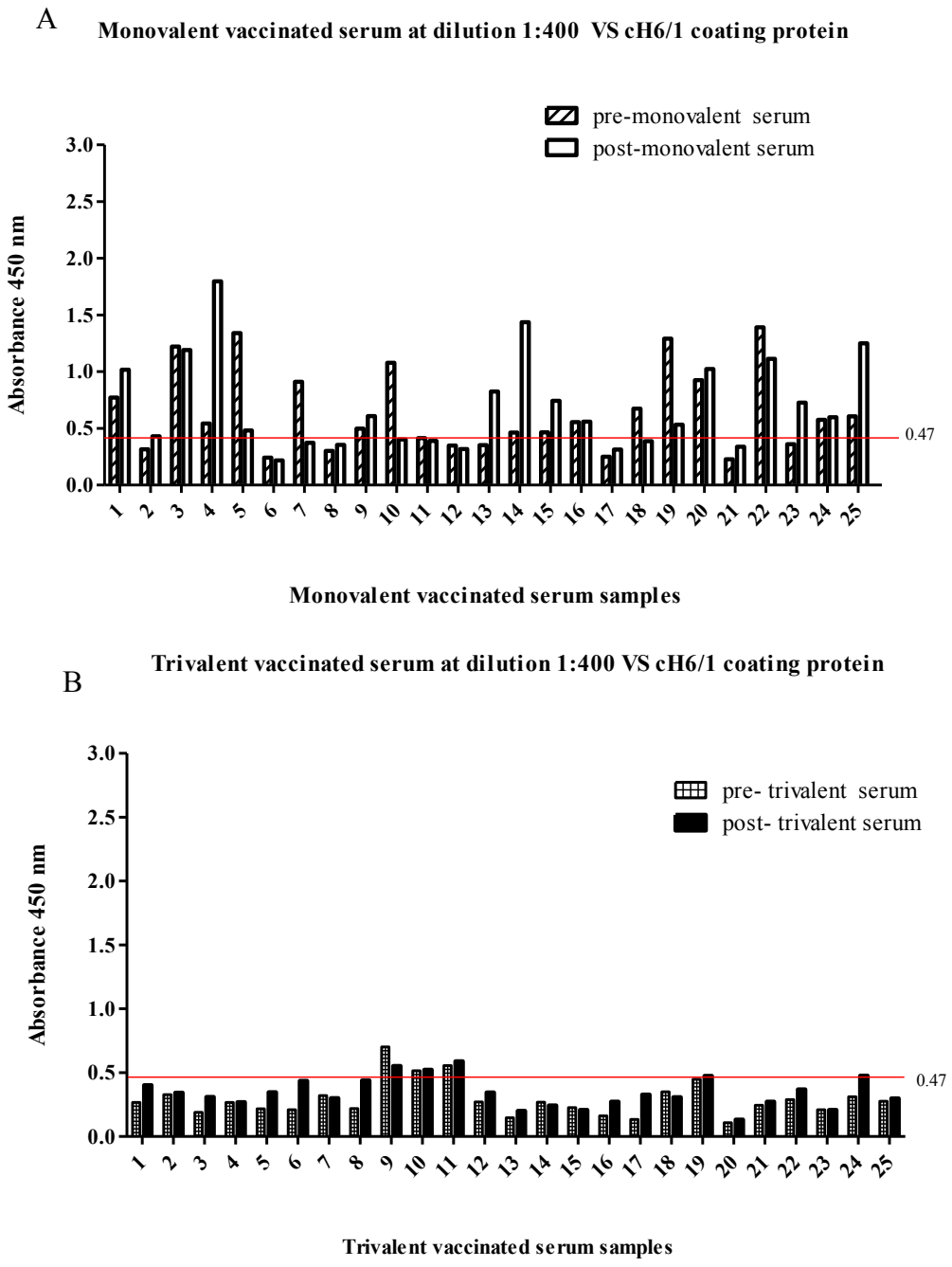


Figure 3 Reactivity of HA-stalk antibody in human sera against chimeric influenza protein (cH6/1) at sera dilution 1:400. Y axis showed number of pair sera (2 bars); two-tone pattern bars were Pre-vaccinated sera (D0), light bar and dark bars were Post-vaccinated sera (D30). The cross line represents the cut off; 0.47.Upper graph (A) showed the OD of the monovalent vaccinated serum. Lower graph (B) showed the OD of the trivalent vaccinated serum.

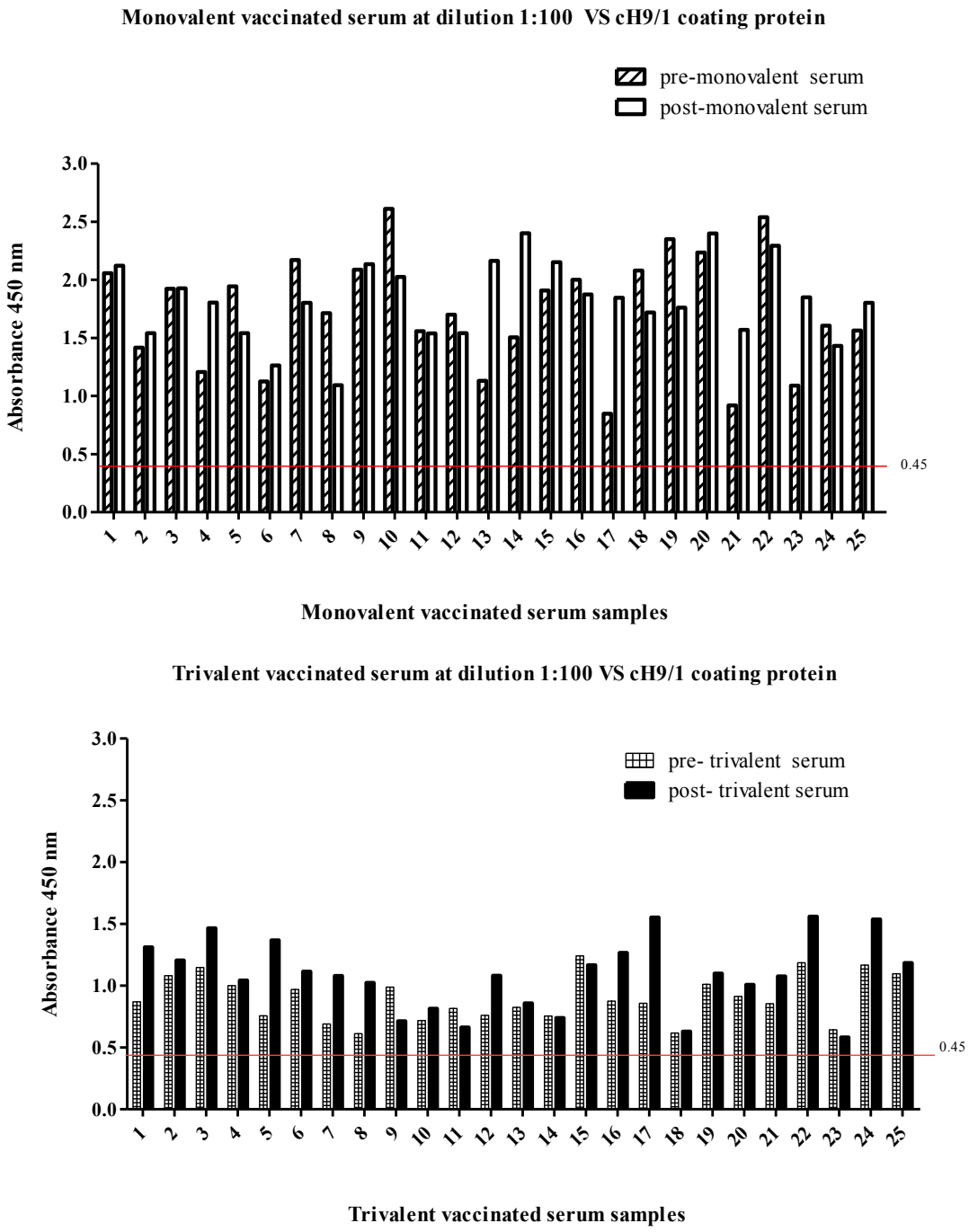


Figure 4 Reactivity of HA-stalk antibody in human sera against chimeric influenza protein (cH9/1) at sera dilution 1:100. Y axis showed number of pair sera (2 bars); two-tone pattern bars were Pre-vaccinated sera (D0), light bar and dark bars were Post-vaccinated sera (D30). The cross line represents the cut off; 0.45. Upper graph (A) showed the OD of the monovalent vaccinated serum. Lower graph (B) showed the OD of the trivalent vaccinated serum.

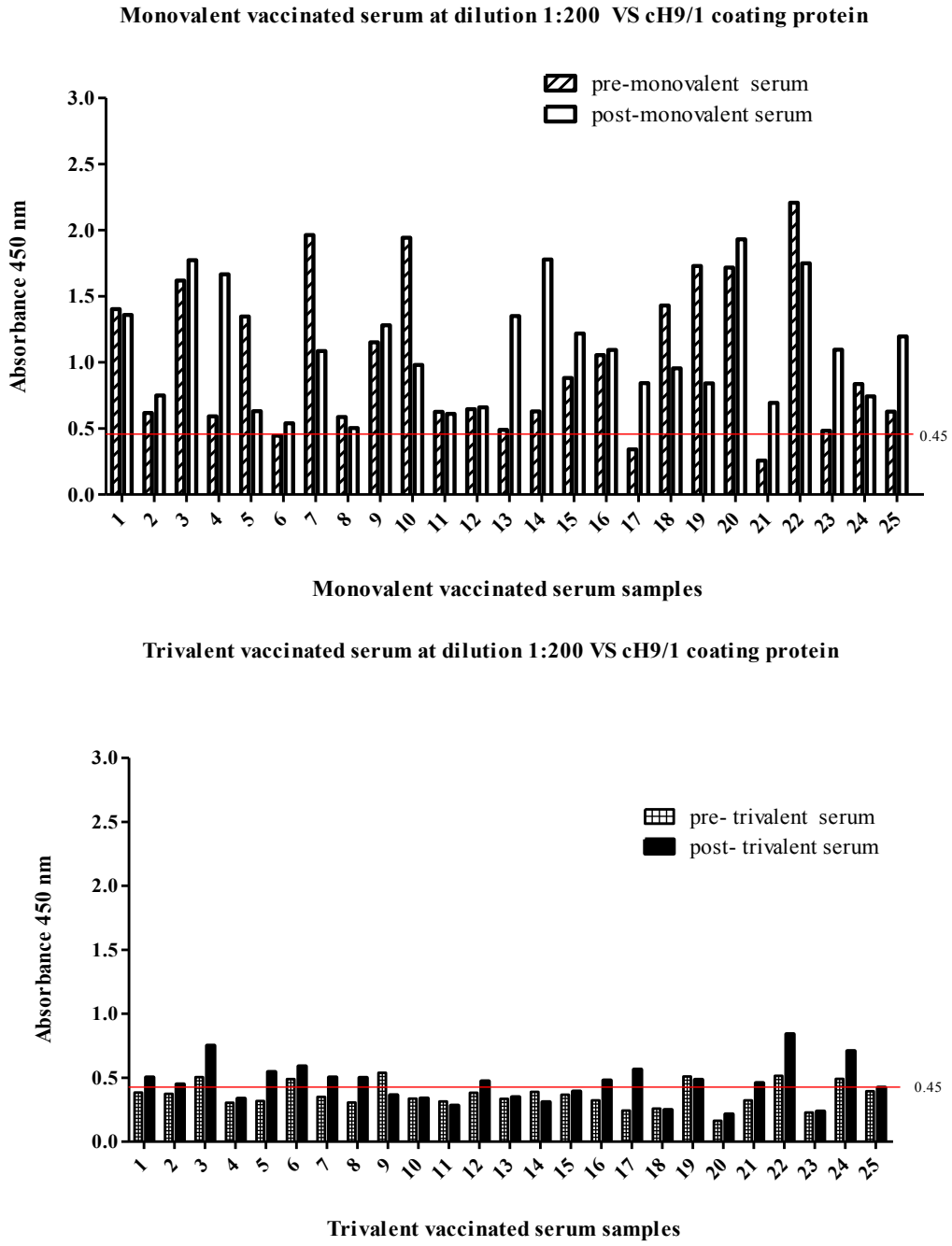


Figure 5 Reactivity of HA-stalk antibody in human sera against chimeric influenza protein (cH9/1) at sera dilution 1:200. Y axis showed number of pair sera (2 bars); two-tone pattern bars were Pre-vaccinated sera (D0), light bar and dark bars were Post-vaccinated sera (D30). The cross line represents the cut off; 0.45. Upper graph (A) showed the OD of the monovalent vaccinated serum. Lower graph (B) showed the OD of the trivalent vaccinated serum.

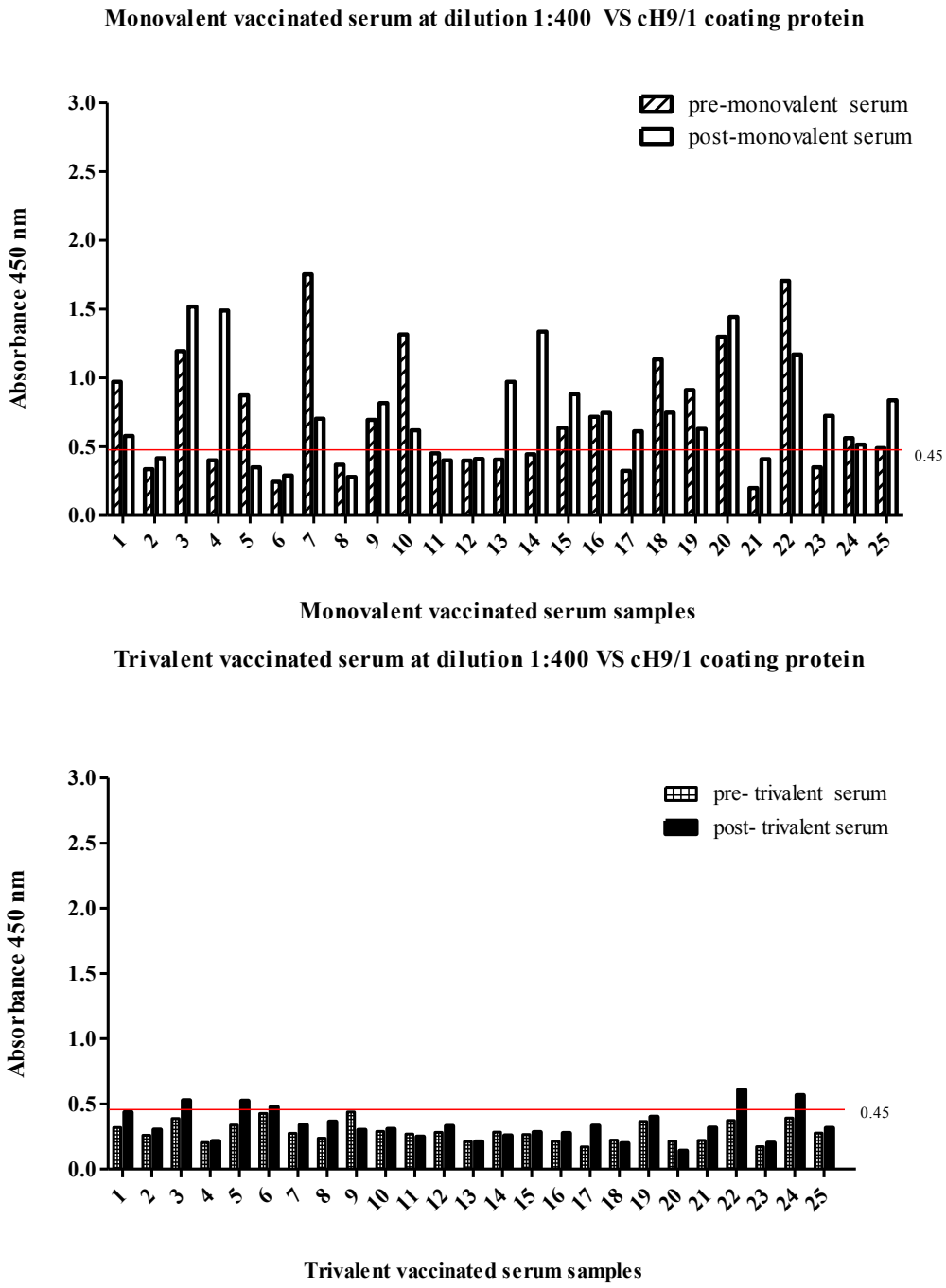


Figure 6 Reactivity of HA-stalk antibody in human sera against chimeric influenza protein (cH9/1) at sera dilution 1:400. Y axis showed number of pair sera (2 bars); two-tone pattern bars were Pre-vaccinated sera (D0), light bar and dark bars were Post-vaccinated sera (D30). The cross line represents the cut off; 0.45. Upper graph (A) showed the OD of the monovalent vaccinated serum. Lower graph (B) showed the OD of the trivalent vaccinated serum

The seroconversion rate after vaccination with monovalent (pandemic) vaccine by ELISA assay

Monovalent vaccinated serum pairs showed some seroconversion against the CH6/1 and cH9/1 proteins. Seroconversion can be defined either by having the OD value changing from negative in pre-vaccinated to positive in post-vaccinated sera at a fixed dilution or by having significant increase in end-point titer (>4 folds) in post-vaccinated sera as compared to pre-vaccinated sera. At dilution 1:200, 1 and 4 pairs of monovalent and trivalent vaccinated sera, respectively, showed seroconversion (Fig. 2A and B). The low seroconversion rate in monovalent vaccinated sera was due to the high positive rate in pre-vaccinated sera. For influenza protein cH9/1, 3 and 8 pairs of monovalent and trivalent vaccinated sera, respectively showed seroconversion (Fig. 3A and B).

The seroconversion rate of both inactivated vaccinated sera using endpoint titer is shown in Table 1 to 4. There was seroconversion against influenza protein cH6/1 and cH9/1 in 8 and 11 pairs of monovalent vaccinated sera, respectively, as shown in Table 1 and 2, respectively. And there was seroconversion against influenza protein cH6/1 and cH9/1 in 3 and 7 pairs of trivalent vaccinated sera, respectively, as showed in Table 3 and 4, respectively. The monovalent vaccinated sera showed Geometric Mean Titer (GMT) of pre-vaccinated at 622 and 692, while post vaccinated had 676 and 1054 for cH6/1 and cH9/1, respectively. On the other hand, the trivalent vaccinated sera showed Geometric Mean Titer (GMT) of pre-vaccinated at 143 and 127, while post vaccinated had 127 and 194 for cH6/1 and cH9/1, respectively. The binding ability between pre-monovalent and pre-trivalent vaccinated sera against cH6/1 and cH9/1 showed significant difference at $p<0.05$, while only post-monovalent and post-trivalent vaccinated sera against cH9/1 showed significant difference at $p<0.05$ (Table 5). The seroconversion of monovalent vaccinated sera was present at higher percentage compared to trivalent vaccinated sera (Table 6).

Table 1 End–point dilution of ELISA of the monovalent vaccinated sera against to influenza protein cH6/1.

	Serum Dilution	
number of serum	Monovalent D0	Monovalent D30
1	1600	1600
2	400	400
3	6400	6400
4	12500	12500
5	1600	800
6	200	200
7	1600	800
8	800	800
9	400	800
10	1600	200
11	400	400
12	200	200
13	100	800
14	200	1600
15	200	800
16	400	400
17	100	400
18	6400	1600
19	800	200
20	400	800
21	100	100.
22	12500	1600
23	100	400
24	200	100
25	800	3200

The end-point dilution showed the seroconversion rate between pre- and post- of monovalent vaccinated sera. 8 pair of monovalent vaccinated sera showed the seroconversion rate as represent with shading.

Table 2 End–point dilution of ELISA of the monovalent vaccinated sera against to influenza protein cH9/1.

	Serum Dilution	
number of serum	Monovalent D0	Monovalent D30
1	400	1600
2	200	400
3	3200	6400
4	400	6400
5	1600	400
6	400	400
7	3200	400
8	400	400
9	400	400
10	1600	200
11	100	200
12	100	200
13	400	3200
14	400	3200
15	800	3200
16	1600	3200
17	400	3200
18	12500	12500
19	1600	800
20	12500	3200
21	100	400
22	6400	3200
23	100	400
24	400	100
25	400	3200

The end-point dilution showed the seroconversion rate between pre- and post- monovalent vaccinated sera. 11 pair of monovalent vaccinated sera showed the seroconversion rate as represent with shading.

Table 3 End–point dilution of ELISA of the trivalent vaccinated sera against to influenza protein cH6/1.

	Serum Dilution	
number of serum	Trivalent D0	Trivalent D30
1	100	100
2	200	100
3	100	200
4	100	100
5	400	400
6	100	400
7	100	200
8	200	200
9	400	400
10	100	100
11	100	100
12	200	200
13	200	400
14	100	100
15	100	100
16	200	800
17	200	3200
18	100	100
19	200	200
20	100	200
21	100	200
22	100	100
23	100	100
24	100	100
25	400	3200

The end-point dilution showed the seroconversion rate between pre- and post- trivalent vaccinated sera. 4 pair of trivalent vaccinated sera showed the seroconversion rate as represent with shading.

Table 4 End–point dilution of ELISA of the trivalent vaccinated sera against to influenza protein cH9/1.

	Serum Dilution	
number of serum	Trivalent D0	Trivalent D30
1	200	100
2	100	100
3	100	400
4	100	200
5	100	100
6	100	200
7	800	1600
8	100	100
9	200	100
10	100	100
11	100	100
12	200	1600
13	100	400
14	100	100
15	100	100
16	100	400
17	100	800
18	100	100
19	200	200
20	100	100
21	100	100
22	100	200
23	100	100
24	100	100
25	400	800

The end-point dilution showed the seroconversion rate between pre- and post- trivalent vaccinated sera. 7 pair of trivalent vaccinated sera showed the seroconversion rate as represent with shading.

Table 5 The end–point dilution of the monovalent and trivalent vaccinated tested with the chimeric influenza protein (cH6/1 and cH9/1) in ELISA

Serum samples	NO. of serum samples	GMT	T-Test
Chimeric influenza protein cH6/1			
Monovalent (D0)	25	622	<0.05
Trivalent(D0)	25	143	
Monovalent(D30)	25	676	>0.05
Trivalent(D30)	25	217	
Chimeric influenza protein cH9/1			
Monovalent (D0)	25	695	<0.05
Trivalent(D0)	25	128	
Monovalent(D30)	25	1054	<0.05
Trivalent(D30)	25	194	

GMT = Geometric Meant Titer, t-test; two-tail, un pair t-test <0.05 = significant

D0 =pre-vaccinated sera, D30 = post-vaccinated sera

Table 6 The percentage of seroconversion of the monovalent and trivalent vaccinated tested with the chimeric influenza protein (cH6/1 and cH9/1) in ELISA

Serum samples	Chimeric influenza proteins No. with≥4 folded [a] rise in end-point titer (%)	
	cH6/1	**cH9/1**
Monovalent	8 (16)	11 (22)
Trivalent	4 (8)	7 (14)

[a] Seroconversion with the convalescent vaccination antibody titer≥40 to the chimeric influenza virus.

Haemagglutinin Inhibition assay titers

All vaccinated sera were screened for stalk-reactivity against chimeric influenza protein (cH6/1 and cH9/1) in ELISA assay. These chimeric influenza proteins contained globular head from non-human influenza subtype of H6 and H9 while stalk domain remain from H1 influenza subtype. The principle of HI assay involves binding of globular HA domain to sialic acid receptor on cell surface. This assay was performed to exclude the anti-chimeric HA-globular head antibody which may interfere with the neutralization assay against chimeric virus. Influenza A viruses H1N1, H6N8, H9N2 and cH9/1 were used in this studied. H1N1 virus is the strains which circulate among human population, while the H6N8 and H9N2 viruses were non-human and isolated from avian species, and were used as HI controls. Therefore, the monovalent vaccinated sera should show positive HI titer only with influenza H1N1 wild type and remain negative for the avian influenza and chimeric influenza cH9 viruses, while the trivalent vaccinated sera should show negative HI titer for all influenza viruses tested in this study. As predicted, The HI results are negative for chimeric influenza virus cH9/1 and avian influenza viruses; H6N8 and H9N2 while having HI titer of 1:400-1:800 for H1N1 virus in the monovalent vaccinated sera and all negative HI titer for the trivalent vaccinated sera (Figure 7).

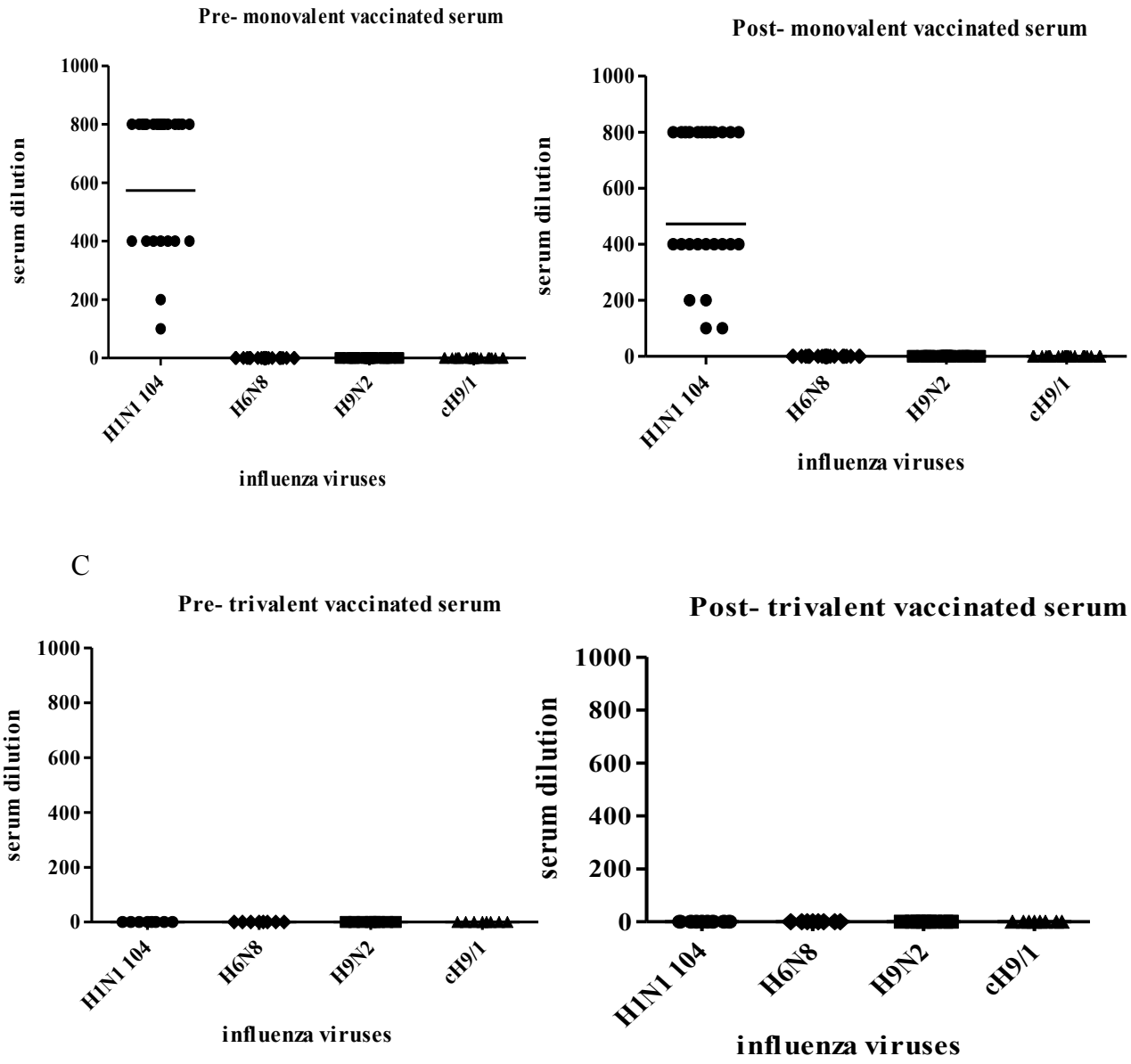


Figure 7 Hemagglutination inhibition assay. X axis represent influenza virus strains, Y axis shows HI titers. A: the result showed HI titer of pre-monovalent vaccinated sera and B: the result showed HI titer of post- monovalent vaccinated sera. C the result showed HI titer of pre- trivalent vaccinated sera and D: the result showed HI titer of post- trivalent vaccinated sera.

Plaque reduction neutralization test (PRNT)

The neutralization of influenza viruses was determined using plaque reduction neutralization test (PRNT). This method was performed against chimeric influenza virus (cH9/1) to study the neutralization activity of HA-stalk antibody in human serum. Seasonal influenza virus (H1N1) and avian influenza virus (H9N2) were used as a positive and negative control respectively. Each influenza control plaques showed 60-100 PFU (Figure 8). The trivalent vaccinated sera against chimeric cH9/1 virus showed fewer neutralization inhibition than the monovalent vaccinated sera with 50% inhibition in 4 of 50 trivalent and 11 of 50 in monovalent vaccinated sera at the dilution 1:40 (Figure 9 and 10). In monovalent vaccinated sera, the 50% inhibition tigers were 1:160, 1:80 and 1:40 in 4, 4 and 3 samples respectively. The comparison of neutralizing ability between 2 groups of vaccinated sera showed similar results with almost all samples showed at least 10% inhibition. However, none of the pre-trivalent vaccinated sera and only 4 of the post-trivalent vaccinated sera showed 50% inhibition level at dilution 1:40. In contrast, 7 of the pre-monovalent vaccinated sera and 4 of the post-monovalent vaccinated sera showed 50% inhibition level at dilution 1:40.

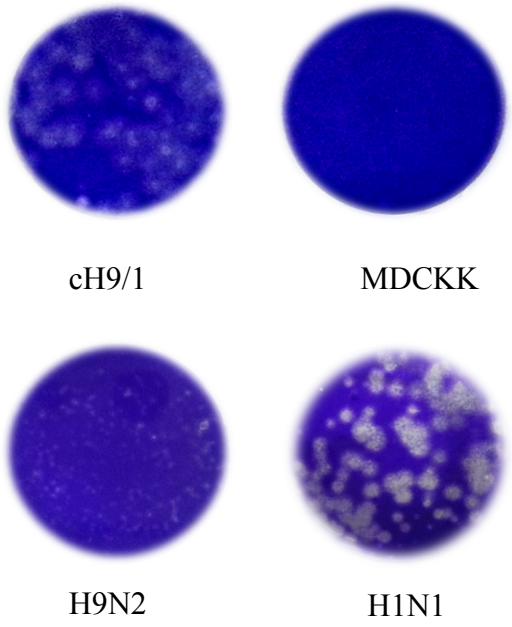


Figure 8 Plaque morphology influenza virus of cH9/1, H9N2 and cell control (MDCK) under 0.8% low melting agarose overlay medium

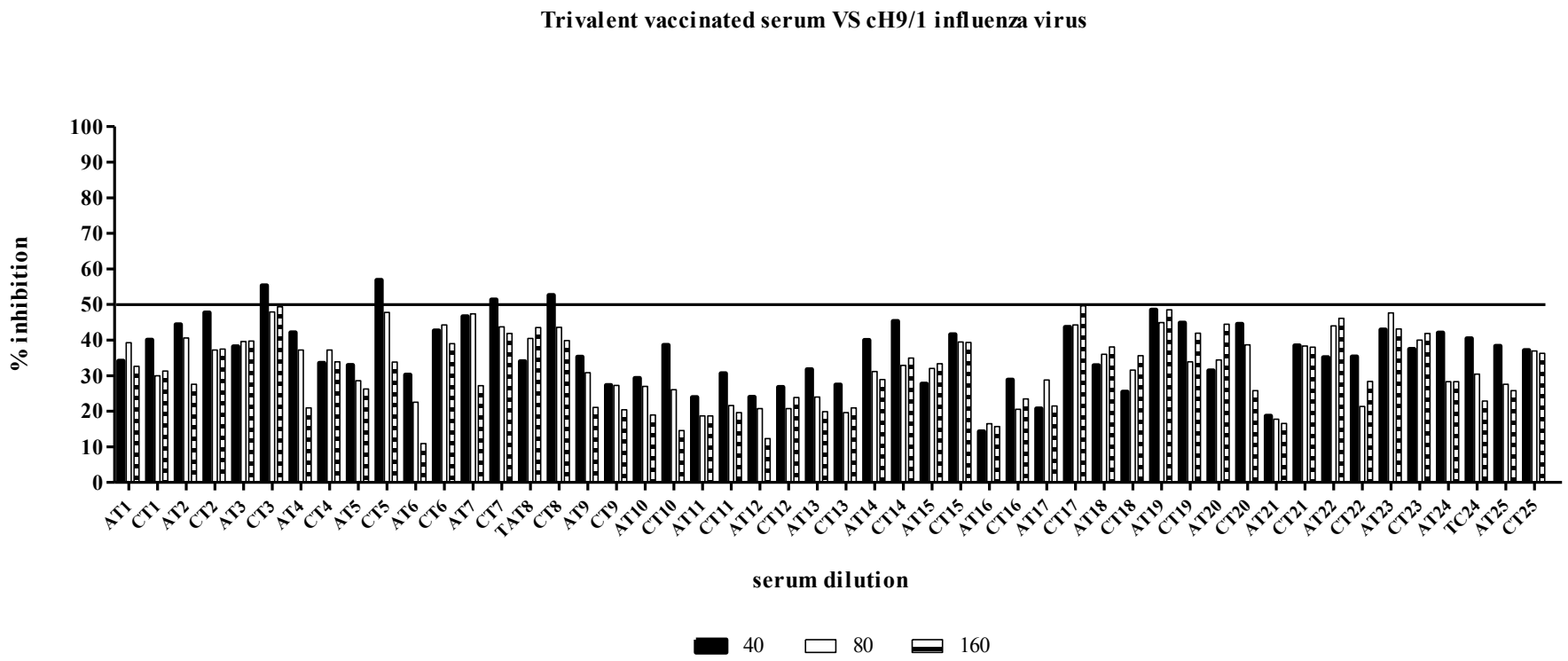


Figure 9 The neutralizing antibody to chimeric influenza virus cH9/1. X axis represent serum dilution. Y axis represents % inhibition.

The cross line represents 50% inhibition. The result showed the percentage of neutralizing inhibition of trivalent vaccinated sera

AT = pre-trivalent vaccinated sera

CT = post-trivalent vaccinated sera

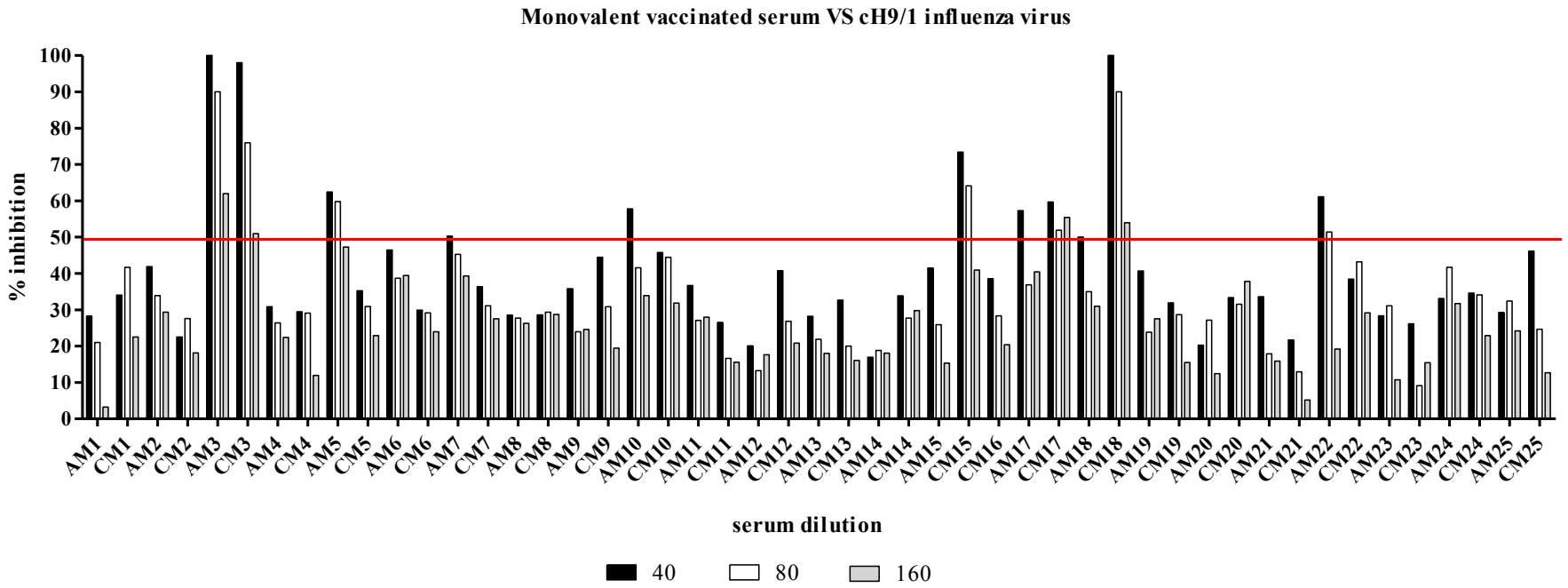


Figure 10 The neutralizing antibody to chimeric influenza virus cH9/1. X axis represent serum dilution. Y axis represents % inhibition.

The cross line represents 50% inhibition. The result showed the percentage of neutralizing inhibition of monovalent vaccinated sera.

AM = pre-monovalent vaccinated sera

CM = post-monovalent vaccinated sera

Discussion

Human and animal serum contains various non-specific inhibitors against influenza virus. Non-specific inhibitors in serum can interfere with experimental results. Therefore, it is important to remove these non-specific inhibitors before carrying out any serological diagnosis of influenza A virus (24, 25). There are three classes of inhibitors present in sera; alpha, beta and gamma (26-28). Gamma inhibitor is heat stable, mostly found in horse serum while beta inhibitor is present in guinea pig, mice and human sera. In human serum, the beta inhibitor belongs to mannose binding lectin protein (MBP). This beta inhibitor is heat labile hence can be destroyed by heat treatment at 56°C. Potassium periodate (KIO_4) can inactivate inhibitors from mucoprotein and therefore used for treatment of human serum before detecting antibody to influenza virus (29). There are some sugar specific inhibitors in mouse and guinea pig serum that are close to human MBP (30). Those non-specific inhibitors in both animal and human serum can also be inactivated by KIO_4 (31). Many researchers previously treated human serum with KIO_4 reagent (24). But more recently, most studies only treated serum with only heat or heat-RDE. However, we tried the inactivation condition for these sera based on the previous study that treated vaccinated sera with KIO_4. Therefore, we compared the inactivation method; standard heat-RDE method and the method with the additional KIO_4 treatment. The result showed no difference between the vaccinated sera treated with KIO_4 and heat-RDE using ELISA and HI assay but KIO_4 showed cell toxicity in microneutralization assay. Thus, in our study, the vaccinated sera treated with KIO_4 were used only in ELISA and HI assay.

In a previous study, anti HA-stalk antibody could be induced by A/California/04/2009 (Cal/09) and A/New Jersey/1976 (NJ/76) vaccine. The H1N1 1976 swine flu or A/New Jersey/76 (Hsw1n1) was an influenza virus that caused outbreak at Fort Dix, New Jersey in 1976. NJ/76 vaccine was quickly produced and widely administered in the USA. However, the outbreak did not spread beyond Fort Dix and the pandemic did not happen (32). A/California/04/2009 (Cal/09) contain HA segment that closely relate to A/New Jersey/1976 (NJ/76) and 1918, Spanish Flu. Both NJ/76 and Cal/09 vaccinated sera result showed the elevated of endpoint IgG titer of anti HA–stalk against chimeric influenza protein cH6/1 (globular HA domain from H6 and stalk domain from the HA of PR8). The NJ/76 and monovalent Cal/09-like vaccine showed similar activation of anti HA-stalk, even though these vaccines contained different HA globular head (24). It is believed that this activation was cause by anamnestic response. Effective vaccinations against influenza viruses rely on the induction of antibody responses and the generation of memory B cell that will provide specific protection against influenza infection. Memory B cell are usually produced following infection or vaccination, which will readily proliferate and differentiate into antibody-secreting plasma cell, known as anamnestic response (33), upon re-encountering with the specific antigen. The immunodominant site of influenza virus is located on the globular head of HA protein. The globular head region is highly variable and prone to changing. This enables the virus to efficiently escape neutralization by the pre-existing antibodies. In contrast, the stalk domain of HA protein is more conserved but less immunogenic, therefore the immune response towards this region is usually masked by the anti-globular head response. It was previously shown that anti-stalk antibody can broadly neutralized various strains of influenza virus. Anti-stalk antibody can be elevated through anamnestic response by infection or vaccination with

influenza strains containing HA protein with globular heads that differ substantially from seasonal influenza virus while the stalk domain remains similar (24).

This study aimed to examine the anti HA-stalk in 2009 H1N1 (monovalent) influenza vaccinated sera in comparison to vaccinated sera before 2009 (trivalent). The monovalent vaccinated sera in this study were collected during the clinical trial by the Ministry of Public Health. These sera were collected from people who had received the pandemic H1N1 (pH1N1) vaccine strain (A/California/7/2009(H1N1)) from 2009 to 2010. The entire volunteers were healthcare workers from hospitals in Thailand. The trivalent vaccinated sera were collected before year 2009 from Thai volunteer. Both vaccinated sera were tested for HA stalk binding using ELISA. All monovalent vaccinated sera showed anti HA-stalk binding to both cH6/1 and cH9/1 influenza proteins. The chimeric influenza protein was constructed by engineering method that globular HA domain encode with H6 or H9 and stalk domain from the HA of PR8 (H1) (34). Even though the chimeric influenza protein contained different HA globular head, they contained the same stalk from PR8. Thus, the positive titers of monovalent vaccinated sera were anti HA-stalk as expected. Previous studies showed that of the 2009 pandemic virus could boost antibody specific to conserved regions of HA stalk (24, 35). However, some pre-vaccinated monovalent sera were found to have higher anti HA-stalk antibody than post-vaccinated sera. As previously mentioned, the monovalent vaccinated sera were collected from healthcare worker in hospital. Those volunteers had high risk of exposure to pH1N1 before vaccination. Some volunteers may have natural infection and developed anti HA-stalk Abs before the vaccination. A previous study in Thai population showed that after the first wave of outbreaks in 2009, around 48% of the population had been already exposed to the pandemic virus. (36). In this study, the monovalent vaccinated sera were collected after the first wave of the pandemic in 2009. The very high prevalence of anti-HA stalk in the pre-vaccinated sera in this group was likely due to the fact that the subjects were healthcare workers and therefore highly exposed to the pandemic virus. On the other hand, trivalent vaccinated sera were collected before 2009. Therefore, the trivalent vaccine receivers had never been exposed to pH1N1. The trivalent vaccinated sera showed little binding to both cH6/1 and cH9/1 chimeric influenza proteins. The lack of binding antibody to globular head of H6 and H9 was confirmed by negative hemagglutination inhibition test with wild type avian influenza viruses.

HA-stalk antibody titer was confirmed using Hemagglutination inhibition (HI) assay. In this study, positive ELISA results against cH9/1 and cH6/1 chimeric protein and negative HI result against cH9/1 influenza virus would indicates presence of anti-stalk antibody. In general, the HI was performed to determine antibodies titers in serum that can inhibit erythrocyte agglutination by virus (37). Vaccine usually induce antibodies that are strain-specific and target the HA globular head. Therefore, biding of antibodies to this region will block sialic acid binding site and hence prevent erythrocyte agglutination. The HI negative result showed agglutination of red blood cells whereas HI positive showed non-agglutinated pattern as a button shape. HI assay was a traditional method to test protective antibody after vaccination (38). Monovalent and trivalent sera were tested against wild type H1N1 influenza virus, avian influenza virus and chimeric influenza virus H9 N3 (NA from A/Swine/Missouri/4296424/06 virus) (39). All sera were treated with KIO_4 reagent before performing HI assay (26). The monovalent vaccinated sera showed HI positive titer (≥ 400) against H1N1 (pH1N1) influenza virus, whereas the trivalent vaccinated showed HI negative titer. The antibodies of monovalent vaccinated sera were reactive to globular head of H1N1 influenza virus. However, the trivalent vaccinated sera that were

vaccinated before 2009 cannot react to the HA globular head of pH1N1. H6N8 and H9N2 are Low pathology avian influenza (LPAI) that do not circulate in human population. Therefore, both monovalent and trivalent sera showed negative HI titer because they did not contain antibody against the globular head of H6 and H9. Both monovalent and trivalent vaccinated sera showed negative HI titer against cH9/1. The results were as predicted and confirm that positive titer from ELISA was HA-stalk antibody. This is because the sera could not inhibit binding of chimeric influenza virus that contain a different HA globular head from the vaccine strain to red blood cells (40-41).

PRNT assay is based on inhibition of viral infection in cell culture and inhibition of plaque formation. In a previous study, a monoclonal antibody 6F12 (mAb6F12) was used as a positive control for anti HA-stalk. They compared the concentrations of mAb 6F12 and purified human IgG from pooled samples of infected sera that yield similar neutralizing activity against cHA virus. The result showed that about 7% of total IgG were specific to HA-stalk (34). In this study, PRNT was performed to determine neutralization capability of monovalent and trivalent vaccinated sera. The vaccinated sera that showed positive ELISA test and negative HI titer were used in this assay. cH9/1 N3 influenza virus was used to test for the neutralizing capability of both monovalent and trivalent sera. The result showed that some monovalent vaccinated sera had neutralizing activity, while the trivalent sera did not show any neutralization capability against cH9/1 N3 virus. The positive neutralization capability was confirmed with H9N2 (avian influenza virus). The result showed no neutralizing Abs ability to H9N2 because the H9N2 contained different HA stalk from human influenza virus. The serum samples from healthcare workers who received monovalent pH1N1 were investigated for the presence of binding HA-stalk antibodies. From ELISA test, 48 (95%) of monovalent vaccinated sera showed binding positive titer to two chimeric influenza protein cH6/1 and cH9/1 but only 11 (22%) showed neutralization capability. The low neutralizing activity of anti-stalk antibodies is in concordance with previous studies (34). This indicated that neutralizing HA antibodies are not common. However, anti HA-stalk antibodies can inhibit influenza virus via different mechanisms. It can block at the entry site of proteases and prevent cleavage HA0 into the HA1 and HA2. This prevents HA from changing the conformation necessary for fusion to cell membrane and viral entry. Anti HA-stalk can keep the HA on the cell surface and inhibit progeny budding from host cell (42-43). In vivo, interestingly, anti HA-stalk can induce antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity against infected cells (43-44).

In conclusion, this study successfully found HA-stalk Abs binding activity in monovalent vaccinated sera from healthcare workers in Thailand. However, neutralizing anti HA-stalk antibody was not detected in most of the vaccine receivers. Nevertheless, the anti HA-stalk non-neutralizing antibodies induced by the vaccine might play role in preventing infection via other mechanisms.

Suggestions for Future Research

This study shows comparisons between anti-stalk antibodies levels in pre-2009 and 2009 vaccinated sera, and their neutralization capabilities. The results showed that 2009 monovalent vaccine can boost anti-stalk antibodies more efficiently than the pre-2009 trivalent vaccine. Although the 2009 vaccinated sera show high level of HA-stalk antibodies in ELISA, only 22% of the stalk reactive sera showed neutralization ability. This suggests that the anti HA-stalk antibodies that were boosted by monovalent vaccine are mostly non-neutralizing. However, these binding antibodies may have the ability to prevent influenza virus infection via other mechanisms. The role of anti HA-stalk antibody in preventing influenza infection needs further studies.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ
   ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติจำนวน 3 เรื่อง

- Sirinonthanawech N, Surichan S, Namsai A, Puthavathana P, Auewarakul P, **Kongchanagul A***. Titration of individual strains in trivalent live-attenuated influenza vaccine without neutralization. J Virol Methods. 2016 Nov;237:154-158. doi: 10.1016/j.jviromet.2016.09.001. Epub 2016 Sep 3. PMID: 27596269
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- **Kongchanagul A**, Samnuan K, Wirachwong P, Surichan S, Puthavathana P, Pitisuttithum P, Boonnak K*.

2. การนำผลงานวิจัยไปใช้ประโยชน์
   เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

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- Tayong K, Auewarakul P, Puthavathana P, **Kongchanagul A*.** The effects of additional potassium periodate treatment in combination with the standard heat-RDE inactivation of post influenza vaccination sera using ELISA, HI and microneutralization assay. The 40th Congress on Science and Technology of Thailand: “Science and Technology towards ASEAN Development”. 2014 Dec 2 - 4, Hotel Pullman Khon Kaen Raja Orchid, Khon Kaen, Thailand.

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Titration of individual strains in trivalent live-attenuated influenza vaccine without neutralization

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Formulation and quality control of trivalent live-attenuated influenza vaccine requires titration of infectivity of individual strains in the trivalent mix. This is usually performed by selective neutralization of two of the three strains and titration of the un-neutralized strain in cell culture or embryonated eggs. This procedure requires standard sera with high neutralizing titer against each of the three strains. Obtaining standard sera, which can specifically neutralize only the corresponding strain of influenza viruses and is able to completely neutralize high concentration of virus in the vaccine samples, can be a problem for many vaccine manufacturers as vaccine stocks usually have very high viral titers and complete neutralization may not be obtained. Here an alternative approach for titration of individual strain in trivalent vaccine without the selective neutralization is presented. This was done by detecting individual strains with specific antibodies in an end-point titration of a trivalent vaccine in cell culture. Similar titers were observed in monovalent and trivalent vaccines for influenza A H3N2 and influenza B strains, whereas the influenza A H1N1 strain did not grow well in cell culture. Viral interference among the vaccine strains was not observed. Therefore, providing that vaccine strains grow well in cell culture, this assay can reliably determine the potency of individual strains in trivalent live-attenuated influenza vaccines.

1. Introduction

Live-attenuated influenza vaccine (LAIV) is being widely used in many countries (Wareing and Tannock, 2001; Jin and Subbarao, 2015). The manufacturing process is less complicated and the yield per embryonated egg is much higher than inactivated vaccines (Rudenko and Isakova-Sivak, 2015). This is an attractive option for vaccine production in a pandemic, when the number of embryonated eggs is the main limiting factor for total production capacity (Luke and Subbarao, 2006; Rudenko and Isakova-Sivak, 2015). The World Health Organization has been supporting establishment of LAIV production capacity in developing countries using the A/Leningrad/134/17/57 master donor strain as part of the pandemic preparedness plan (Friede, 2011; Rudenko et al., 2011; Surichan et al., 2011).

The production of LAIV involves growth of individual vaccine strains in embryonated eggs, partial purification of the viruses from allantoic fluid, titration of monovalent concentrated bulks and formulation into trivalent vaccine by mixing the separate strains to obtain the desired amount of infectious virus (WHO, 2013). The trivalent vaccine preparation needs to be checked for the infectivity of each strain. The titration of individual strains in the trivalent vaccine preparation is also needed for evaluating stability of the vaccine preparation after specified storage conditions.

Titration of individual strains in trivalent LAIV is usually performed by selective neutralization of two of the three strains by standard sera and end-point titration of the un-neutralized strain in embryonated eggs as EID_{50} (50% egg infectious dose) or alternatively in cell culture as $TCID_{50}$ (50% tissue culture infectious dose). This procedure requires specific sera with very high neutralizing titer against each of the vaccine strains (Yeolekar and Dhere, 2012). The standard sera are produced by immunizing specific pathogen free animals with purified hemagglutinin of the respective vaccine strains (WHO, 2013). This can be time-consuming and presents an important obstacle for vaccine production in particu-

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lar with the rapid spread of a new strain. To provide an alternative assay without the requirement of standard neutralizing sera, a new approach using subtype-specific antibodies to detect viruses in cell cultures simultaneously infected with the trivalent vaccine strains was developed.

2. Materials and methods

2.1. Monovalent and trivalent LAIV preparation

Influenza type A (H1N1 and H3N2) and type B live-attenuated reassortant vaccine strains, designated A/17/California/2009/38, A/17/Perth/09/87 and B/60/Brisbane/08/83, respectively, were obtained from the Institute of Experiment Medicine (IEM), Russian Academy of Medical Sciences, St. Petersburg, Russia. The master donor strains were used to provide six internal genes for the vaccine strains. A/Leningrad/134/17/57 (H2N2) was used for the generation of the two Influenza A H1N1 and H3N2 vaccine strains and a clone of B/USSR/60/69 for the Influenza B vaccine strain. The hemagglutinin and neuraminidase genes were from wild-type antigenic strains. Each monovalent vaccine is antigenically similar to A/California/07/09 (H1N1), A/Perth/16/08 (H3N2) and B/Brisbane/06/08. The trivalent vaccine was prepared as a mixture of the three monovalent vaccine strains.

2.2. Infectivity titration by EID_{50} assay

50% Egg Infectious Dose (EID_{50}) assay was used to determine the infectivity titers of vaccine strains in allantoic fluids of embryonated chicken eggs. Each monovalent vaccine was serially diluted ten-fold. Each dilution was inoculated into the allantoic cavity of an embryonated egg using 10 eggs per vaccine dilution. Eggs were incubated at 32° C for 72 h in egg incubator with 1 h automatic turning system and 75% humidity. After the incubation period, infected eggs were chilled at 4° C overnight before harvesting to kill the embryo and constrict the blood vessels to obtain fluids free from blood. The infectivity titers were estimated by testing the egg allantoic fluid for hemagglutination (HA) activity using the standard hemagglutination assay. Allantoic fluids were transferred into 96-well plate and then 0.5% of goose red blood cells (GRBCs) were added into each well plate. The titer was calculated using Reed and Muench method (Reed and Muench, 1938) to determine the concentrations of viral suspension that cause 50% infection of eggs. Hemagglutination assay for each test was performed in duplicate.

2.3. Subtype-specific and type-specific antibodies

The level of H1N1 infection was detected by H1-specific mouse monoclonal antibodies, ED9, while H3N2 infection was detected by H3-specific monoclonal antibodies, 12D1. The H1 and H3 specific monoclonal antibodies were obtained through the courtesy of Professor Peter Palese, Icahn School of Medicine at Mount Sinai, New York. Type specific antibody against Influenza A viral nucleoprotein, Anti-FluA-NP (#MAB8257, Millipore) and against Influenza B viral nucleoprotein, Anti-FluB-NP (#MAB8259, Millipore) were used to detect the level of influenza A infection and influenza B infection, respectively.

2.4. Infectivity titration by $TCID_{50}$ assay

The Madin-Darby Canine Kidney (MDCK) cells were used in the assay. Cells were maintained in Modified Eagle Medium (MEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 100U/ml penicillin and streptomycin (Gibco). 50% tissue culture infectious dose ($TCID_{50}$) titer was estimated for each monovalent vaccine strain in MDCK cell monolayers, with 0.25 μg/ml tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma), in 96-well plates and incubated at 33 °C, 5% CO_2 for 22–24 h. Infectivity was detected with subtype-specific anti-influenza HA antibodies, anti-influenza A or anti-influenza B nucleoprotein (NP) antibodies. The viral $TCID_{50}$ titers were calculated using Reed and Muench method.

2.5. ELISA for determining infectivity titer

Infected cells were fixed with 80% cold acetone at 4 °C for 1 h, and then blocked with 3% hydrogen peroxide, to inhibit endogenous peroxidase activity, for 30 min at room temperature. Specific anti-influenza mouse monoclonal antibody, diluted to of 1 μg/ml in blocking buffer (PBS pH 7.2 with 1% BSA and 0.1% Tween-20), was added to each well and incubated at 37 °C for 1hr, washed, followed by goat anti-mouse antibody conjugated to horseradish peroxidase (Southern Biotech Associates, USA), diluted 1:2000 in blocking buffer, and further incubated at 37 °C for 1 h, then washed. TMB peroxidase substrate system (KPL, USA) was added into each well and incubated in the dark for 10 min at room temperature. 1 M H_2SO_4 was used to stop the reaction and read at OD 450/630. Any test well with an OD > 2 times OD of cell control wells (CC) was scored positive for virus growth.

2.6. Standard sera and selective neutralization

Sheep antisera used in this study were obtained from the National Institute for Biological Standards and Control (NIBSC), UK, consisting of anti-influenza A/California/7/2009(H1N1) HA serum (NIBSC code: 11/238); anti-Influenza A/Perth/16/09 HA serum (NIBSC code: 11/206); and anti-Influenza B/Brisbane/60/08 HA serum (NIBSC code: 11/136).

The antisera were pre-treated with 3 vol of receptor destroying enzyme (RDE II) (DENKA SEIKEN,UK) at 37 °C for 18 h to remove non-specific inhibitor, followed by incubation at 56 °C for 1 h to inactivate complement. The treated antisera were then adsorbed with equal volume of 50% goose red blood cells (GRBCs) at 4 °C for 1 h to remove nonspecific agglutinins.

Effective dilutions of each antisera against each monovalent vaccine were determined. Effective dilution is the antibody titer of specific antiserum that completely neutralizes the homologous influenza virus vaccine strain by neutralization assay. The specific antiserum was 2-fold-serially diluted and mixed with fixed amount of the homologous influenza virus. The effective titer of the test antiserum is defined as the highest antiserum dilution that completely inhibits influenza hemagglutinin activity. This effective antibody titer will be further used in the experiment on determining the potency of individual vaccine component present in a dose of trivalent live attenuated influenza vaccine based on blocking the infectivity of the other two influenza vaccine strains with specific antisera, and followed by determining for the infective titers of the remaining non-neutralized strain.

Three mixtures of Anti (H1 + H3), Anti (H1 + B) and Anti (H3 + B) were prepared at a concentration of five times the effective dilution. Selective neutralization was carried out by mixing trivalent vaccine with each mixture of antisera. The titer of the remaining strain that has not been neutralized was measured using the previously described ELISA and estimated using Reed and Muench method.

2.7. Titration of vaccine strains in trivalent LAIV preparation

$TCID_{50}$ titers of individual vaccine strain in trivalent vaccine were determined by ELISA using specific antibody without prior neutralization by antisera mixtures. The trivalent vaccine preparation was serially diluted and inoculated on to MDCK cell monolayer in 96-well micro culture plate, then incubated at 33 °C, 5% CO_2 for

Table 1
$TCID_{50}$ titers using subtype-specific antibodies vs. EID_{50} titers.

Monovalent Bulk Vaccine (Strain & Lot no.)	$TCID_{50}$ titer/0.1 ml	EID_{50} titer/0.1 ml
MB-H1N1-5401	<1	6.69
MB-H3N2-5403	3.58	6.94
MB-B-5402	5.05	7.45

22–24 h. Infectivity of H3 strain was detected by subtype-specific anti-influenza H3 HA antibody, while type B strain was detected by anti-influenza B nucleoprotein (NP) antibody. The assay was carried out as in the standard $TCID_{50}$ titration method as described in Section 2.4.

2.8. Cross-reactivity assay

The cross reactivity between the specific antibodies to other vaccine strains that were used in the trivalent vaccine preparation was assayed with a panel of infected MDCK cells with individual monovalent vaccine strains.

2.9. Linearity

The accuracy of the assay was confirmed by plotting mean viral potency values of three independent experiments against the concentrations. Monovalent H3 and B vaccine were diluted in a serial 10-fold dilution to determine the potency range between the upper and lower concentrations. The linear relationships were evaluated across four dilutions within the potency range using TCID50 titration assay with subtype-specific antibodies. The linear regression analysis was performed using Microsoft Excel linear regression tool. The high correlation coefficient (r) of the regression line indicates high degree of correlation where the actual titer and dilution of the virus show linear relationship.

3. Results

3.1. Titration of monovalent LAIV preparation in cell culture

The influenza A H3N2 and influenza B monovalent LAIV viruses yielded acceptable $TCID_{50}$ titers in MDCK cells, although 2–3 logs lower than the EID_{50} titers, while the influenza A H1N1 strain, grew very poorly in MDCK cells and showed no detectable $TCID_{50}$ titer when using anti-HA subtype-specific antibody ED9 (Table 1). In an attempt to find a susceptible cell line for the H1N1 strain, various cell lines viz. CEF, VERO, DF-1, HEK293T and MDCK were tested. All cells were tested in the presence and absence of TPCK-trypsin but MDCK with TPCK-trypsin was found to be the most permissive (data not shown). This is in concordance with previous data showing poor adaptability of the 2009 H1N1 influenza hemagglutinin to growth in cell culture (Wörmann et al., 2016). The $TCID_{50}$ titers were measured using subtype-specific versus type-specific antibodies for detection of viral growth (Table 2). The $TCID_{50}$ titers of the influenza A H3N2 strain using the anti-HA subtype-specific antibodies 12D1 was comparable to that of anti-FluA NP type-specific antibody used in the standard $TCID_{50}$ assay. This suggested that 12D1 subtype-specific antibodies provided sufficient sensitivity for viral detection. Due to the poor growth of the H1N1 virus in MDCK cells, it was excluded from further titration experiments.

Table 2
Comparison between $TCID_{50}$ titers using subtype-specific antibodies against FluA viral hemagglutinin and the standard $TCID_{50}$ assay using type-specific antibody against FluA viral nucleoprotein.

Monovalent Bulk Vaccine	Detecting Antibody	$TCID_{50}$ titer/0.1 ml
MB-H1N1-5401	ED9 (H1-specific)	<1
	Anti-FluA-NP	2.75
MB-H3N2-5403	12D1 (H3-specific)	4.65
	Anti-FluA-NP	4.75

Table 3
Cross-reactivity between subtype-specific antibodies and other virus subtypes.

Monovalent Bulk Vaccine	Detecting Antibody	$TCID_{50}$ titer/0.1 ml
MB-H1N1-5401	12D1 (H3-specific)	<1
	Anti-FluB-NP	<1
MB-H3N2-5403	12D1 (H3-specific)	3.75
	Anti-FluB-NP	<1
MB-B-5402	12D1 (H3-specific)	<1
	Anti-FluB-NP	5.17

3.2. Titration of vaccine strains in trivalent LAIV preparation

The accurate measurement of $TCID_{50}$ titers using subtype-specific antibodies suggested that the method can be used for titration of multiple strains. The actual titers of H3N2 and B plotted against the virus dilutions showed linear relationship. The results showed high correlation coefficient (r) of the regression line within the range of 0.99–1.0 for both H3N2 and B indicating high degree of correlation between the virus titers and virus dilution (Fig. 1). The antibodies were checked for cross-reactivity and shown to have no reactivity against heterologous strains of the trivalent LAIV preparation (Table 3). However, viral interference among strains in competitive infection may theoretically be a problem. The trivalent LAIV vaccine preparation was titrated without prior selective neutralization and subtype-specific anti-HA H1 (ED9) and H3 (12D1) antibodies were used instead of the influenza A-specific anti-NP antibody. While H1N1 strain showed no titer due to its poor growth in cell culture (data not shown), both H3N2 and B strain $TCID_{50}$ titers were comparable to those of monovalent stocks before mixing and the EID_{50} titers (Table 4). This indicated that $TCID_{50}$ titration using subtype-specific antibodies of individual vaccine strains in the mixed virus population without prior selective neutralization of the other two strains can reliably measure the infectivity titers of the vaccine strains, and that viral interference did not interfere with the measurement.

3.3. Selective neutralization and individual strain titration

Individual vaccine strain was titrated using the subtype-specific antibody after selective neutralization of the other two vaccine strains. The titers were found to be similar to those without selective neutralization (Table 4). This indicated that co-infection of the three strains did not lead to a viral interference phenomenon at the level that interfered with the viral titer measurement.

Table 4
$TCID_{50}$ titers of viral growth with or without prior neutralization by antisera.

Tested Vaccine	Neutralizing Antisera	Detecting Antibody	Titer
MB-H3N2-5403	Anti-(H1 + B)	12D1 (H3-specific)	3.33
	None		3.54
TB-H1N1-H3N2-B	Anti-(H1 + B)		3.25
	None		3.04
MB-B	Anti-(H1 + H3)	Anti-B	4.83
	None		5
TB-H1N1-H3N2-B	Anti-(H1 + H3)		4.21
	None		4.61

TB: Trivalent Bulk Vaccine; MB: Monovalent Bulk Vaccine.

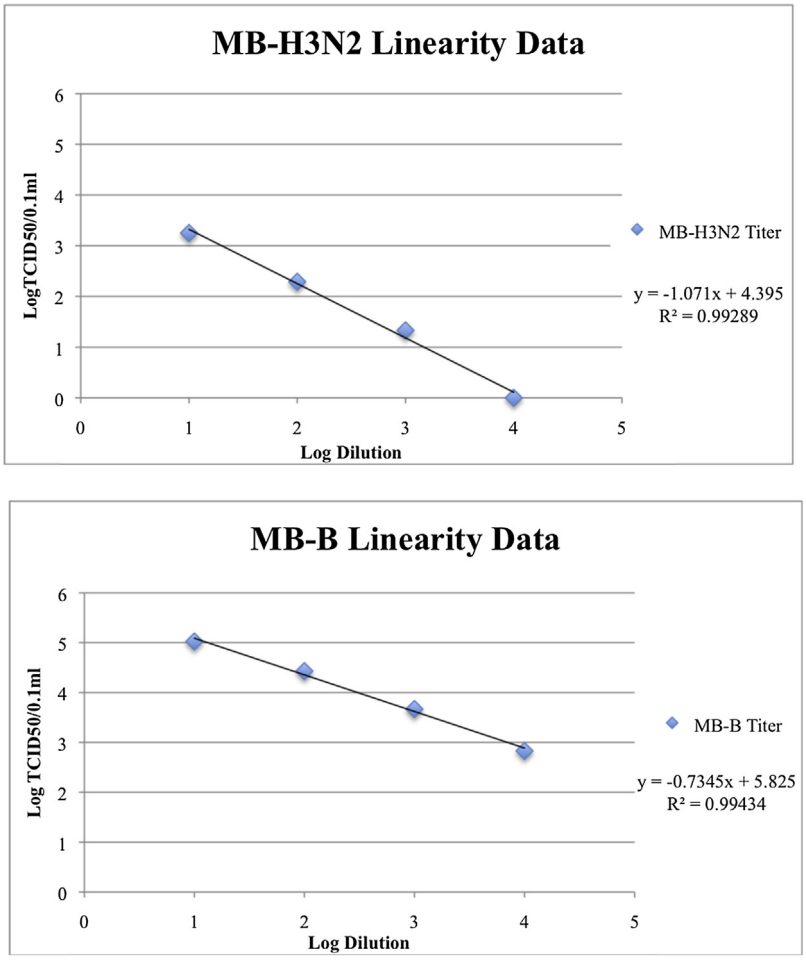

Fig. 1. Linearity—Potency vs. concentration of serially diluted samples.

4. Discussion

EID_{50} is a standard assay commonly used for determining infectious titer of viruses that grow well in embryonated eggs. However, the EID_{50} assay is more laborious and less flexible in terms of egg supply and work schedule compared to cell culture-based assay. The traditional method also requires antiserum specific to influenza A H1N1, H3N2 and influenza B that are potent in neutralization and do not cross react with other virus type and subtype.

It was observed that the replication times of each influenza strain in the trivalent vaccine are not equal in cell culture, particularly that of the H1N1 vaccine strain in comparison to the H3N2 and influenza B strains. The influenza A H3N2 and influenza B monovalent LAIV viruses yielded acceptable $TCID_{50}$ titers in MDCK cells, although 2–3 logs lower than the EID_{50} titers. This is not surprising, as it has been shown that donor strains A/Leningrad/134/17/57 and B/USSR/60/69 yields in MDCK cells were about 10–100-fold lower than that of the embryonic chicken eggs (Audsley and Tannock, 2005).

For this assay, that is a non-neutralizing assay where the three strains of viruses are allowed to co-infect, it is critical that the viruses are not exceedingly different in their fitness. The H3 and B strains are about 1.5 log different in their titers and could be accurately co-titrated. This indicated that strains with titer difference within 1–2 log could be co-titrated using this method. On the other hand, the H1 strain did not show detectable titer at 24 h post inoculation, when the peak signals of H3 and B strains were detected. The signal of H1 infection was detected when cells infected with single strain were maintained for 3-5 days. This indicated that the kinetics of H1 infection was slower than that of H1 and B strains, and that the level of viral replication at an early time point should be at least 3 log lower. We tried to extend the incubation period to 48 h without success because at 48 h there were massive cell death, which interfered with the readout of the assay. Therefore, viruses with 2-fold difference in replication kinetics cannot be co-titrated either. Such viruses would have titer difference at an early time point of at least 2-fold in log scale.

In an attempt to find the most suitable cell culture system that would allow the three vaccine strains to grow equally well, we had tested the infectivity of H1N1 vaccine strain against the other two strains in CEF, VERO, DF-1, HEK293T and MDCK, though all without success. H1-specific monoclonal antibodies, ED9 and 6F12, as well as FluA-specific anti-NP antibody were used to detect the level of infection of H1N1 strain, using ELISA, IFA and flow cytometry method, it was found that the level of infection of the H1 strain is very low in comparison to H3 and B strains in all cell types tested (data not shown). This proved to be a major problem in the titration of this set of trivalent vaccine. Therefore, H1N1 strain was excluded from the study because its replication rate in the cell culture is very low when compared with two other strains in the trivalent vaccine.

Interference effect was not observed in H3 and B in this study. However, we could not test the H1 strain for interference effect due to its inefficient growth, both in the trivalent and single strain infection condition. It was previously observed in several studies that when influenza A and influenza B viruses with comparable titers were allowed to co-infect, the intertypic reassortants were

not detectable and that growth of influenza A virus can be significantly suppressed in the presence of some strains of influenza B virus (Aoki et al., 1984; Kaverin et al., 1983; Mikheeva and Ghendon, 1982). It is not known whether influenza B proteins are involved in this inhibitory effect. However, even without H1, the newly developed assay can be used for H3 and B strains in combination with conventional assay for H1 strain.

This assay provides vaccine manufacturers an alternative method for titrating individual strains in trivalent LAIV by using subtype-specific antibodies to detect viruses of specific subtypes in cell cultures simultaneously infected with the trivalent vaccine preparation, therefore eliminating the need for standard neutralizing sera.

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The effects of additional potassium periodate treatment of serum in combination with the standard heat-RDE inactivation using ELISA, HI and microneutralization assay.

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Abstract

It is known that normal human sera contain non-specific inhibitors of influenza viruses. These non-specific inhibitors can interfere with antibody pattern in immune influenza sera and falsify the experimental results. It has been long suggested that sera should be pre-treated to inactivate these inhibitors. Standard protocol uses heat inactivation at 56°c followed by further treatment with receptor destroying enzyme (RDE). However, some studies attempted to further improve the inactivation method by using chemical solution such as potassium periodate, potassium iodide, trypsin, or sodium citrate. Many laboratories use different methods of serum inactivation best suited for the types of serum and strains of influenza viruses used. This study investigates the effect of additional potassium periodate (KIO_4) treatment of serum in combination with the standard heat-RDE inactivation using Enzyme-Linked Immunosorbent Assay (ELISA), haemagglutination-inhibition (HI) assay and microneutralization assay. The comparison of serum treated with standard heat-RDE and heat-RDE in combination with KIO_4 showed no significant results in HI assay and ELISA, though sera treated with additional KIO_4 give a slightly more stable ELISA result. However, microneutralization experiment showed that KIO_4 can non-specifically inhibit viral infection. Therefore while treatment of sera with additional KIO_4 might be beneficial for ELISA and HI assay, the method is unsuitable for microneutralization and the standard heat-RDE inactivation is recommended.

Introduction

Sera from both human and animals usually contain various non-specific inhibitors that may cross interfere experimental results such as haemagglutination-inhibition (HI) assay and give a false positive antibody pattern (1). The HI assay is a standardized serological method for detecting anti-influenza haemagglutinin (HA) antibodies. In order to accurately assess HA-specific antibody in sera and to prevent fault positive result pattern of antibody, it is necessary to inactivate/remove these non-specific inhibitors (2). The presence of non-specific inhibitors in human and animal sera was first described by Francis in 1947. There are three classes of inhibitors present in human sera – alpha, beta and gamma (3-5). Different laboratories use different methods of inactivation. The most common method is through heat inactivation at 56°c followed by further treatment with receptor destroying enzyme. (1, 6-8).

Receptor Destroying Enzyme (RDE) was found by Sir Frank Macfarlane Burnet. The enzyme was obtained from *Vibrio cholerae*. Macfarlane and his colleague collected some purification of *V. cholerae*'s enzyme (strain 4 Z); they found that the filtrates of *V. cholerae* made human cells able to inagglutination by influenza viruses (9). Heat inactivation at 56°c followed by further treatment with receptor RDE has long been used as standard protocol to treat serum for used in the serological diagnosis of influenza A virus. Some older studies tried

to compare the efficacy of inactivation serum by using several chemical solutions such potassium periodate (KIO_4) (2, 10), trypsin (2) and sodium citrate(11). Many laboratories use different methods of serum inactivation best suited for the types of serum and strains of influenza viruses used. Some study attempted to further improve the inactivation method by using chemical solution such as potassium periodate in addition to the standard heat inactivation and RDE treatment method. This reason led to our objective that aimed to investigate the effect of potassium periodate in addition to the heat-RDE inactivation to remove non-specific inhibitor of influenza virus in human sera using Enzyme-Linked Immunosorbent Assay (ELISA), haemagglutination-inhibition (HI) assay and microneutralization assay.

Materials and Methods

Cell and Viruses

MDCK cells (Madin-Darby Canine Kidney cell line) were grown in EMEM (Eagle's Minimum Essential Medium) supplemented with 10% heat-inactivated FCS (fetal calf serum) (Gibco BRL, USA) containing antibiotics included penicillin, gentamycin and fungizone. The cells were kept at 37°c with 5% CO2 condition. Influenza A virus strain A/Siriraj/03/06, A/Siriraj/08/07 and H9N2 (A/Chicken/Hong Kong/G9/97) were provided by Professor Pilaipan Puthavathana. Influenza H1 protein was obtained through BEI Resources, NIAID, NIH: H1 Hemagglutinin (HA) Protein with C-Terminal Histidine Tag from Influenza Virus, A/California/04/2009(H1N1)pdm09, Recombinant from Baculovirus, NR-15749. Influenza H3 protein obtained through BEI Resources, NIAID, NIH: H3 Hemagglutinin (HA) Protein with C-Terminal Histidine Tag from Influenza Virus, A/Perth/16/2009 (H3N2), Recombinant from Baculovirus, NR-19442.

Human serum samples

Sera were collected from healthcare personnel at Faculty of Medicine Siriraj Hospital, Mahidol University. The volunteer received a seasonal vaccination annually. The sera were collected in year 2008 and stored at -20°c were utilized (non-specific inhibitor) healthcare personnel with an age range of 30 – 50 years.

Serum Inactivation

Heat & RDE inactivation

Sera were incubated with 3 volumes of RDE at 37°c overnight and heat-inactivated at 56°c for 30 min. The sera were allowed to cool down at room temperature before 50% Goose Red Blood Cells (GRBCs) and normal saline solution were added to the treated sera to make GRBCs dilution of 1:10 and the final serum dilution of 1:10. GRBCs were allowed to incubate adsorb with sera at 4°c for 1 hour, inverting every 15 minutes. The adsorbed sera were centrifuged at 5000 rpm for 1 minute and collected.

Heat, RDE and additional potassium periodate (KIO_4) inactivation

Sera were incubated with 3 volumes of RDE at 37°c overnight and heat-inactivated at 56°c for 30 min. The sera were allowed to cool down to room temperature before 3 volumes of 11 mM potassium periodate solution was added into the mixture and incubate at room temperature for 15 minutes. Then 3 volumes of 1% of glycerol saline solution and was incubated at room temperature for another 15 minutes. 50% Goose Red Blood Cells (GRBCs) were allowed to adsorb with sera at dilution of 1:10 volume of sera, at 4°c for 1 hour, inverting every 15 minutes. The adsorbed sera were centrifuged at 5000 rpm for 1 minute

and collected. The final dilution for each serum was 1:71. 1XPBS was added to make the serum dilution of 1:100 for used in the experiment.

Enzyme-linked immunosorbent assay (ELISA)

100ng of purified influenza HA protein (H1 and H3 protein) diluted in coating buffer (50mM of Na_2CO_3/$NaHCO_3$; pH9.6) was coated onto microliter plates (Inmulon 2; Nunc) and incubated overnight at 4°c. The coated plates were washed twice with 0.025% tween/1X PBS and blocked with 5% of non-fat milk diluted in 1X PBS for 30 minutes at room temperature. The serum was serially diluted in 5% non-fat milk/PBS and incubated at room temperature for one hour in the coated plate. The plates were then washed with 1XPBS. Fifty microliters of secondary antibody (Goat-human IgG-horseradish peroxidase (HRP)) (Meridian life Science Inc.) at 1:5000 dilution in 5% non-fat milk was added in each well and incubated at room temperature for 1 hour. Then the plates were washed again by using washing-buffer (1XPBS/0.5%Tween 20), then fifty microliters of substrate (TMB peroxidase substrate system) (KPL, USA) was added into each well after washing. The plates were kept in the dark for ten minutes at room temperature. The reaction was stopped by adding 1M H_2SO_4 into the plate; the plates were read at OD 450 by using an ELISA plate reader.

Haemagglutination Assay

The hemagglutination assay will be used for determining the hemagglutination titers of viruses. Fifty microliters of 1xPBS (pH 7.2) will be added into each well of 96 well microplate. Fifty microliters of each culture supernatant will be added to the first column. Serial two fold dilutions will be made by transferring 50 µl from the first well. Final 50 µl was discarded. Goose red blood cells (GRBCs) suspension will be added 50 µl to each well on the plate. The plate will be incubated at 4°C for 30 min. GRBCs control will be prepared by adding 50 µl of PBS instead of culture supernatant. The GRBCs control will be checked for complete settling of RBCs. The hemagglutination titer will be read at the highest virus dilution that caused complete hemagglutination.

Haemagglutination Inhibition (HAI) Assays

HAI assay is a serological technique use to determine antibodies titers in serum that can inhibit viral agglutination to erythrocyte. 25 microliters of serially sera were allowed to pre-incubation with 25 microliters of 8 HAU/50 viruses at room temperature for 30 minutes. Then 50 microliters of 0.5% GRBCs were added into each well and incubator at 4 °C for 30 minutes. HAI titer was read from a button shape at final dilution before haemagglutination.

Microneutralization assay

Microneutralization assay was used to determine neutralization ability of sera to specific virus. Sera and viruses were diluted in 1XMEM supplemented with 1 µg/ml trypsin. Serially diluted sera were allowed to pre-incubate with 200 TCID50/100µl of virus at 37°c for 2 hour. After incubation, MDCK cells (Madin-Darby Canine Kidney cell line) previously plated onto 96-well plate were washed 1X MEM/trypsin. Then serum and virus mixtures were transferred into each wells containing MDCK cells, and incubated at 37°c for 24 hours. The cells were then fixed with 80% ice-cold acetone for 1 hour at 4°c. Then ELISA for detection of viral infectivity was performed. Briefly, after the cells were fixed, the plate will be washed twice and blocked with 3% hydrogen peroxide for 30 minutes at room temperature. Primary antibody (mouse monoclonal antibody to influenza A nucleoprotein) (Chemicon, USA) was diluted to 1:1000 with a blocking buffer (PBS pH 7.2 with 1% BSA and 0.1 % Tween-20) and added into each well, then allowed to incubate at 37°c in a

humidified incubator for 1 hour. After incubation, plate was washed four times, then Secondary antibody (goat anti-mouse antibody conjugated to horseradish peroxidase) (Southern Biotech Associates,USA) at a dilution 1:2000 were added and incubated at 37°c for 1 hour, and then washed. The substrate (TMB peroxidase substrate system) (KPL, USA) was added into each well and incubate at room temperature in the dark for 10 minutes. After 10 min, 1M H2SO4 will be added to stop the reaction. The plate will be taken for read at OD 450/630 by using ELISA reader.

Results

Comparison of inactivation methods between the standard heat-RDE inactivation versus additional potassium periodate (KIO_4) treatment of serum in combination with the standard heat-RDE inactivation using ELISA

In this study, ELISA was used to assay the binding activity of HA-specific antibodies in volunteer sera inactivated using two different methods. Each of the three sera from volunteers was inactivated using the standard heat-RDE method and the method with the additional KIO_4 treatment. The sera were serially diluted and allowed to react with influenza A H1 or H3 proteins previously coated onto 96-well plate. Level of binding was determined by measuring of the absorbance in each well at OD450, where the higher binding activity correlates to the higher absorbency. Figure 1 shows the ELISA results of the 3 sera inactivated using two different methods against H1 and H3 proteins. The results show no significant differences in the binding activity regardless of which of the two methods used.

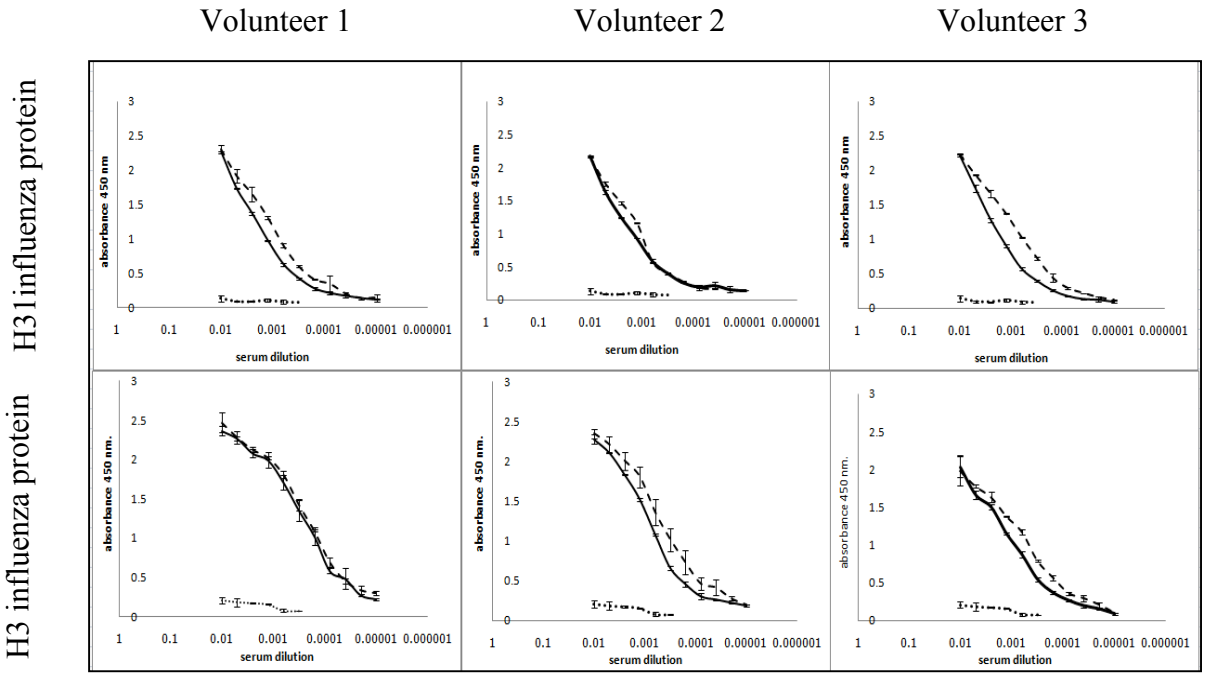

Figure1: – – – Standard heat-RDE inactivation ——— RDE additional KIO_4 inactivation

..... Negative control (secondary antibody)

Comparisons of inactivation methods between the standard heat-RDE inactivation versus additional potassium periodate (KIO_4) treatment of serum in combination with the standard heat-RDE inactivation using Haemagglutinin Inhibition assay.

This study used the same set of inactivated sera as the previous ELISA experiment, HI assay is used to determine level of antibodies in serum that can inhibit viral agglutination to erythrocyte. Influenza A viruses H1N1, H3N2 and H9N2 were used in this studied. H1N1 and H3N2 viruses are the strains which circulate among human population, while the H9N2 virus was isolated from chicken. As H9N2 is not a human virus, it was used as a HI control and human sera should not have any HI activity against this virus. The HI result in Table 1 showed, as predicted, a HI negative result for H9N2 while having HI titer of 1:800-1:1600 for H1N1 virus and 1:600 for H3N2 virus. However, there were no differences in HI titer between each serum that were inactivated by the standard method using heat-RDE inactivation and the method with the additional KIO_4 treatment.

HI Titer						
Viruses / Serum (Dilution)	**Volunteer1**	**Volunteer1 +KIO_4**	**Volunteer2**	**Volunteer2 +KIO_4**	**Volunteer3**	**Volunteer3 +KIO_4**
H1N1	1:800	1:800	1:1600	1:1600	1:800	1:800
H3N2	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600
H9N2*	0:0	0:0	0:0	0:0	0:0	0:0

Table1: Comparison of HI results using sera inactivated by the standard heat-RDE inactivation versus additional potassium periodate (KIO_4) treatment

Effect of potassium periodate from neutralization activity

Microneutralization assay is used to determine the neutralization ability of serum for specific viruses. In this study, the inactivated sera and the viruses used were the same set as those in the previous experiments. The microneutralization results are as shown in Table 2. It was interesting to see that all sera treated with additional KIO_4 showed higher neutralization titers than that of sera treated with the standard heat-RDE inactivation method for H3N2 and H9N2 virus and only serum form volunteer 1 for H1N1 virus. However, H9N2 virus which should not have any neutralization titer, show level of neutralization at 1:400 in the sera treated with additional KIO_4 while displayed no neutralization titer in sera using the standard heat-RDE inactivation method. Therefore the experiment was repeated with the additional group of KIO_4 control without serum. The result showed that KIO_4 at the same concentration used during serum inactivation can non-specifically inhibit virus infection which can be seen from the neutralization titer of 1:200-1:400 (Table 2).

NT Titer							
Viruses / Serum (dilution)	**Volunteer 1**	**Volunteer 1 +KIO_4**	**Volunteer 2**	**Volunteer 2 +KIO_4**	**Volunteer 3**	**Volunteer 3 +KIO_4**	**KIO4**
H1N1	1:800	1:1600	1:3200	1:3200	1:1600	1:1600	1:400
H3N2	1:400	1:1600	1:200	1:800	0:0	1:400	1:400
H9N2	0:0	1:800	0:0	1:400	0:0	1:400	1:200

Table2: Effects of potassium periodate in microneutralization assay

Discussion

Human or animal serum contains various non-specific inhibitors. Non-specific inhibitors in serum can interfere with experimental results. Therefore it is important to remove these non-specific inhibitors (10, 11) before carrying out any serological diagnosis of influenza A virus. Several previous studies showed that by heating sera at 56°c, by adding RDE or chemical reagents such as sodium citrate, potassium periodate and potassium iodine can help remove non-specific inhibitors. Currently, the standard method used for serum treatment by heat inactivation at 56°c followed by further treatment RDE. In this study, we want to investigate the effect of additional KIO_4 treatment of serum in combination with the standard heat-RDE inactivation using ELISA, HI and microneutralization assay. The experiments were performed using 3 volunteer sera against H1N1, H3N1 and N9N2 viruses. The volunteer sera were treated using 2 methods of inactivation, method one was to inactivate serum using standard heat-RDE inactivation, and method two was to inactivate serum using the standard heat-RDE inactivation with an additional KIO_4 treatment. For both method, 3 volumes of RDE was added to all serum samples and incubated at 37°c overnight and heat-inactivated at 56°c for 30 min. In addition, for the second method, potassium periodates and glycerol saline were added. All treated sera were adsorbed with 50% red blood cells and diluted to the same final serum dilution before used.

For ELISA, plates were coated with influenza A HA proteins (H1 or H3 protein) (BEI Resources) and sera were serially diluted allowed to react with the proteins. The result shows that there is no significant different in absorbance at OD450 for both method of serum inactivation. However, we observed that sera treated with additional KIO_4 treatment gave slightly more stable results than standard heat-RDE inactivation as can be seen from the smoother curve shown in Figure1. The same set of inactivated sera was also used in HI assay against H1N1, H3N2 and H9N2 influenza viruses. Both methods of inactivation gave the same HI results for all sera and virus tested.

On the other hand, microneutralization results, using the same set of inactivated sera and the viruses as the two previous experiments, show that most sera treated with additional KIO_4 displayed higher neutralization titers than that of sera treated with the standard heat-RDE inactivation method. Upon further investigation, it was found that KIO_4 at the same concentration used during serum inactivation can non-specifically inhibit virus infection.

Previous studies suggested to inactivated serum with KIO_4 to decreased non-specific inhibitor before going further to do haemagglutinin inhibition test (2, 7, 12). However, there are no references as to whether it is suitable to use KIO_4 treated serum in microneutralization assay. Therefore from the result of this study, treatment of serum with KIO_4 at the concentration used in this study is not recommended for use in microneutralization assay and the standard heat-RDE inactivation should be performed.

Conclusion

This study investigates the effect of additional potassium periodate (KIO_4) treatment of serum in combination with the standard heat-RDE inactivation using Enzyme-Linked Immunosorbent Assay (ELISA), haemagglutination-inhibition (HI) assay and microneutralization assay. The comparison of serum treated with standard heat-RDE and heat-RDE in combination with KIO_4 showed no significant results in HI assay and ELISA, though sera treated with additional KIO_4 give a slightly more stable ELISA result. However, microneutralization experiment showed that KIO_4 can non-specifically inhibit viral infection. Therefore while treatment of sera with additional KIO_4 might be beneficial for ELISA and HI assay, the method is unsuitable for microneutralization and the standard heat-RDE inactivation is recommended.

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รายงานสรุปการนำผลงานวิจัยไปใช้ประโยชน์

สัญญาเลขที่: MRG5680078
ชื่อโครงการ: วัคซีนต่อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 ในการกระตุ้นแอนติบอดีจะเพาะต่อก้านของโปรตีน hemagglutinin ในคน
หัวหน้าโครงการ: ดร.อลิตา คงชนะกุล **หน่วยงาน:** ศูนย์วิจัยและพัฒนาวัคซีน สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล
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สถานะผลงาน ✔ ปกปิด

ความสำคัญ / ความเป็นมา

วัคซีนไข้หวัดใหญ่เป็นเครื่องมือสำคัญในการป้องกันการเจ็บป่วย และบรรเทาผลกระทบจากการระบาด ถึงแม้จะมีการใช้วัคซีนอย่างกว้างขวางมาเป็นเวลานาน แต่วัคซีนในปัจจุบันยังมีปัญหาสำคัญที่ไม่สามารถครอบคลุมเชื้อสายพันธุ์ที่มีการเปลี่ยนแปลงไปจากไวรัสสายพันธุ์ที่ใช้ผลิตวัคซีนที่เรียกว่า antigenic drift ทำให้ต้องมีการเปลี่ยนแปลงสายพันธุ์วัคซีน antigenic sites หลักของไวรัสไข้หวัดใหญ่คือโปรตีน HA ซึ่งประกอบไปด้วย 2 ส่วน คือ ส่วนหัว (globular head) และ ส่วนก้าน (stalk/stem) โดย neutralizing epitopes จะอยู่บนส่วนยอดที่เรียกว่า globular head ซึ่งมีการเปลี่ยนแปลงได้ง่าย ขณะที่ในส่วนของ stalk มีการเปลี่ยนแปลงน้อยระหว่างไวรัสต่างสายพันธุ์ แต่การกระตุ้นแอนติบอดีต่อ stalk นั้นเป็นไปได้ยากกว่า ปัจจุบันมีความพยายามศึกษาวิจัยการสร้างวัคซีนที่มีความครอบคลุมกว้างต่อเชื้อไข้หวัดใหญ่ได้ทุกสายพันธุ์ ไม่นานมานี้มีรายงานวิจัยว่าการติดเชื้อและการได้รับวัคซีน pandemic 2009 H1N1 สามารถกระตุ้นการสร้าง antibody ต่อส่วน stalk ทั้งนี้เชื่อว่าเป็นผลมาจากการที่ผู้ที่ติดเชื้อหรือได้รับวัคซีนนั้นมี memory ต่อ stalk domain อยู่ก่อนแล้วจากการติดเชื้อหรือได้รับวัคซีน seasonal H1N1 ก่อนหน้านั้น และเมื่อได้รับไวรัส pandemic 2009 ที่มีส่วน stalk คล้ายกัน แต่ส่วน head แตกต่างกันมาก ก็ทำให้มีการกระตุ้น anamnestic response เฉพาะต่อส่วน stalk ทั้งนี้ไวรัส pandemic 2009 H1N1เป็นไวรัสที่มีต้นกำเนิดของยีน HA จากไวรัส 1918 pandemic (Spanish flu) เช่นเดียวกับไวรัส seasonal H1N1 แต่แยกสายวิวัฒนาการไปอยู่ในสุกร โครงการวิจัยนี้เพื่อต้องการศึกษาว่า วัคซีนต่อไวรัสไข้หวัดใหญ่ pandemic 2009 H1N1 จะมีความสามารถในการกระตุ้นแอนติบอดีจะเพาะต่อ stalk ของโปรตีน HA ได้มากน้อยเพียงใดในคน โดยซีรั่มของอาสาสมัครทั้งกลุ่มที่เคยได้รับวัคซีน Pandemic 2009 H1N1 influenza vaccine และ กลุ่มที่เคยได้รับวัคซีน Seasonal trivalent influenza vaccine ก่อนปี 2009 จะถูก screen ELISA โดยใช้โปรตีน HA ที่ถูกดัดแปลงให้ส่วนหัวเป็นของไวรัสสายพันธุ์ H9 และ H6 ที่ไม่ระบาดในคน แต่มียังคงส่วนก้านของ HA ไว้เป็น H1 เหมือนเดิม เพื่อดูปฏิกิริยาจำเพาะของซีรั่มแต่ละกลุ่มที่มีต่อก้านของโปรตีน HA หลังจากนั้นซีรั่มที่มีแนวโน้มที่มีแอนติบอดีต่อก้านโดยการทำ ELISA จะถูกนำไปทำ HAI (Hemagglutinin Inhibition assay) ต่อเพื่อยืนยันว่าซีรั่มนั้นทำปฏิกิริยาต่อส่วนก้านเท่านั้น โดยที่ผลของ HAI ต้องเป็นลบ และซีรั่มจะถูกนำไปทำ neutralization assay เพื่อดูผลของการลบล้างฤทธิ์ของซีรั่มต่อ chimeric virus cH9/1N3 ว่าแอนติบอดีจำเพาะต่อก้านที่ถูกกระตุ้นขึ้นโดยวัคซีน Pandemic 2009 H1N1 influenza vaccine จะมีความสามารถในการลบล้างฤทธิ์หรือไม่

วัตถุประสงค์ของโครงการ

1. เพื่อศึกษาว่าวัคซีนต่อไวรัสไข้หวัดใหญ่ pandemic 2009 H1N1 จะมีความสามารถในการกระตุ้นแอนติบอดีจะเพาะต่อ stalk ของโปรตีน HA ได้มากน้อยเพียงใดในคน
2. เพื่อศึกษาว่าแอนติบอดีจำเพาะต่อก้านที่ถูกกระตุ้นขึ้นนั้นจะมีความสามารถในการลบล้างฤทธิ์หรือไม่

ผลการวิจัย

Hemagglutinin (HA) โปรตีนของเชื้อไวรัสไข้หวัดใหญ่ประกอบไปด้วย 2 ส่วน คือ ส่วนหัว (globular head) และ ส่วนก้าน (stalk) โดย neutralizing epitopes จะอยู่บนส่วนหัวซึ่งเป็นบริเวณที่มีการเปลี่ยนแปลงได้ง่าย ในขณะที่ส่วนก้านจะมีการเปลี่ยนแปลงน้อยระหว่างไวรัสต่างสายพันธุ์ แต่การกระตุ้นแอนติบอดีต่อส่วนก้านนั้นเป็นไปได้ยากกว่าส่วนหัว การศึกษาที่ผ่านมาพบว่า วัคซีนไข้หวัดใหญ่ 2009 สามารถกระตุ้นแอนติบอดีต่อต้านก้านของ HA ได้ โครงการวิจัยนี้จึงต้องการศึกษาแอนติบอดีต่อต้านก้านของ HA ในอาสาสมัครคนไทยที่เคยได้รับวัคซีนไข้หวัดใหญ่ 2009 เปรียบเทียบกับอาสาสมัครที่ได้รับวัคซีนไข้หวัดใหญ่ก่อนปี 2009 โดยซีรั่มของอาสาสมัครจะถูกทดสอบด้วยวิธี ELISA โดยใช้โปรตีน HA cH6/1 และ cH9/1 ที่ถูกดัดแปลงให้ส่วนหัวเป็นของไวรัสสายพันธุ์ H9 และ H6 ที่ไม่ระบาดในคน แต่มียังคงส่วนก้านของ HA สายพันธุ์ H1 เพื่อดูปฏิกิริยาจำเพาะของซีรั่มแต่ละกลุ่มที่มีต่อก้านของโปรตีน HA ผลการทดลองพบว่า แอนติบอดีต่อต้านก้าน HA ในซีรั่มของอาสาสมัครที่เคยได้รับวัคซีนไข้หวัดใหญ่ 2009 ให้ผลการเข้าจับเป็นบวก ในขณะที่อาสาสมัครที่ได้รับวัคซีนก่อนปี 2009 ให้ผลการเข้าจับเป็นบวกน้อยกว่าอาสาสมัครที่เคยได้รับวัคซีนไข้หวัดใหญ่ 2009 กับทั้ง cH6/1 และ cH9/1 จากผลการทดลองแสดงให้เห็นว่าประชากรที่ได้รับวัคซีนหรือติดเชื้อจากไวรัสไข้หวัดใหญ่ 2009 H1N1 สามารถกระตุ้นแอนติบอดีต่อก้าน HA ได้ โดยซีรั่มบวกจะถูกนำไปทำ HAI (Hemagglutinin Inhibition assay) เพื่อยืนยันการไม่จับกับส่วนหัว H6 และ H9 แต่เป็นปฏิกิริยาต่อส่วนก้านเท่านั้น ก่อนจะถูกนำไปทำ neutralization assay เพื่อดูผลของการลบล้างฤทธิ์ของซีรั่มต่อ chimeric virus cH9/1N3 ผลการทดลอง

พบว่าซีรั่มที่ได้รับการฉีดวัคซีนไข้หวัดใหญ่ 2009 แสดงความสามารถในการลบล้างฤทธิ์จากการทดสอบกับไวรัสไข้หวัดใหญ่ลูกผสมเพียงเล็กน้อยเท่านั้น แม้ว่าความสามารถในการลบล้างฤทธิ์ที่ส่วนก้านจะไม่สามารถตรวจสอบได้ แต่ผลการทดลองแสดงให้เห็นว่าวัคซีนสามารถกระตุ้นแอนติบอดีต่อต้านก้าน HA ซึ่งอาจช่วยป้องกันการติดเชื้อไวรัสผ่านกลไกอื่นได้ยังคงต้องการการศึกษาต่อไป

คำสืบค้น (Keywords)

influenza virus, H1N1, vaccine, hemagglutinin, stalk antibodies

การนำผลงานวิจัยไปใช้ประโยชน์

✔ **ด้านวิชาการ**

- นำไปต่อยอดเป็นผลงานตีพิมพ์ในวารสารระดับนานาชาติ จำนวน 3 เรื่อง
- ผลิตนักศึกษาปริญญาโทจำนวน 1 คน
- การเสนอผลงานในที่ประชุมวิชาการจำนวน 1 เรื่อง

✔ **การเผยแพร่/ประชาสัมพันธ์**

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติจำนวน 3 เรื่อง
   - Sirinonthanawech N, Surichan S, Namsai A, Puthavathana P, Auewarakul P, **Kongchanagul A***. Titration of individual strains in trivalent live-attenuated influenza vaccine without neutralization. J Virol Methods. 2016 Nov;237:154-158. doi: 10.1016/j.jviromet.2016.09.001. Epub 2016 Sep 3. PMID: 27596269
   - Suptawiwat O, **Kongchanagul A***, Boonarkart C, Auewarakul P. H1N1 seasonal influenza virus evolutionary rate changed over time. Virus Res. 2018 May 2;250:43-50. doi: 10.1016/j.virusres.2018.03.020. Epub 2018 Mar 30. PMID: 29608996
   - **Kongchanagul A**, Samnuan K, Wirachwong P, Surichan S, Puthavathana P, Pitisuttithum P, Boonnak K*.

2. การเสนอผลงานในที่ประชุมวิชาการจำนวน 1 เรื่อง
   - Tayong K, Auewarakul P, Puthavathana P, **Kongchanagul A*.** The effects of additional potassium periodate treatment in combination with the standard heat-RDE inactivation of post influenza vaccination sera using ELISA, HI and microneutralization assay. The 40th Congress on Science and Technology of Thailand: “Science and Technology towards ASEAN Development”. 2014 Dec 2 - 4, Hotel Pullman Khon Kaen Raja Orchid, Khon Kaen, Thailand.

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A live attenuated H5N2 prime- inactivated H5N1 boost vaccination induces influenza virus hemagglutinin stalk specific antibody responses

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ABSTRACT

Background: The emergence and spread of highly pathogenic avian influenza (H5N1) viruses have raised global concerns of a possible human pandemic, spurring efforts towards H5N1 influenza vaccine development and improvements in vaccine administration methods. We previously showed that a prime-boost vaccination strategy induces robust and broadly cross-reactive antibody responses against the hemagglutinin globular head domain. Here, we specifically measure antibodies against the conserved hemagglutinin stem region in serum samples obtained from the prior study to determine whether stalk-reactive antibodies can also be induced by the prime-boost regimen.
Method: Serum samples collected from 60 participants before vaccination and on days 7, 28 and 90 following boosting vaccination were used in this study. 40 participants received two doses of live attenuated H5N2 vaccine (LAIV H5N2) followed by one dose of inactivated H5N1 vaccine a year later, while 20 participants received only the inactivated H5N1 vaccine. We tested these serum samples for stalk-reactive antibodies via enzyme-linked immunosorbent (ELISA) and microneutralization assays.
Results: Stalk-specific antibody levels measured by both assays were found to be significantly higher in primed individuals than the unprimed group. ELISA results showed that 22.5, 70.5 and 57.5% of primed participants had a four-fold or more increase in stalk antibody titers on days 7, 28 and 90 following boosting vaccination, respectively; whereas the unprimed group had no increase. Peak geometric mean titers (GMT) for stalk antibodies in the LAIV H5N2 experienced group (24,675 [95% CI; 19,531–31,174]) were significantly higher than those who received only the inactivated H5N1 vaccine (8877 [7140–11,035]; $p < 0{\cdot}0001$). Moreover, stalk antibodies displaying neutralizing activity also increased in primed participants, but not in the unprimed group.
Conclusion: Our finding emphasizes the importance of prime-boost vaccination for effectively inducing stalk antibodies, which is an attractive target for developing vaccines that induce stalk reactive antibodies.

1. Background

The emergence of avian influenza H5N1 viruses in Asia has raised concerns about their potential to cause pandemic disease. The most effective strategy for control and prevention of influenza virus infection is through vaccination, thus efforts are put into developing safe and immunogenic vaccines against the avian influenza viruses and other potential pandemic influenza strains [1]. The current influenza virus vaccine usually stimulates the immune system to create antibodies against the globular head domain of the hemagglutinin (HA) protein, which has a high plasticity and is easily subjected to changes, especially changes that are caused by antigenic drift [2–4]. On the other hand, the stalk domain of the hemagglutinin is stable and conserved in many influenza virus strains. The hemagglutinins of influenza A virus subtypes have been phylogenetically separated into two groups with regards to the sequence homology of the stalk domain, namely group 1 and

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group 2. Group 1 consists of influenza subtypes H1, H2, H5, H6, H8, H9, *H*11, H12, H13, H16, H17 and H18; whereas group 2 consists of influenza subtypes H3, H4, H7, *H*10, H14 and H15. It has been shown that the stalk antibodies are capable of broadly neutralizing the influenza virus infection [5,6] and can provide cross protection for experimental animals against many strains of influenza A virus [7,8].

Stalk antibodies are induced by repeated stimulations of the hemagglutinin protein; for instance, when there is a natural infection of different influenza virus subtypes at different time periods or when the vaccine against the virus is administered many times, such as annual seasonal influenza vaccination [9–13]. Studies in both humans and experimental animals have shown that sequential exposure of either natural influenza virus infections or influenza vaccine administration can significantly stimulate the production of stalk antibodies. For example, people who are infected with influenza 2009 H1N1 and have a history of infection with other strains of H1N1 influenza virus, have displayed an increase level in stalk antibodies. Moreover, it has been found that repeated administration of influenza vaccines can also stimulate the production of stalk antibodies, just like being naturally infected [14,15].

Previously, we have evaluated the immune responses following priming with a live attenuated vaccine strain A/17/turkey/Turkey/05/133 (H5N2) and boosting with an inactivated H5N1 vaccine in Thai volunteers. Results showed that the volunteers produced a low level of antibodies against the avian influenza virus after receiving the first vaccination of LAIV H5N2; but after a boost was given using the inactive H5N1 vaccine, the level of antibodies against the virus increased, showing a significant difference in both the quantity and quality of the antibodies. The evaluation on the quality of the vaccine in this previous study was done by detecting antibodies against the globular head domain of the hemagglutinin using HAI assay [16]. In this study, we specifically measured the stalk antibodies in the same serum samples of the volunteers from the previous study. We showed that the response to the inactivated H5N1 in LAIV H5N2 primed subjects includes induction of stalk specific antibodies.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Review Committee of Faculty of Tropical Medicine, Mahidol University (EC number: MUTM 2017–035).

2.2. Serum samples

Serum samples were received from the previous study [16]. 40 serum samples were collected from volunteers who received both live attenuated A/17/turkey/Turkey/05/133 (H5N2); LAIV H5N2 which is produced by classical genetic assortment using A/Leningrad/134/17/57 as donor strain, and inactivated H5N1 vaccine (Orniflu); clade 2.2, whereas 20 serum samples were collected from LAIV H5N2 naïve volunteers who received only the inactivated H5N1 vaccine. Additional information for this clinical trial can be found on ClinicalTrials.gov website (identifier: NCT01841918 and NCT02229357).

2.3. Viruses and recombinant proteins

Recombinant protein, cH6/1 (H6 globular head from A/mallard/Sweden/81/02 (H6N1) and stalk domain from A/Puerto Rico/8/34) and C9/1N3 virus (H9 globular head from A/guinea fowl/ Hong Kong/ WF10/99 (H9N2), neuraminidase from A/swine/Missouri/4296424/06 (H2N3) and stalk domain from A/Puerto Rico/34 (H1N1)) [15] were kindly provided by Dr. Florian Krammer (Icahn School of Medicine at Mount Sinai, NY, USA).

2.4. Virus propagation

C9/1N3 virus was propagated in the allantoic cavities of 10-to 11- day-old embryonated eggs (Kasetsart University, Thailand) at 37 °C. The allantoic fluid was harvested at 72 h post-inoculation and tested for hemagglutinating activity using 0.5% goose red blood cells (Animal Hospital, Mahidol University). Allantoic fluid was pooled, aliquoted, and stored at −80° C until use.

2.5. Anti- stalk antibody ELISA

Flat-bottom 96-well plates (Immulon 4 HBX; Thermo Fischer Scientific, USA) were coated with 2 μg/ml recombinant chimeric 6/1 hemagglutinin (kindly provided by Dr. Florian Krammer, Icahn School of Medicine at Mount Sinai, NY, USA) in coating solution (KPL, USA) at 4 °C for 16 h. Plates were washed three times with PBS containing 0.1% Tween 20 (PBS-T) (ThermoFisher Scientific, USA) and were blocked with 200 μl of 0.5% skim milk powder for 1 h at room temperature. After removal of the blocking solution, serum samples at 1:500 starting concentration were 2-fold diluted in blocking solution. Following 2 h incubation at room temperature, the plates were washed four times with PBS-T. Goat anti-human IgG (Fab specific) horseradish peroxidase (HRP)-conjugated antibody (Sigma, Germany) at 1:2000 dilution in blocking solution was added to all wells (50 μl/well) and incubated at room temperature for 1 h. The plates were washed four times with PBS-T and developed with 50 μl/well of TMB peroxidase substrate for 10 min. The reaction was stopped with 50 μl /well of 1 M H_2SO_4 and was then subsequently read at an absorbance of 490 nm with SpectraMax ELISA reader (Molecular device, USA)

2.6. Microneutralizaton assay

Flat bottom 96-well tissue culture plates were seeded with 200 μl of 1×10^5/ml MDCK cells containing MEM media with 10% FCS and were incubated in 5% CO_2 at 37 °C overnight. Testing serum samples were heat inactivated at 56 °C for 30 min. The treated serum samples were diluted at 1:10 and were serially diluted by 2-fold in MEM supplemented with TCPK-treated trypsin. Diluted sera were incubated with 100 $TCID_{50}$/50 μl of cH9/1N3 (kindly provided by Dr. Florian Krammer) for 1 h in 5% CO_2 at 37 C. After incubation, cells were washed with plain MEM and 200 μl of MEM containing L-glutamine and Anti-anti solution (ThermoFisher Scientific, USA) was added into each well. The cells were then incubated at 37 °C in 5% CO_2 for 4 days. To measure microneutralization activity, 50 μl of culture supernatant was transferred into a separate V-bottom 96 well plate, and 50 μl of 0.5% goose red blood cells in PBS were added. The samples were read after 45 min incubation at room temperature.

2.7. Hemagglutination inhibition assay (HAI)

The HAI assay was performed as previously described [17]. Briefly, serum samples treated with receptor-destroying enzyme (RDE) were serially diluted by 2-fold in 96 well V-bottom plates starting at a 1:10 dilution. Four HA units of C9/1N3 or A/California/07/09 (H1N1) or A/heron/Hong Kong/LC10/2002 (H6N8) viruses in 25 μl was added. After 30 min of incubation at room temperature, 50 μl of 0.5% goose red blood cells in PBS were added to the virus-serum mixture. The HAI activity was read after 45 min incubation at room temperature. HAI antibody titers were

recorded as the inverse of the highest antibody dilution that inhibited hemagglutination.

2.8. Statistics

The significance of difference between different groups was assessed with unpaired Student's t test using Prism 6 (GraphPad Software Inc., USA); $p < 0.05$ were considered significantly different.

3. Results

3.1. Vaccination with LAIV (H5N2) prime followed by an inactivated H5N1 vaccine boost regimen elicits stalk antibody responses

A total of 60 serum samples from our previous study [16] were used for the current study. Of the 60 subjects, 40 subjects were given two doses of LAIV strain A/17/turkey/Turkey/05/133 H5N2 by intranasal route and 20 subjects were placebo. One year later, all subjects were administered one dose of the inactivated H5N1 vaccine by intramuscular route. Serum samples were collected on day 0 before the administration of the booster vaccine, and sequentially on days 7, 28 and 90 post booster vaccination (Fig. 1). We have previously shown that two intranasal doses of LAIV H5N2 followed by an inactivated H5N1 boost can induce robust antibody responses to the head domain of H5 hemagglutinin proteins as demonstrated by HAI assay. In this study, we hypothesized that this prime-boost regimen would increase the stalk reactive antibodies. To test our hypothesis, we evaluated whether the antibodies elicited by these prime-boost vaccinations could react to chimeric HA molecule (cH6/1), a chimeric HA that combines H1 stalk domain with an exotic head domain from H6 virus. Since humans are naïve to the H6 head domain, reactivity measured by this anti-HA stalk enzyme-linked immunosorbent assay (ELISA) would indicate reactivity to H1 stalk. Indeed, the serum samples were tested for the presence of reactive antibodies against H6 globular head domain by HAI assay. The results showed that none of the serum samples display reactive antibodies against the H6 globular head domain as demonstrated by HAI assay using A/heron/Hong Kong/LC10/2002 (H6N8) (Supplementary Fig. 1). The endpoint titer in ELISA was used to quantitatively detect the stalk-reactive antibodies. On day 0 (prior to boost vaccination), the geometric mean titer (GMT) is 7000 – 8000 for both groups (Fig. 2). Booster vaccination significantly increased the stalk-reactive antibodies in LAIV experienced volunteers on day 7, day 28 and day 90 post boosting with $p = 0.05$, $p < 0.001$ and $p < 0.001$, respectively (Fig. 2A), but not in the volunteers who received only inactivated H5N1 vaccination (Fig. 2B). For LAIV experienced group, the titers of stalk reactive antibody following boosting vaccination dramatically increased to 10,196, 24,675 and 21,644 on days 7, 28 and 90, respectively. However, the stalk-reactive antibody titers remain at similar levels for LAIV naïve group, with GMT of 7464, 8877 and 8574 on days 7, 28 and 90, respectively. The seroconversion rates of participants at 7-, 28- and 90-days post boost by ELISA and microneutralization assays are summarized in Table 1. An increase in stalk antibody titers can be detected as early as 7 days after boosting with inactivated H5N1 vaccine. Of the LAIV H5N2 experienced individuals, 22.5%, 70.5% and 57.5% had a 4-fold increase in stalk antibody titers measured by stalk-based ELISA assay on days 7, 28 and 90 after receiving the inactivated H5N1 vaccine, respectively.

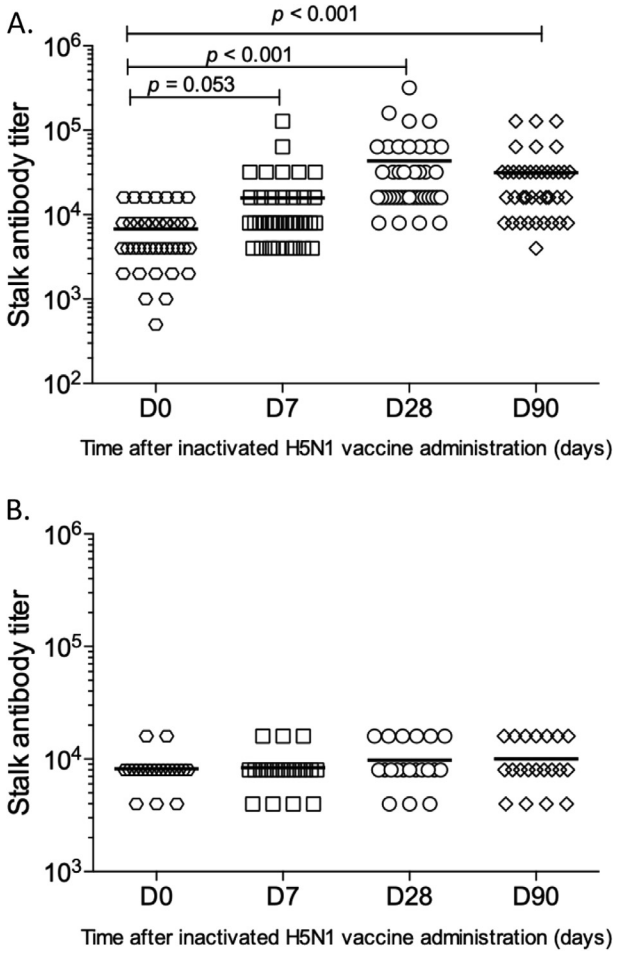

Fig. 2. The stalk reactive antibody titers measured by ELISA in LAIV H5N2 experienced group (A) and LAIV H5N2 naïve group (B) prior to boost vaccination and 7-, 28- and 90-days post boosting with inactivated H5N1 vaccine**.** The stalk reactive antibody titers significantly increased in LAIV H5N2 experienced group post boosting vaccination with $p = 0.05$, $p < 0.001$ and $p < 0.001$ for day 7, day 28 and day 90, respectively; while LAIV H5N2 naïve individuals had no significant increase.

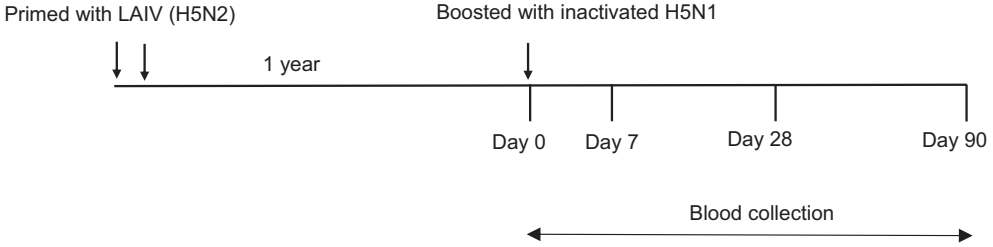

Fig. 1. The timeline of when the subjects were vaccinated with each vaccine and when the serum samples were collected**.** Out of 60 subjects in total, 40 subjects were primed with two doses of LAIV (H5N2) and 20 subjects were not. One year later, all 60 subjects were boosted with inactivated H5N1. Serum samples were collected prior to boosting vaccination (Day 0), and 7-, 28- and 90- days after boosting vaccination.

Table 1
The comparison of stalk antibody titers in percentage and GMT values between LAIV naïve group and LAIV H5N2 experienced group using ELISA and microneutralization assays.

Assay	Group	Day 7 after H5 IIV administration		Day 28 after H5 IIV administration		Day 90 after H5 IIV administration	
		Subjects with 4-fold Ab Rise		Subjects with 4-fold Ab Rise		Subjects with 4-fold Ab Rise	
		Percentage	GMT (95% CI)	Percentage	GMT (95% CI)	Percentage	GMT (95% CI)
ELISA assay	Group I (N = 20) (H5 IIV alone)	0%	7464 (6239–8929)	0%	8877 (7140–11035)	0%	8574 (6640–11071)
	Group II (N = 40) (LAIV H5N2 + IIV)	22.5%	10,196 (7798–13333) $P < 0.001$	70.5%	24,675 (19531–31174) $P < 0.001$	57.5%	21,644 (16413–28540) $P < 0.001$
MN assay	Group I (N = 20) (H5 IIV alone)	0%	16.21 (14.18–18.53)	0%	16.21 (14.18–18.53)	0%	16.21 (14.18–18.53)
	Group II (N = 40) (LAIV H5N2 + IIV)	15.0%	29.90 (21.53–38.80) $P < 0.001$	37.50%	51.27 (39.02–67.37) $P < 0.001$	42.50%	55.90 (41.40–75.47) $P < 0.001$

Abbreviations: IIV, Inactivated influenza vaccine; LAIV, Live attenuated influenza vaccine; CI, Confidence interval; GMT, Geometric mean titer; MN, Microneutralization assay; Ab, Antibody.

3.2. Stalk reactive antibodies can neutralize cH9/1 chimeric virus

Since endpoint ELISA technique showed that prime-boost vaccination can significantly induce HA stalk-specific binding antibodies, we further investigated whether these antibodies also display the inhibitory function by performing microneutralization assay using a chimeric HA, cH9/1 virus. This chimeric virus contains both an irrelevant H9 head domain, as well as a neuraminidase from a non-human virus, while still maintaining the H1 stalk domain. None of the serum samples display reactive antibodies against the H6 globular head domain as demonstrated by HAI assay using chimeric cH9/1 virus (Supplementary Fig. 2). Therefore, the virus neutralizing effects should directly be contributed by the stalk-reactive antibodies. In agreement with the stalk-reactive antibody titers assessed by ELISA, prime-boost vaccination resulted in a significant increase in stalk-neutralizing antibody titers following booster vaccination in LAIV experienced individuals but not in LAIV naïve group. The significant increase in stalk-neutralizing antibody titers were detected on day 7 ($p = 0.002$), day 28 ($p < 0.001$) and day 90 ($p = 0.001$) following booster vaccination (Fig. 3). In concordance to the stalk antibody titers measured by ELISA, we also observed a 4-fold rise of neutralizing stalk antibodies in the LAIV H5N2 experienced subjects. The seroconversion rates detected by microneutralization assay were 15%, 37.5% and 42.5% on days 7, 28 and 90 post booster vaccination, respectively (Table 1). There were no significant differences in stalk-neutralizing antibody titers at any days after booster vaccination in LAIV H5N2 naïve individuals.

3.3. No correlation between HA head domain specific antibodies and HA stalk reactive antibodies

To better understand the relationship between stalk antibodies measured by ELISA and HAI assays, the correlation between the stalk antibody titers and the HAI antibody titers against the H1 head domain were calculated. Because the H1 virus is classified as group 1 influenza virus based on the similarity of the stalk domain, we determined whether pre-existing antibody responses against H1N1 virus had any effect on stalk antibody induction in our study. No correlation between HAI antibody titers against H1N1 and stalk antibody titers was observed in this study (Fig. 4).

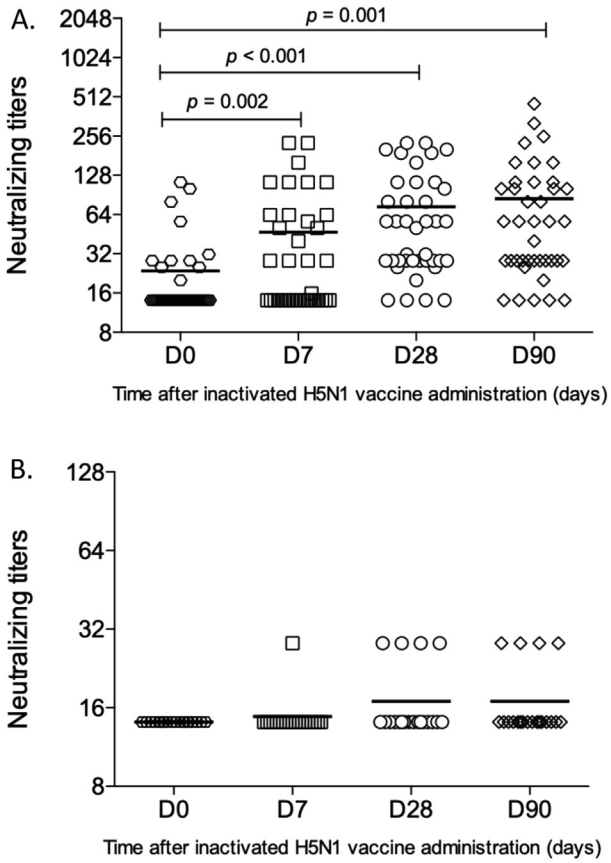

Fig. 3. The neutralizing stalk antibody titers measured by microneutralization assay in LAIV H5N2 experienced group (A) and LAIV H5N2 naïve group (B) prior to boost vaccination and 7-, 28- and 90-days post boosting with inactivated H5N1 vaccine. The neutralizing stalk antibody titers significantly increased in LAIV H5N2 experienced group following boosting vaccination with $p = 0.002$, $p < 0.001$ and $p = 0.001$ for day 7, day 28 and day 90, respectively; while LAIV H5N2 naïve individuals had no significant increase.

4. Discussion and conclusion

We have previously published that a vaccination strategy using LAIV H5N2 as a prime and an inactivated H5N1 as a boosting vaccine can induce equally robust HAI and neutralizing antibody responses against the globular head domain [16]. However, stalk-reactive antibody responses following these prime-boost vaccinations have not been studied. In general, the stalk domain is immuno-subdominant compared to the head domain and is usually not targeted by the immune system following vaccination [18]. So far, induction of stalk antibodies can be achieved by immunizing with a headless HAs vaccine or sequential vaccination with chimeric HAs that contain conserved stalk domain and distinct

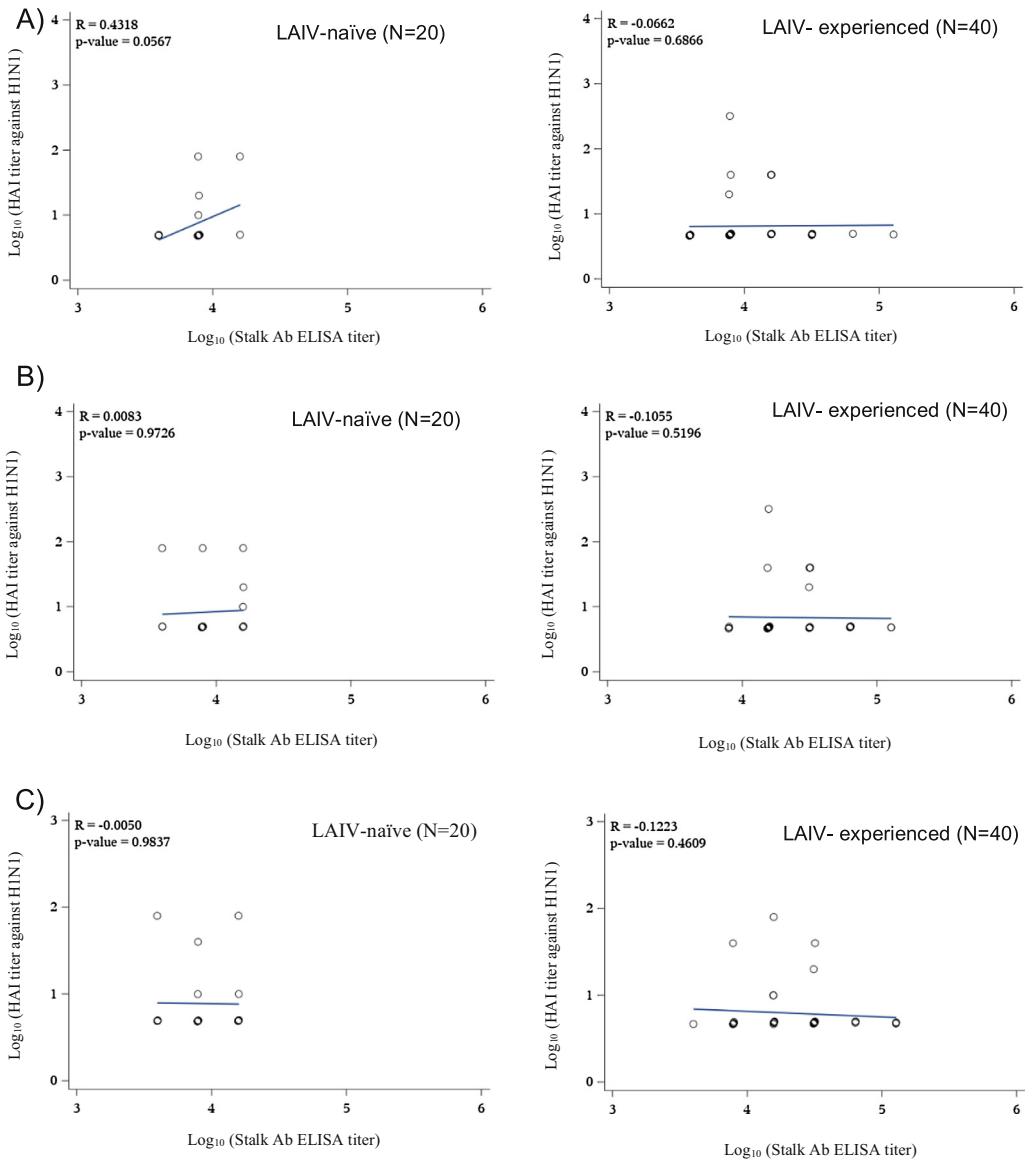

Fig. 4. The correlation between the stalk antibody titers and the HAI antibody titers against the A/California/07/09 (H1N1) on days 7 (A), 28 (B) and 90 (C) post boosting vaccination in LAIV naïve and LAIV experienced groups. No correlations were shown between the stalk antibody titers and the HAI antibody titers against the head domain of H1 on any of the days post boosting vaccination.

head domains [19]. These concepts have proven to be successful in naïve mouse and ferrets [15,20–22]. Because LAIVs are known to induce all arms of the adaptive immune response, it is interesting to explore whether using the LAIV platform can effectively induce stalk antibody production. Recently, a trend in a boosting of H1N1 stalk antibodies was found in children who received two doses of seasonal LAIV but not in adults who had high levels of pre-existing H1 stalk antibodies [23].

Although our initial study was not specifically designed to induce stalk-based immunity, it was important to explore whether the prime-boost strategies with different vaccine platforms can induce stalk antibody responses. We decided to use a sub-optimal dose of IIV H5N1 (15 μg of Influenza H5N1 hemagglutinin) to determine whether prior priming with LAIV H5N2 would enhance antibody responses. This setting allows us to demonstrate whether LAIV H5N2 would immunologically prime subjects for robust responses upon administration of a sub-optimal dose of IIV H5N1. The inactivated IIV H5N1 used in this study was previously demonstrated to be only moderately immunogenic in the phase I clinical trial. (https://www.who.int/immunization/diseases/influenza/107_abstractkrasilnikov1_line229.pdf?ua = 1). In agreement with other study [24], we also observed low seroconversion rates following IIV administration in LAIV naïve participants. In general, unadjuvanted inactivated influenza H5N1 vaccines are poorly immunogenic in humans. To achieve a HAI titer of 1:40 in > 50% of subjects, two doses of 90 μg each of IIV H5N1 would be required [25,26].

In this study, we measured the stalk-reactive antibodies in serum samples collected from volunteers who participated in our previous prime-boost study [16]. In contrast to the seasonal influenza vaccine studies, where the antibody response is exclusively directed to the globular head domain of HA [2], our analysis demonstrated that a single dose of inactivated H5N1 vaccine induces a stalk specific antibody response in H5N2 LAIV primed subjects. Recent report of human antibody responses to H7N7 LAIV prime- inactivated H7N7 boost study described similar findings, in which subjects primed to generate strong anti- H7 head antibody responses to the inactivated H7N7 vaccine also generated a strong anti-stalk antibody

response [27]. However, Hallilley et al. demonstrated vigorous stalk-specific antibody responses in both primed and unprimed groups [27], whereas the significant increase in stalk antibody titers were mainly detected in primed subjects in our study.

In addition, we also found that the baseline stalk antibody titers assessed by ELISA were high in both groups in our study, which may result from the history of natural H1 infection or vaccination that can induce some levels of antibodies against the stalk domain in humans [12,15]. Virtually all adults have been exposed to Influenza multiple times and such exposure have profound effect on the immune response to vaccination. Each encounter with natural infection or vaccination shapes the immune repertoire, and the potential induction of broadly influenza reactive antibodies will be regulated by the existing immunological memory. It has been nicely demonstrated that children generally have very low baseline titer of stalk specific antibodies, whereas the elderlies have the highest stalk-reactive antibodies. Moreover, middle-aged individuals who were shown to have high levels of baseline stalk antibodies mounted a moderately strong response against the stalk domain upon vaccination with seasonal influenza virus vaccine [28]. The presence of baseline stalk-reactive antibodies can potentially be boosted with the right vaccine. The increase in stalk-reactive antibodies upon vaccination is strongly associated with pre-existing influenza immunity.

Because the stalk antibody responses were detected as early as seven days following vaccination with inactivated H5N1 vaccine, it is unlikely that the antibodies measured was elicited by the inactivated H5N1 vaccine; rather, the immune memory was established after priming with LAIV H5N2. In agreement with the stalk antibody titers measured by ELISA, we detected an increase in neutralizing antibody titers but to a lesser extent. We hypothesize that the antibodies targeting the stalk region may contribute to the protection *in vivo* through post Fc mediated antibody dependent cell cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC), resulting in the killing of infected cells [29,30].

It will be important to further explore these biologically active and protective antibodies in passive transfer-experiment to ensure protective activity of these antibodies *in vivo*. However, it will be challenging to discriminate biological activities of the head and stalk reactive antibody responses since the H5N2 LAIV prime and inactivated H5N1 boost vaccination elicits strong anti-H5 head and anti-stalk antibody responses. Moreover, the specificity of stalk antibodies observed in this study needs to be further characterized by performing competition assays with well characterized broad neutralizing stalk targeting antibodies.

Our current study extends previous study demonstrating that H5N2 LAIV primes for HAI and microneutralization antibody responses to a single dose of inactivated H5N1 vaccine. Here we show that H5N2 LAIV also primes for a stalk specific antibody response, which emphasize a proof of concept in using LAIV prime as an effective pre-pandemic priming strategy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.10.084.

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H1N1 seasonal influenza virus evolutionary rate changed over time

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ABSTRACT

It was previously shown that the seasonal H1N1 influenza virus antigenic drift occurred at a slower rate than the seasonal H3N2 virus during the first decade of the 21th century. It was hypothesized that the slower antigenic evolution led to a decrease in average ages of infection, which in turn resulted in lower level of global viral circulation. It is unclear what caused the difference between the two viruses, but a plausible explanation may be related to the fact that the H1N1 virus had been in human population for much longer than the H3N2 virus. This would suggest that H1N1 antigenic drift in an earlier period may have been different from a more recent period. To test this hypothesis, we analyzed seasonal H1N1 influenza sequences during various time periods. In comparison to more recent H1N1 virus, the older H1N1 virus during the first half of the 20th century showed evidences of higher nonsynnonymous/synonymous ration (dN/dS) in its hemagglutinin (HA) gene. We compared amino acid sequence changes in the HA epitopes for each outbreak season and found that there were less changes in later years. Amino acid sequence diversity in the epitopes as measured by sequence entropy became smaller for each passing decade. These suggest that there might be some limit to the antigenic drift. The longer an influenza virus has drifted in human population, the less flexibility it may become. With less flexibility to adapt and escape the host immunity, the virus may have to rely more on younger naïve population.

1. Introduction

Seasonal influenza viruses are highly variable. Antigenic drift occurs frequently and makes necessary the annual update of vaccine strains. Hemagglutinin (HA) is the major viral envelope protein and contains major antigenic determinants. Influenza HA evolves under a strong positive selective pressure from the host immune response (Fitch et al., 1991). Because of this strong immune pressure, old influenza strains neutralizable by herd immunity become regularly extinct. This strong immune pressure allows antigenically escape strains to outgrow old strains and cause antigenic drift and new outbreaks (Hay et al., 2001). This antigenic drift is a global event, in which a new variant spreads out globally (Rambaut et al., 2008). Although it is generally accepted that seasonal influenza viruses evolve under strong positive selection exerted by host immunity, the degree of the positive selection can be different. It has been previously shown that during the first decade of the 21st century the H1N1 and influenza B viruses had lower rates of antigenic escape than the H3N2 virus with (Bedford et al., 2015). While the H3N2 virus strictly followed the single lineage phylogeny with periodic emergence of new global strains and extinction of old strains, the H1N1 and influenza B viruses had lower level of global viral circulation and higher level of local persistence resulting in a transient multi-branch phylogeny (Bedford et al., 2015). It was hypothesized that the lower rates of immune escape of influenza B and H1N1 may have led to younger average ages of infection as compared to H3N2. It was also hypothesized that the lower average ages of infection may explain the reduced global viral circulation as children travel long-distances less frequently than adults (Bedford et al., 2015). However, it is unclear why influenza B and the H1N1 virus showed lower rates of antigenic escape. It is possible that this is a difference in intrinsic properties of the viruses. However, as influenza B and H1N1 were older than H3N2, it is also possible that this difference is related to the length of time that the viruses have circulated in human population. The H1N1 virus entered human population in 1918 and influenza B had circulated in human population for much longer, whereas the H3N2 virus came into human population in 1968 (Cox and Subbarao, 2000).

Influenza viruses are believed to be in an optimal balance with their natural hosts. In water fowls, influenza A viruses were shown to be in an evolutionary stasis. This is probably a result of a long-term co-evolution of the viruses and hosts (Webster et al., 1992). Accordingly, the

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Table 1
The values of dN/dS and LRT tests for HA1 sequences by CodeML analysis.

	dN/dS	LRT (M7–M8)	P-value
1918–1957 (72 strains)	0.436	56.21	< 0.00001
1977–2008 (71 strains)	0.271	27.88	< 0.00001
1933–1943 (22 strains)	0.485	14.09	0.00017
1945–1957 (45 strains)	0.387	46.22	< 0.00001
1977–1984 (48 strains)	0.286	21.35	< 0.00001
1985–1997 (83 strains)	0.339	63.66	< 0.00001
1998–2008 (107 strains)	0.256	31.46	< 0.00001
2009–2015 (106 strains)	0.237	8.10	0.00442

longer a virus circulates in a host species, the more it should become closer to this evolutionary stasis. This hypothesis would predict that antigenic drift of a seasonal influenza virus will decrease with time. It is, however, unclear how long it would take to see this effect. The evolutionary stasis of avian influenza A viruses in water fowls may have taken thousands of years. A century is a relatively short time period in comparison. However, if the observed difference in the antigenic drift of H1N1 and H3N2 was a direct result of the difference in time length of the viral circulation, half a century must have been sufficient to cause an observable difference. Comparing old and recent H1N1 evolution may provide some insight into this hypothesis. Since the H1N1 epidemic was interrupted between 1950s to 1977 by H2N2 and H3N2, we decided to compare the evolution of H1N1 before and after 1977.

2. Materials and methods

2.1. Sequences

We focused our analyses on the HA1 gene because it codes for the main antigenic protein of the virus that showed antigenic drift and its sequences are the most abundantly available. Four hundred and eleven full length sequences of H1N1 HA1 were retrieved from NCBI Influenza virus database of the full length sequences were selected and aligned with Bioedit program. After alignment, sequences with 100% similarity were excluded from the analyses. All available sequences from 1918 to 1987 were included, and sequences were randomly selected from the more the recent years (1988–2015) to cover all geographical regions. The sequences were arbitrarily divided into 6 groups by decade (Table 1).

2.2. Phylogenetic analyses

Phylogenetic trees based on HA1 nucleotide sequences were constructed by maximum likelihood method implemented in PAUP version 4.0. The resulted trees were further used as guide trees for estimating selection pressure in CodeML application program in Phylogenetic Analysis by Maximum Likelihood (PAML) package (Yang, 2007). We used models M7 and M8, where M7 contains 10 ω categories to describe ω amongst sites, all constrained to be < 1; M8 differs from M7 only in that it estimates ω for an extra class of sites (p10) at which ω can be > 1 (Yang, 1997). Models were compared using a likelihood ratio test and the Bayes Empirical Bayes (BEB) method was used for a posteriori estimation of individual codons under positive selection (Yang et al., 2005). Phylogenetic tree based on HA1 amino acid sequences were constructed by maximum likelihood method in MEGA program version 6.0.

2.3. Hamming distance and $P_{epitope}$

Hamming distance of HA1 epitopes was calculated by comparing consensus sequence of each year to that of the previous year. $P_{epitope}$, which has been previously shown to correlate with antigenic distance (Deem and Pan, 2009), was also calculated by comparing consensus sequences of the epitope residues of two consecutive years. For each epitope, the P-value is defined as the proportion of different amino

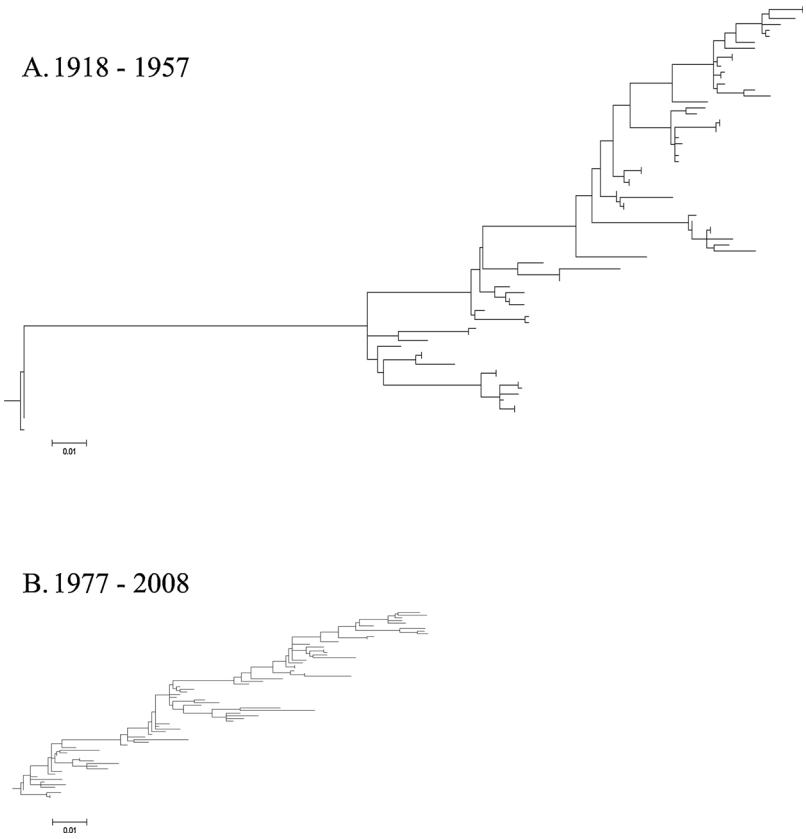

Fig. 1. Phylogenetic analysis of H1N1 HA1 nucleotide sequences. The phylogeny trees were generated by maximum likelihood from HA1 nucleotide sequences of human influenza A viruses isolated from 1918 to 1957 (a) and 1977 to 2008 (b). Branch lengths are drawn to scale. The scale bar represents 0.01 units of nucleotide change per site.

Fig. 2. Maximum likelihood trees of H1N1 HA1 nucleotide sequences (a) and amino acid sequences (b). The H1N1 HA1 sequences were arbitrarily divided into 6 groups by decade. All available sequences from 1918 to 1987 were included, and sequences were randomly selected from the more the recent years (1988–2015) to cover all geographical regions. Branch lengths are drawn to scale. The scale bar represents 0.01 units of nucleotide or amino acid change per site. Tree lengths are provided in parentheses under the time periods.

acids between these two sequences. The largest of the five P-values is defined as $P_{epitope}$, and the corresponding epitope is defined as the dominant epitope (Deem and Pan, 2009). We used previously described 62 amino acid residues in 5 epitopes (A–E) for these analyses (Huang et al., 2012).

2.4. Sequence entropy

Shannon entropy is a measure of uncertainty or unpredictability of information content. It has been used to describe diversity of amino acid sequences and identify antigenic sites of influenza HA (Huang et al., 2012; Pan and Deem, 2011). To measure entropy of the HA

Table 2
The values of dN/dS and LRT tests for HA1 sequences, which were deleted the egg-adapted positions, by CodeML analysis.

	dN/dS	LRT (M7–M8)	P-value
1918–1957 (72 strains)	0.404	42.54	< 0.00001
1977–2008 (71 strains)	0.259	29.16	< 0.00001
1933–1943 (22 strains)	0.456	8.65	0.00327
1977–1984(48 strains)	0.300	27.59	< 0.00001
1985–1977 (83 strains)	0.333	66.89	< 0.00001
1988–1997 (72 strains)	0.308	39.63	< 0.00001
1998–2008 (107 strains)	0.228	15.85	< 0.00001
2009–2015 (106 strains)	0.232	6.23	0.01256

antigenic sites, alignment was compiled for each time period containing all amino acid positions that were previously mapped in antigenic sites (Caton et al., 1982; Deem and Pan, 2009; Huang et al., 2012). Shannon entropy of each amino acid position in the alignment, which is defined as $H = \sum_{I=1}^{20} Pil$, where *Pi* is proportion or probability of an amino acid, was then calculated by a web-based entropy calculator at http://imed.med.ucm.es/PVS/.

3. Results

3.1. Evolution of H1N1 HA during difference periods

It was previously shown that seasonal H1N1 virus in 2000–2008 had a slow rate of antigenic drift than H3N2 virus in the same period (Bedford et al., 2015). We suspected that this might be because H1N1 had been in human population for much longer. This would mean that earlier H1N1 would have had faster antigenic drift than the more recent H1N1 virus. To test this hypothesis, HA1 gene sequences of seasonal H1N1 viruses from the period before and after the interruption by H2N2 and H3N2 viruses during 1957–1977 were compiled and analyzed. We chose to analyze only the HA1 part of the gene because it is more variable than the HA2 and contains all the variable antigenic epitopes of the HA globular head. Although both the phylogenetic trees of HA1 before 1957 and after 1977 showed the typical monophyletic pattern indicating positive selection, the tree before 1957 has a much longer main trunk (Fig. 1). The longer trunk suggests that the HA1 gene before 1957 had more mutations accumulating in the length of the main trunk. Shorter main trunk, on the other hand, suggests that more mutations were lost in the dead-end side branches and did not contribute to the main trunk length. We next generated both nucleotide and amino acid phylogenetic trees of the H1 HA1 by decade (Fig. 2). Only the main trunk length of amino acid tree significantly shrank down for each passing decade (one-tail Spearman correlation of amino acid tree; *P*-value = 0.0292, nucleotide tree *P*-value = 0.0681), except for the period of 1998–2008. Nevertheless, there was a clear trend toward shorter main trunk in the more recent history of the H1N1 virus. This includes that 2009 pandemic virus, which surprisingly had little changes during the last 7 years since its emergence.

The nature of selection was determined by the dN/dS ratio of the HA1 nucleotide sequence alignment separately constructed from the sequences before 1957 and after 1977. Although the log-likelihood ratios indicate significant positive selection for both periods, the HA1 gene of early 20th century before 1957 showed a higher dN/dS ratio of 0.436, whereas the more recent HA1 after 1977 had dN/dS ratio of only 0.271 (Table 1). Because the dN/dS ratio of the sequences before 1957 includes the 1918 pandemic virus and because a pandemic virus may evolve rapidly in order to adapt to the new transmission condition in human population, the ratio might be biased toward higher positive selection. In order to clearly see changes in the selective pressure over time, the dN/dS ratio was determined for HA1 sequences of each decade (Table 1). Excluding the 1918 pandemic virus, the evolution of early HA1 during 1933–1943 showed a dN/dS ratio of 0.485, clearly higher than those of the following decades. The period of 1945–1957 also showed a moderately higher dN/dS ratio than the more recent periods. These indicate that the HA1 of seasonal H1N1 virus may have evolved with a gradually increasing negative selective pressure over a period of about half a century and then continued to evolve with a steadily level of selection over the subsequent period (one-tail Spearman correlation; *P*-value = 0.0083). Because virus isolation in cell culture was only available after 1950s, earlier sequences were therefore derived solely from egg-cultured viruses. To provide a supporting evidence that the observed difference in the dN/dS ratio among the time periods was not due to a bias of having egg-derived sequences in the earlier period, we deleted amino acid position 129, 163, 187, 190, 225 and 226, which were shown to contain egg-adapted mutations (Gambaryan et al., 1999; Robertson et al., 1991), from the alignments and reanalyzed for dN/dS ratio. Similar decrease in dN/dS ratio with time was observed after deletion of the egg-adapted positions (one-tail Spearman correlation; *P*-value = 0.0514) (Table 2).

3.2. Changes in antigenic residues

Amino acid difference in 5 epitopes of HA1 was shown to correlate with antigenic distance and reduced vaccine efficacy against mismatched strains (Deem and Pan, 2009; Huang et al., 2012). The difference in epitope amino acid sequence can be quantified either by Hamming distance or $P_{epitope}$ (Deem and Pan, 2009; Huang et al., 2012). To understand the trend in antigenic evolution, Hamming distance and $P_{epitope}$ were calculated by comparing consensus epitope sequences between two consecutive years. The values were used to represent levels of antigenic change in each outbreak season. Both Hamming distance and $P_{epitope}$ showed a clear decreasing trend with time (Fig. 3). For each individual epitope, the P values of most epitopes except for epitope C showed the decreasing trend (Fig. 3).

3.3. Diversity in the antigenic sites

The decreasing dN/dS in the more recent H1N1 HA1 suggested that the more recent virus had slow antigenic drift. This would mean less diversity in the antigenic sites of the HA1. In order to determine the amino acid diversity in the HA1 antigenic sites, amino acid alignment at positions of previously described antigenic sites was constructed for HA1 sequences of each decade. Sequence entropy, which is a quantitative measure of uncertainty of amino acid at each position, was then calculated (Pan and Deem, 2011). In accordance to the decreasing dN/dS, the entropy of the amino acid positions in the HA1 antigenic sites showed a clear decreasing trend over the decades (Fig. 4). This indicates lower diversity in the antigenic sites, which may link to slower antigenic drift, in the more recent viruses. Because the number of available old strains used in the analysis was much lower than the more recent strains, the different sample sizes might cause a bias in the analysis. To ensure the validity of the entropy results, recent strains were randomly selected for 22 strains per decade and reanalyzed for entropy. The random resampling was performed for 10 times. The mean and maximum entropy of the resampling sets of recent strains still showed similarly lower levels than those of older strains (Fig. 5).

4. Discussion

Our data are in agreement with previously published data showing that amino acid substitution rate and positive selective pressure of seasonal influenza virus H1N1 during 1918–1957 were higher than during 1977–2009 (Furuse et al., 2010), suggesting that evolutionary rate of the H1N1 seasonal influenza virus has slowed down with time. However, the analyses included viruses from the beginning of the pandemic in 1918, and it was shown that for many emerging viruses a rapid increase of viral diversity was a phenomenon usually observed at the beginning of an epidemic when expansion of viral population was

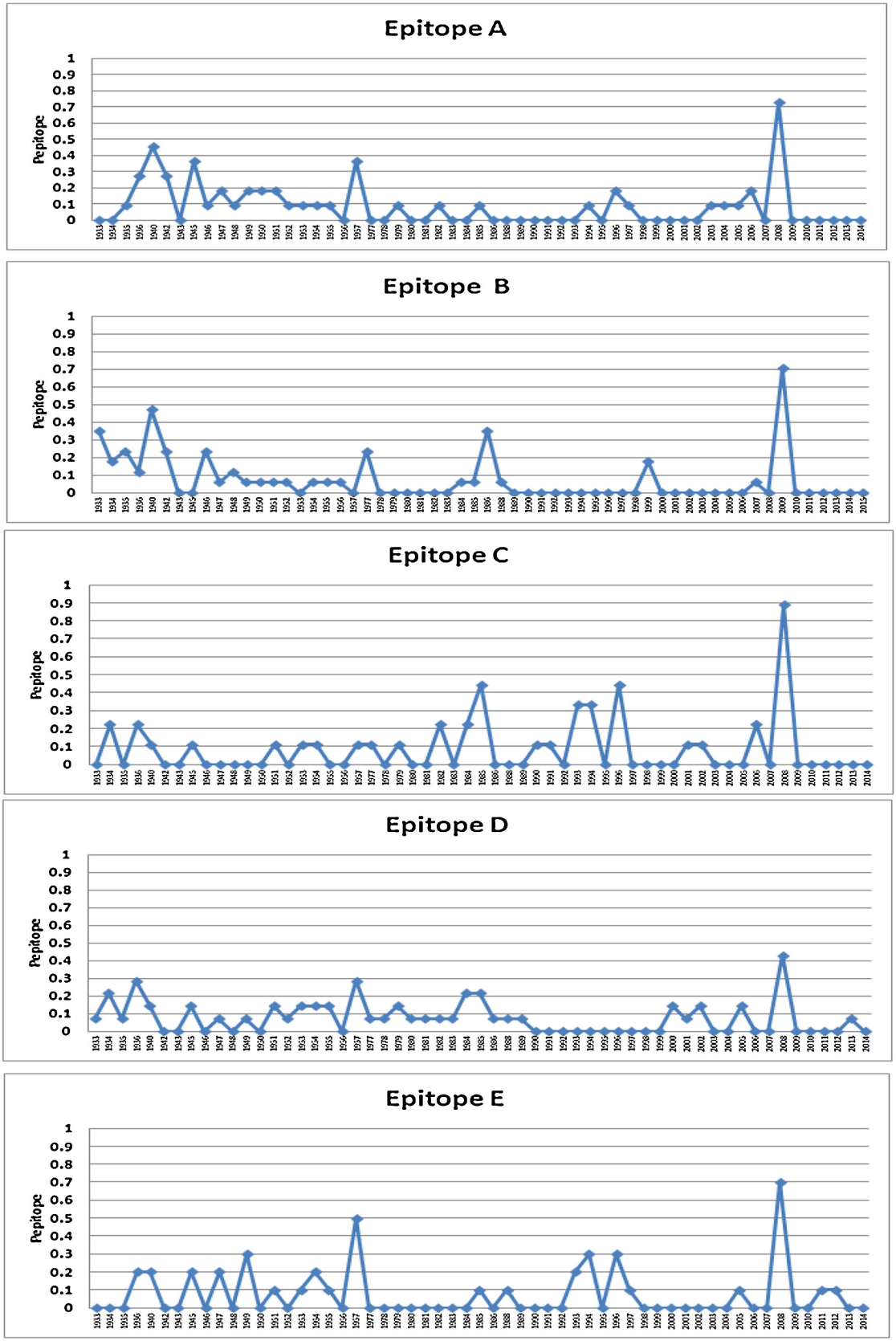

Fig. 3. P epitope values of the HA antigenic sites represent antigenic changes by year. The values were calculated by comparing consensus sequences of the epitope residues of two consecutive years. The P epitope is defined as the proportion of different amino acids between these two sequences.

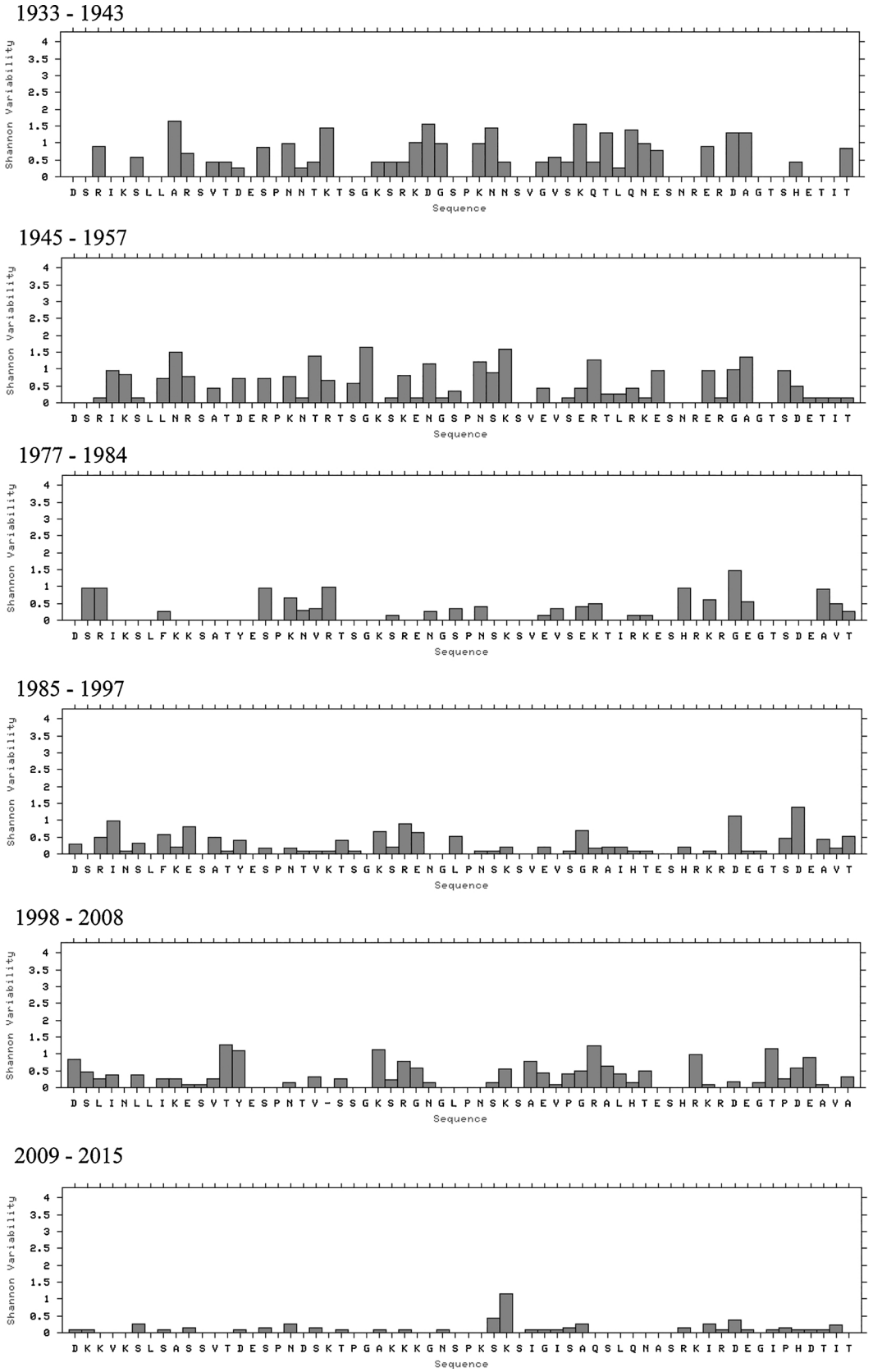

Fig. 4. Shannon entropy of 62 amino acid residues in the HA antigenic sites by decade. Amino acid residues previously identified as antigenic sites were obtained from HA1 sequences arbitrarily divided into 6 groups by decade. Shannon entropy was then calculated by a web-based entropy calculator at http://imed.med.ucm.es/PVS/.

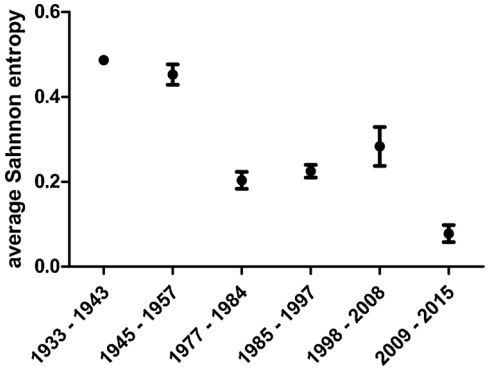

Fig. 5. Mean Shannon entropy of the HA antigenic sites by decade. Ten sets of randomly selected 22 strains per decade were analyzed for Shannon entropy of the HA antigenic sites, except for the 1933–1943 decade where only one set of sequences was available. Mean ± SD of the entropy values are shown for each decade.

unhindered by competition among viral variants, some of which may be later selected out by purifying selection resulting in reduced diversity when the epidemic became more mature (Faria et al., 2016; Gire et al., 2014; Meyer et al., 2015). Our decade-wise analyses, on the other hand, included only viruses starting more than a decade after the pandemic, and should be much less affected by the initial phase of epidemic.

The antigenic drift as a global event is understandable in the view of current global community when any corner of the world can be reached within a couple of days and a large number of people travel across continents and countries in airliners every day. This is in sharp contrast to the first few decades of the history of influenza in humans. In the early 20th century, traveling around the world in 80 days was still a scientific fiction. International traveling had not become a part of everyday life until the 1950s when commercial airliners began to operate. The rapid expansion of international travel thereafter has transformed human society in many ways including the risk of rapid spread of new pathogens. Massive international travel is probably a pivotal factor in the thorough mixing and global circulation of seasonal influenza strains, which play an important role in the antigenic drift and evolution of seasonal influenza (Lemey et al., 2014). It was previously proposed that slower antigenic drift of H1N1 seasonal influenza virus as compared to H3N2 virus was associated with lower ages of infection that lead to reduced global circulation and increased local persistence of viral strains (Bedford et al., 2015). Given constant viral and host factors, massive increase in international travel should have resulted in a more rapid antigenic drift. However, while international travel was dramatically increasing through commercial air travel after 1970s, the antigenic drift of H1N1 seasonal influenza virus was getting slower. This suggests that the decreasing antigenic drift of H1N1 HA was an intrinsic property of the virus rather than a result of changing transmission environment.

The reason for the decrease in the antigenic evolutionary over time is unclear. It is possible that structural constraint may restrict changes for preservation of the HA function and viral fitness. Although it has been previously shown that the HA was highly flexible to changes and could accommodate more mutations than other viral proteins (Heaton et al., 2013), it was also shown that there was preference of certain amino acids in some positions in HA antigenic sites (Kryazhimskiy et al., 2008). This implies that there may be some restriction in the changes of HA antigenic sites. In addition, it was proposed that in addition to immune escape the evolution of HA may also direct toward optimization of receptor binding avidity (Hensley et al., 2009).

The current H1N1 virus seemed to follow the trend of the old seasonal H1N1 virus with slow antigenic drift. There were little changes in antigenic property of the virus since its emergence as a pandemic virus (Koel et al., 2015). Previous data showed that the HA of this virus was mutating toward stability and optimal receptor binding rather than immune evasion (Castelan-Vega et al., 2014; de Vries et al., 2013). Complex interaction with host immunity targeting highly conserved epitopes has been proposed to be responsible for the extinction of the old seasonal H1N1 virus and may play a role in limiting antigenic escape of the current H1N1 virus (Pica et al., 2012).

Antigenic drift of influenza B virus was shown to be even lower than H1N1 virus (Bedford et al., 2015). In addition to antigenic drift rate, nucleotide mutation and amino acid substitution rates in influenza B was observed to be lower than those of influenza A H3N2 and H1N1 viruses (Bedford et al., 2015). There was also observation that mutation rate in HA1 of H1N1 was lower than that of H3N2 during 1980–2000 (Ferguson et al., 2003). These indicate that influenza B and H1N1 viruses are evolving at lower rates than H3N2 virus. Because influenza B had been in human population much longer than H1N1, it suggests that the decrease of evolution rate over time applies to both influenza B and H1N1 viruses. This implies that H3N2 may follow the same pattern. Understanding the changes in evolution rate of seasonal influenza viruses may help us foresee the future trend of seasonal influenza viruses.

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