

Impaired Vaccine-Induced Antibody Response Against Clade 6B H1N1 Viruses in Individuals Before Viral Emergence

Kuan-Ying A. Huang,^{1,2,3} Yhu-Chering Huang,¹ Cheng-Hsun Chiu,^{1,2} Kuo-Chien Tsao,⁴ and Tzou-Yien Lin^{1,5}

¹Division of Pediatric Infectious Diseases, Department of Pediatrics, Chang Gung Memorial Hospital, Taoyuan, Taiwan, ²Molecular Infectious Disease Research Centre, Chang Gung Memorial Hospital, Taoyuan, Taiwan, ³Research Center for Emerging Viral Infections, College of Medicine, Chang Gung University, Taoyuan, Taiwan, ⁴Department of Laboratory Medicine, Chang Gung Memorial Hospital, Taoyuan, Taiwan, and ⁵College of Medicine, Chang Gung University, Taoyuan, Taiwan

Background. Clade 6B H1N1 pdm09 influenza viruses cause substantial morbidity and mortality worldwide. Human antibody profiles elicited upon vaccination against the clade 6B virus are largely unclear before viral emergence.

Methods. Healthy volunteers, including children aged 3–8 years, adolescents aged 9–17 years, and adults, were enrolled before the clade 6B H1N1 outbreak and received the 2013–2014 inactivated influenza vaccine. We determined antibody responses before and after vaccination. Vaccine-induced plasmablast-derived antibodies were tested against H1N1 pdm09 reference and clade 6B viruses.

Results. The majority of the subjects generated robust hemagglutination inhibition and neutralizing antibody responses upon vaccination across the different age groups. Nevertheless, a subset of young adults preferentially produced antibodies that failed to neutralize clade 6B viruses that emerged and circulated in 2014–2016. The hemagglutinin K163Q change at the Sa antigenic site, one of the substitutions that define clade 6B viruses, was responsible for resistance to neutralization by both postvaccination sera and vaccine-induced plasmablast-derived antibodies.

Conclusions. Vaccine-induced antibody immunity is compromised by the antigenic change of H1N1 pdm09 virus in a subset of adults, and this may warrant the incorporation of human serology in the antigenic characterization of virus and vaccine strain selection.

Keywords. inactivated influenza vaccine; human serum; plasmablast-derived polyclonal antibodies; clade 6B H1N1 pdm09 virus; antigenic drift.

Seasonal influenza imposes a huge burden to human health, and ~300 000 influenza-associated deaths occur per year worldwide [1]. The inactivated vaccine has been available since the 1940s, and it has become the mainstay of seasonal influenza prevention [1].

The induction of neutralizing antibodies against hemagglutinin (HA), the head of influenza surface protein, by inactivated vaccine correlates with protection against homologous virus infection [2]. Despite the presence of HA-targeting neutralizing antibodies, the influenza virus could continuously evolve through antigenic drift and escape from the existing antibody

immunity [3]. In vitro studies have shown that single residue substitutions near the receptor-binding site can facilitate the escape of the virus from polyclonal neutralizing antibodies [4, 5]. Evidence also shows that antigenically mismatched vaccine is associated with reduced effectiveness against laboratory-confirmed influenza [1, 6].

Seasonal influenza vaccines are reformulated annually because of frequent antigenic drift in circulating viruses [7]. Determining if circulating viruses are antigenically similar to the reference virus (candidate vaccine virus) primarily relies on the characterization of viruses with postinfection ferret antisera using the hemagglutination inhibition (HI) assay [7]. The antigenic variant is distinguishable from the reference virus with postinfection ferret sera, suggesting that the variants could have epidemic potential.

The 2009 H1N1 pandemic (pdm09) virus emerged in 2009, replaced previous seasonal H1N1 viruses, and has since been circulating in humans. In Taiwan, substantial H1N1 outbreaks occurred in early 2014, resulting in thousands of influenza-associated severe cases [8]. The isolated H1N1 viruses were dominated by the clade 6B clade with a featured residue substitution from lysine (K) to glutamine (Q) at position 163 (H1

Received 4 November 2019; editorial decision 23 November 2019; accepted 6 January 2020.

Correspondence: Kuan-Ying A. Huang, MD, PhD, Division of Pediatric Infectious Diseases, Department of Pediatrics, Chang Gung Memorial Hospital, No. 5, Fu-Shin Street, Kweishan 333, Taoyuan, Taiwan (arthur1726@cgmh.org.tw).

Open Forum Infectious Diseases®

© The Author(s) 2020. Published by Oxford University Press on behalf of Infectious Diseases Society of America. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
DOI: 10.1093/ofid/ofz513

numbering) in the Sa antigenic site of HA [8, 9]. The clade 6B virus was considered antigenically similar to the pdm09 reference virus (A/California/07/2009-like), as determined by HI test with postinfection ferret antisera [10, 11]. However, information on human antibody profiles elicited by the A/California/07/2009-like virus in an inactivated vaccine against the clade 6B virus is limited.

The effectiveness of inactivated vaccine may vary among recipients depending on the age and immune status of the population, as well as antigenic match between circulating and vaccine strains [12]. It has remained unclear if the effectiveness of inactivated vaccine is higher for older or younger children [13, 14]. A few studies have suggested that young children have poorer immunogenicity to influenza vaccine, compared with adolescents or adults [15]. It is intriguing to investigate the effect of age on the immunogenicity of inactivated influenza vaccination for children and adults.

Here, we examined antibody profiles before and after the 2013–2014 inactivated influenza vaccination in young children, adolescents, and adults with HI and microneutralization (MN) assays. We showed that each of the 3 age groups had significantly elevated antibody titers against H1N1 pdm09, H3N2, and type B viruses at day 21 postvaccination compared with baseline titers. Nevertheless, we found that a subset of adults had a predominant antibody response that exhibited reduced or absent neutralizing activities against clade 6B viruses at both the serological and plasmablast-derived antibody levels. The characteristic change of KI63Q at the Sa antigenic site of H1N1 pdm09 virus was responsible for the abolition of antibody binding and neutralization.

METHODS

Study Subjects and Vaccination

The study was in accordance with both Good Clinical Practice guidelines and the ethical standards of the Helsinki Declaration of the World Medical Association. The study protocol and informed consent were approved by the ethics committee of the Chang Gung Memorial Hospital. Healthy volunteers were recruited at the Chang Gung Memorial Hospital, Taiwan, in

September 2013, and written informed consent was obtained from all subjects.

All the enrolled subjects had no history of acute respiratory tract infection or influenza-like illness in the 3 months before recruitment. No subjects developed febrile illnesses or acute respiratory infections during the study period.

Adult subjects were vaccinated with a dose of trivalent split virion, inactivated influenza vaccine (TIV; Adimmune Corporation, Taichung, Taiwan). Adolescent subjects aged 9–17 years were vaccinated with a dose of quadrivalent split virion, inactivated influenza vaccine (QIV; Sanofi Pasteur, Lyon, France). Child subjects aged 3–8 years were vaccinated with 2 doses of QIV (Sanofi Pasteur, Lyon, France), with an interval of 21 days between vaccinations (ClinicalTrials.gov identifier: NCT01967784). The demographic information of enrolled subjects and viral antigens for TIV and QIV is listed in Table 1.

Sample Collection

Blood samples were obtained, in triplicate, before vaccination (day 0), 7 days (day 7), and 21 days (day 21) after a single dose of vaccination for the adult and adolescent groups. For the child group, blood samples, also in triplicate, were obtained before the first dose of vaccination (day 0) and 7 days (day 7) and 21 days (day 21) after the second dose of vaccination. The sera were stored frozen at -80°C until they were analyzed simultaneously. Peripheral blood mononuclear cells were separated, cryopreserved, and stored in liquid nitrogen.

Viruses

Influenza clinical strains from the years 2008–2016, including H1N1 viruses (08-70013, 09-71567, 11-70062, 12-90172, 13-305, 14-50, 14-1581, 16-50007, and 16-50121), H3N2 virus 12-50654, and influenza B viruses (13-456 and 09-71805), were obtained from the Clinical Virology Laboratory, Department of Laboratory Medicine, Chang Gung Memorial Hospital (Taoyuan, Taiwan) (Supplementary Table 1). All viruses were isolated, propagated, and plaque-purified in Madin-Darby Canine Kidney cells with virus growth medium (Dulbecco's Modified Eagle Medium/10% fetal bovine serum/penicillin and

Table 1. Demographic Characteristics of Vaccinated Subjects and Components of the Inactivated Influenza Vaccine

Group	Children 3–8 y	Adolescents 9–17 y	Adults
No.	30	30	25
Age, mean \pm SD (range), y	4.8 \pm 1.3 (3–8)	14.4 \pm 2.5 (9–17)	31.3 \pm 4.4 (24–39)
Male:female	19:11	15:15	6:19
Vaccine antigens	A/California/07/2009 (H1N1) pdm09-like virus; A/Texas/50/2012 (H3N2)-like virus; B/Massachusetts/02/2012-like virus (Yamagata lineage); B/Brisbane/60/2008-like virus (Victoria lineage)	A/California/07/2009 (H1N1) pdm09-like virus; A/Texas/50/2012 (H3N2)-like virus; B/Massachusetts/02/2012-like virus (Yamagata lineage); B/Brisbane/60/2008-like virus (Victoria lineage)	A/California/07/2009 (H1N1) pdm09-like virus; A/Texas/50/2012 (H3N2)-like virus; B/Massachusetts/02/2012-like virus (Yamagata lineage)

streptomycin). The viral titer was determined by hemagglutination assay using human blood group O erythrocytes.

The variant of 09-71567 virus was selected with H1N1 pdm09-neutralizing human monoclonal antibody T3-3A (produced in-house) as previously described [9]. The anti-H1N1 pdm09 activities of T3-3A were tested against the parental strain 09-71567 and the escape variant in the HI and MN assays.

The viral RNA was extracted and purified according to the manufacturer's instruction (QIAamp Viral RNA MiniKit, Qiagen, Hilden, Germany). Reverse transcription and cDNA synthesis were conducted with SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, California, USA). Polymerase chain reaction (PCR) of the HA1 region was conducted using cDNA product with the KOD-PLUS kit (Toyobo, Osaka, Japan). The primers used were as follows: H1-1F 5'-ATCGACTAGCAAAGCAGGGG-3', H1-943R 5'-GAAAKGGGAGRCTGGTGT'TTA-3', H1-736F 5'-AGRATGRACTATTACTGGAC-3', H1-1300R 5'-CCAGGAAACCAATCATCAAC-3'. The PCR product was purified, and sequencing was performed.

HI Assay

Serum antibody titers were determined by HI assay using human blood group O erythrocytes. The protocols were adapted from the World Health Organization (WHO) Laboratory Influenza Surveillance Manual [16]. All sera were treated with receptor-destroying enzyme and then heat-inactivated at 56°C for 30 minutes before testing. A serial 2-fold dilution of sera was made from 1:20 to 1:1280 in PBS in 96-well U-bottom microtiter plates. The virus was adjusted to a concentration of 8 hemagglutination units (HAU)/50 μ L in PBS, and an equal volume of PBS (25 μ L) was added to each well. The plates were mixed using a laboratory shaker, covered, and incubated at room temperature for 20 minutes. Next, 50 μ L of 0.75% erythrocytes was added. The erythrocytes had been prepared fresh, stored at 4°C, and used within 72 hours of preparation. The plates were mixed using a laboratory shaker and covered, and then the erythrocytes settled for 60 minutes at room temperature. The HI titer was determined by reciprocal dilution of the last well, which contained nonagglutinated erythrocytes. A back-titration was performed to verify the correct HAU of virus for each assay. Negative and positive serum controls were included for each assay. All sera were tested in duplicate.

Microneutralization Assay

The microneutralization assay detected the inhibition of virus entry into Madin-Darby canine kidney cells in the absence of trypsin, with antibody present throughout the assay, as previously described [9]. The assay measured antibodies that block binding of HA to sialic acid and antibodies that block the fusion activity of HA. Sera samples were heat-inactivated at 56°C for

30 minutes without the receptor destroying enzyme treatment. All sera were tested in duplicate.

B-Cell Sorting and Plasmablast-Derived Polyclonal Antibodies

Peripheral IgM-negative plasmablasts (CD3^{neg}/CD20^{neg&low}/CD19^{pos}/CD27^{high}/CD38^{high}/IgM^{neg}) and T cells (CD3^{pos}) at day 7 postvaccination were identified and sorted by flow cytometry, as previously described [9, 17]. Freshly sorted plasmablast B cells were resuspended with sorted T cells at 3×10^6 cells/mL and cultured in complete medium at 37°C for 7 days. The medium with secreted plasmablast-derived polyclonal antibodies (PPAb) was collected and stored at -20°C.

Statistics

Serum HI titer $\geq 1:40$ indicated seropositivity. The seropositivity rate was defined as the percentage of subjects with an HI titer $\geq 1:40$. Serum samples with an HI titer $< 1:20$ were assigned a titer of 10. The ratio of geometric mean titers at day 21 postvaccination to the titer prevaccination was defined as the fold increase of the antibody titer. For antibody titers and fold increases in antibody titers, statistical significance was determined by the Mann-Whitney test or the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. The chi-square or Fisher exact test was applied to the seropositivity data for significance testing. *P* values $< .05$ were considered statistically significant. Graphs were prepared by GraphPad Prism software, and statistical analyses were performed by GraphPad Prism and SPSS.

RESULTS

Comparable Hemagglutination Inhibition Antibody Responses Upon Influenza Vaccination Among Different Age Groups

A total of 85 subjects were enrolled, and they received the inactivated 2013–2014 northern hemisphere influenza vaccine during the study period. The enrolled subjects were classified into 3 groups: children aged 3–8 years, adolescents aged 9–17 years, and adults (Table 1).

The majority of subjects possessed a baseline geometric mean HI titer against the H1N1 pdm09 (A/California/07/2009-like) virus, and the adolescent group had a higher prevaccination titer than the other 2 groups ($P = .002$, Kruskal-Wallis test; adolescents vs adults $P < .01$, adolescents vs children $P < .01$, post hoc analysis) (Table 2, Figure 1A). There was no significant difference in the seropositivity rate against the H1N1 pdm09 virus before vaccination among the groups ($P = .0778$, chi-square test) (Table 2). The children group had lower seropositivity rates against type B viruses than the other 2 groups at baseline (Yamagata lineage, $P = .0005$, and Victoria lineage, $P = .0125$, chi-square test) (Table 2). By contrast, the adult group had a lower seropositivity rate against the H3N2 virus than the other 2 groups at baseline ($P = .0075$, chi-square test) (Table 2).

Table 2. Serological Antibody Levels at Baseline, 7 Days After Vaccination, and 21 Days After Vaccination

Group	Children 3–8 y	Adolescent 9–17 y	Adults	P ^a
Hemagglutination inhibition geometric mean titer (lower–upper 95% confidence interval) ^b				
H1N1				
Day 0	52.8 (39.7–70.2)	96.5 (74.3–130.5)	51.3 (37.7–69.8)	.0020 ^c
Day 7	188.1 (141.3–250.3)	145.9 (110.5–192.6)	102.7 (74.7–141.2)	.0350 ^c
Day 21	163.7 (121.3–221.0)	167.6 (127.8–219.8)	117.9 (87.5–159.0)	.1400
H3N2				
Day 0	44.9 (28.3–71.2)	60.6 (45.6–80.6)	28.7 (19.8–41.5)	.0151 ^c
Day 7	167.6 (118.5–236.9)	108.0 (79.9–146.0)	65.9 (43.8–99.2)	.0102 ^c
Day 21	130.0 (92.4–182.7)	94.0 (71.9–123.1)	71.6 (48.3–106.1)	.0112 ^c
B Yamagata				
Day 0	16.3 (12.8–20.6)	48.1 (33.6–68.9)	42.3 (29.2–61.1)	<.0001 ^c
Day 7	72.9 (43.7–121.7)	94.0 (64.9–136.2)	69.6 (48.6–99.8)	.4353
Day 21	66.5 (38.0–116.4)	98.5 (70.5–137.5)	80.0 (55.3–115.7)	.3226
B Victoria				
Day 0	14.1 (10.8–18.5)	23.0 (17.8–29.7)	24.3 (17.5–33.6)	.0015 ^c
Day 7	50.4 (35.1–72.4)	40.9 (29.9–56.1)	31.2 (22.2–43.8)	.1555
Day 21	43.9 (31.7–60.7)	55.3 (40.6–75.3)	32.0 (22.6–45.4)	.0805
Fold increase in hemagglutination inhibition titer, mean ± SD ^d				
H1N1				
Day 7	4.4 ± 3.2	1.6 ± 0.7	3.0 ± 3.5	<.0001 ^c
Day 21	3.9 ± 3.0	1.9 ± 1.3	3.2 ± 3.4	.0013 ^c
H3N2				
Day 7	6.3 ± 11.6	2.2 ± 1.8	3.0 ± 3.1	.0180
Day 21	4.7 ± 6.2	1.7 ± 0.9	3.5 ± 3.9	.0154
B Yamagata				
Day 7	8.0 ± 11.8	2.9 ± 3.8	3.6 ± 6.3	.0014 ^c
Day 21	9.2 ± 15.7	2.4 ± 1.8	4.8 ± 8.5	.0414
B Victoria				
Day 7	4.4 ± 2.6	2.3 ± 1.9	1.4 ± 0.5	<.0001 ^c
Day 21	3.8 ± 2.4	2.9 ± 2.0	1.4 ± 0.7	<.0001 ^c
Seropositivity rate ^e				
H1N1				
Day 0	93% (28/30)	93% (28/30)	76% (19/25)	.0778
Day 7	100% (30/30)	100% (30/30)	92% (23/25)	.0856
Day 21	100% (30/30)	100% (30/30)	96% (24/25)	.2969
H3N2				
Day 0	60% (18/30)	87% (26/30)	48% (12/25)	.0075
Day 7	97% (29/30)	97% (29/30)	76% (19/25)	.0120
Day 21	93% (28/30)	97% (29/30)	80% (20/25)	.0884
B Yamagata				
Day 0	23% (7/30)	70% (21/30)	64% (16/25)	.0005
Day 7	73% (22/30)	87% (26/30)	84% (21/25)	.3810
Day 21	63% (19/30)	90% (27/30)	88% (22/25)	.0176
B Victoria				
Day 0	10% (3/30)	43% (13/30)	36% (9/25)	.0125
Day 7	70% (21/30)	70% (21/30)	52% (13/25)	.2860
Day 21	63% (19/30)	83% (25/30)	60% (15/25)	.1163

^aDifferences in antibody titers and fold increases of antibody titers among the 3 groups were analyzed using the Kruskal-Wallis test. Comparisons of seropositivity rates among the 3 groups were conducted using the chi-square test.

^bThe antibody titers were measured against influenza viruses 09-71567 (H1N1 A/California/07/2009-like), 12-50654 (H3N2 A/Texas/50/2012-like), 13-456 (Yamagata lineage B/Massachusetts/02/2012-like), and 09-71805 (Victoria lineage B/Brisbane/60/2008-like).

^cIn Dunn's post hoc analysis of Kruskal-Wallis multiple comparison, the antibody titer or the fold increase of antibody titer in the adult group was significantly different from that of either the children or the adolescent group.

^dThe ratio of geometric mean titers at day 7 or 21 postvaccination to the titer at baseline was defined as the fold increase of the antibody titer.

^eSerum hemagglutination inhibition titer of ≥1:40 was considered seropositivity.

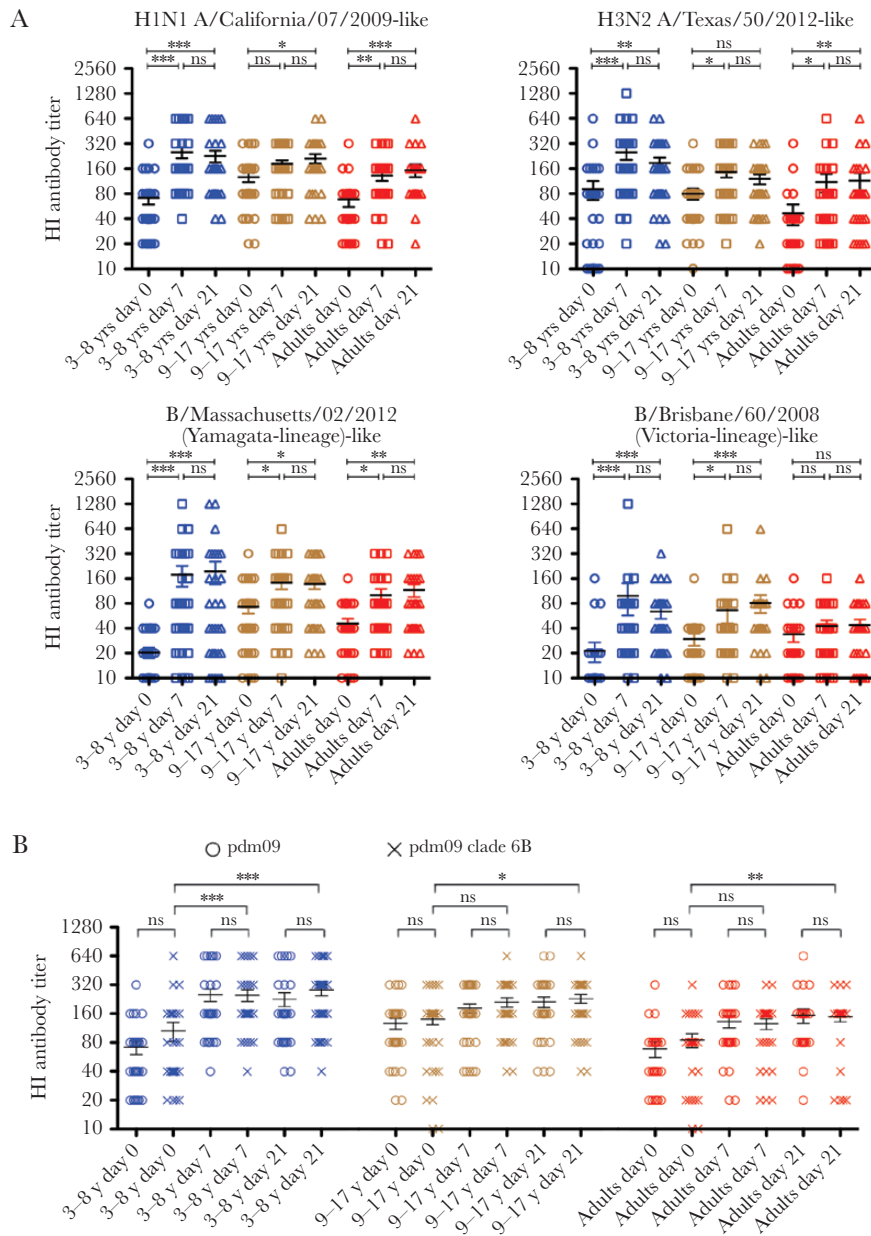


Figure 1. A, Hemagglutination inhibition titers at baseline, day 7 postvaccination, and day 21 postvaccination for children (3–8 years), adolescents (9–17 years), and adults. Adults and adolescents were immunized with the inactivated trivalent 2013–2014 influenza vaccine, and children were immunized with the inactivated quadrivalent 2013–2014 influenza vaccine. The serological antibody level was measured using the hemagglutination inhibition test against influenza viruses 09-71567 (H1N1 A/California/07/2009-like), 12-50654 (H3N2 A/Texas/50/2012-like), 13-456 (Yamagata lineage B/Massachusetts/02/2012-like), and 09-71805 (Victoria lineage B/Brisbane/60/2008-like). B, Hemagglutination inhibition titers against H1N1 viruses 09-71567 (pdm09 strain isolated in 2009) and 14-50 (clade 6B pdm09 strain isolated in 2014) for children (3–8 years), adolescents (9–17 years), and adults. All samples were assayed twice. The bar indicates the mean value \pm standard error of the mean. Difference in the geometric mean titers among the 3 groups was analyzed by Kruskal-Wallis test, with Dunn’s test as the post hoc analysis method. The difference in the geometric mean titers against 09-71567 and 14-50 was analyzed by Mann-Whitney test. * $P < .01$; ** $P < .001$; *** $P < .0001$. Abbreviation: ns, nonsignificant.

After vaccination, elevated geometric mean HI titers against the H1N1 pdm09, H3N2, and type B viruses were detected in all the groups (H1N1 pdm09 day 0 vs day 21, $P < .001$ for children, $P < .05$ for adolescents, and $P < .001$ for adults; H3N2 day 0 vs day 7, $P < .001$ for children, $P < .05$ for adolescents, and $P < .05$ for adults; Yamagata lineage day 0 vs day 21, $P < .001$ for children, $P < .05$ for adolescents,

and $P < .01$ for adults, Kruskal-Wallis with post hoc analysis) (Table 2, Figure 1A). The adult group was administered a trivalent vaccine without the Victoria lineage, and thus the corresponding HI titer change was minimal (Victoria lineage day 0 vs day 21, $P < .001$ for children, $P < .001$ for adolescents, and nonsignificant P value for adults, Kruskal-Wallis with post hoc analysis) (Table 2, Figure 1A).

Similar postvaccination seropositivity rates against the H1N1 pdm09 and H3N2 viruses were detected among the 3 groups (H1N1 pdm09 day 21, $P = .2969$, and H3N2 day 21, $P = .0884$, chi-square test) (Table 2). After vaccination, the child group had a lower day 21 seropositivity rate against type B Yamagata lineage than the other groups (63% in children vs 90% in adolescents vs 88% in adults; $P = .0176$, chi-square test). For type B Victoria lineage, children had a lower day 21 seropositivity rate than adolescents (63% vs 83%), but the difference did not reach statistical significance ($P = .1432$, Fisher exact test).

Antibody Responses to the Clade 6B H1N1 pdm09 Virus Before and After Vaccination

In Taiwan, the H1N1 pdm09 virus was the dominant virus in the 2014–2015 and 2015–2016 seasons, and the circulating strains were designated the clade 6B virus group (Supplementary Table 1) [8]. The clade 6B virus was antigenically indistinguishable from the vaccine reference virus (H1N1 A/California/07/2009-like) using the HI results with postinfection ferret antisera [10, 11]. In this regard, the H1N1 pdm09 antigen for the Northern Hemisphere influenza vaccine remained the same in 2013–2016 [7, 10, 11]. To investigate individual antibody profiles for the clade 6B virus, we compared the baseline and postvaccination antibody titers of clade 6B with those of reference viruses.

At baseline, 70 of the 85 subjects (82%) had positive HI antibody titers (1:40 or more) against the clade 6B virus (Figure 1B). A negative antibody titer (<1:40) at baseline was mostly found in adults (7/25, 28%), followed by adolescents (4/30, 13%) and children (4/30, 13%), but this difference was not statistically significant ($P = .2709$, chi-square test). At baseline, a similar percentage of adults were seronegative to the H1N1 pdm09 virus (7/25 vs 6/25; $P = 1.0000$, Fisher exact test) (Table 2). After vaccination, significantly increased geometric mean HI titers against the clade 6B virus were detected in all 3 groups (day 0 vs day 21, $P < .001$ for children, $P < .05$ for adolescents, and $P < .01$ for adults, Kruskal-Wallis with post hoc analysis) (Figure 1B). Similar geometric mean HI titers against clade 6B and reference viruses were detected in the 3 groups at day 7 and day 21 postvaccination (day 7, $P = .8319$ for children, $P = .4707$ for adolescents, and $P = 1.0000$ for adults; day 21, $P = .1644$ for children, $P = .4323$ for adolescents, and $P = .4362$ for adults, Mann-Whitney test) (Figure 1B). At day 21 postvaccination, 11 out of 15 subjects (4/4 children, 4/4 adolescents, and 3/7 adults) who had a negative baseline HI titer became seropositive for the clade 6B virus (Figure 1B).

As shown in Figure 2A, postvaccination sera raised against the reference virus (A/California/07/2009-like) reacted to the clade 6B virus at titers within 4-fold of the representative homologous titers in children and adolescents. By contrast, 3 postvaccination sera from the adult group (adults C5, C8, and C9; 3/25, 12%) had an 8–16-fold reduction in HI titers. The adults produced robust HI antibody responses to the reference

virus in the 2013–2014 influenza vaccine, but the antibodies failed to react against the clade 6B virus (Figure 2B).

Supplementary Figure 1 shows that the HI antibody titer significantly correlated with the microneutralization titer against the reference virus, indicating that pdm09-neutralizing antibodies elicited after vaccination predominantly targeted the head of pdm09 HA in our subjects.

K163Q Change of pdm09 HA Head Is Responsible for the Reduced Antibody Response

Circulating H1N1 pdm09 viruses that contributed to the 2014–2015 and 2015–2016 seasonal outbreaks carried representative substitutions D97N, S185T, S203T, K283E, and K163Q in the HA globular head domain compared with the pdm09 reference virus (A/California/07/2009-like) [18, 19]. We further tested the sera against a panel of clade 6B viruses isolated between 2014 and 2016 in Taiwan (Supplementary Table 1). Postvaccination sera of adults C5, C8, and C9 had comparable neutralizing titers against pdm09 viruses isolated between 2009 and 2013 that harbored H1 head substitutions D97N, S185T, S203T, or K283E. By contrast, these sera had substantially reduced titers against clade 6B viruses isolated from 2014 to 2016 that harbored additional H1 head substitution K163Q (Figure 2C). For clade 6B viruses isolated in 2016, the additional H1 substitution S162N conferred a potential gain of glycosylation motif at residues 162–164, which also affected the antibody recognition of the Sa antigenic site [20]. In addition, Figure 2C shows that postvaccination control sera from other subjects presented similar inhibitory activities against both earlier pdm09 and recent clade 6B viruses. A control human monoclonal antibody 2-12C, targeting the H1 head residue K130 [9], was not affected by the substitution K163Q in the neutralization assay.

We successfully selected an escape variant of the H1N1 pdm09 virus (09-71567, year 2009 strain) with a neutralizing human monoclonal antibody T3-3A derived from another vaccinated adult [9], and we found a single substitution encoding K163Q in the Sa antigenic site of hemagglutinin. The postvaccination sera of adults C5, C8, and C9 had strong HI and neutralizing activities against parental strain 09-71567 but lost activities against the escape variant (Figure 3A). This result corroborated their serological activities against wild-type clinical isolates, indicating that the H1 head-targeting neutralizing antibodies produced by these vaccinated adults focus on the Sa antigenic site and are sensitive to the HA head K163Q change of pdm09 viruses (Figure 3B).

Plasmablast-Derived Polyclonal Antibodies Associated With Reduced Antibody Response

Plasmablasts are rapidly generated after antigen exposure and transiently circulated through the peripheral blood, and they represent the cellular aspect of antigenic-specific humoral response to vaccination and infection in humans [9, 21].

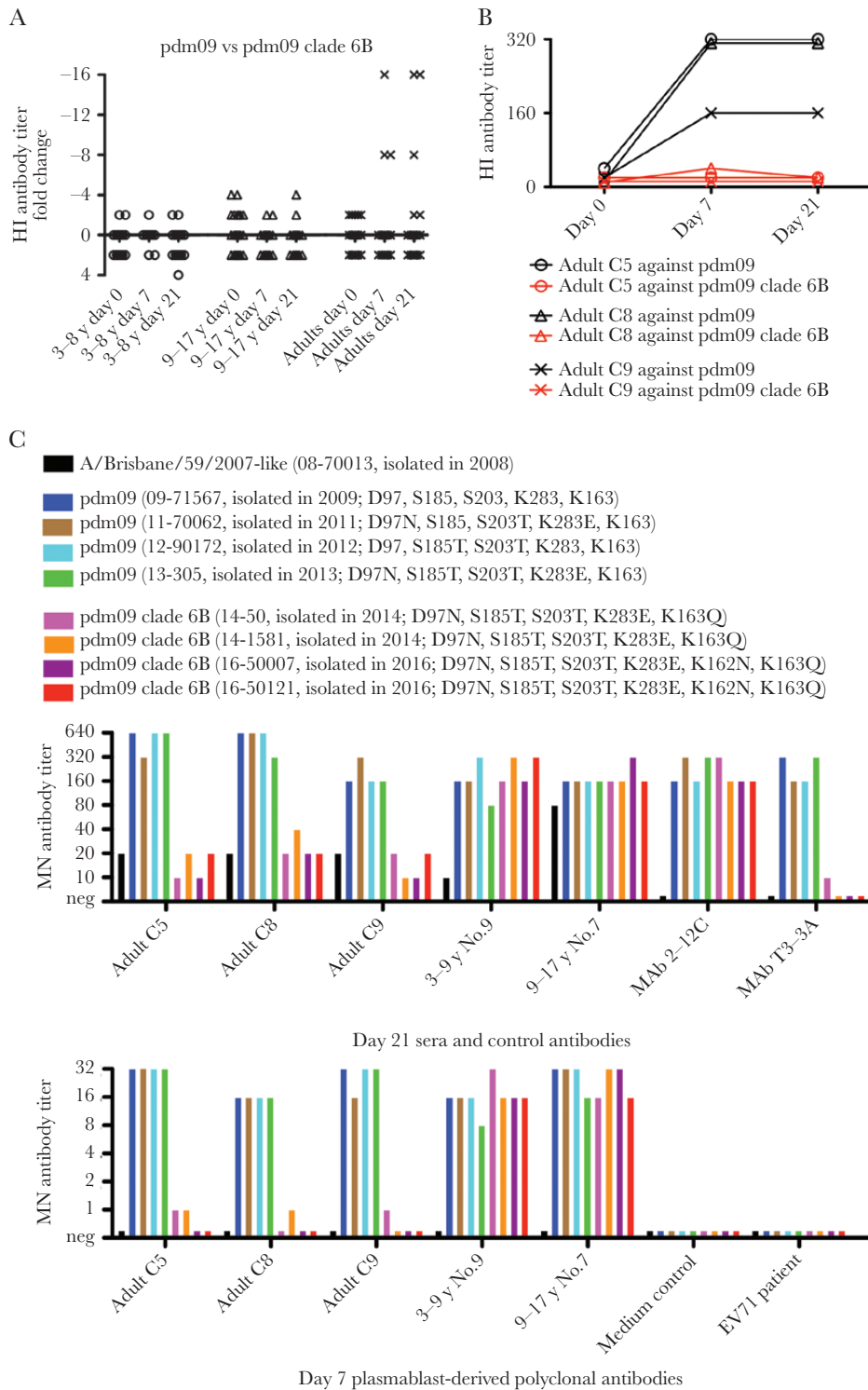


Figure 2. Reduced antibody activities against clade 6B H1N1 pdm09 viruses in adult individuals. A, Postvaccination sera from 3 adults had an 8–16-fold decrease in hemagglutination inhibition titers between the clade 6B virus (strain 14–50) and the reference pdm09 virus (strain 09-71567). B, These 3 adults (adult C5, adult C8, and adult C9, born between 1976 and 1988) generated potent antibody responses to the reference pdm09 virus (strain 09-71567) after vaccination, but these antibodies reacted poorly with the clade 6B virus (strain 14-50). C, Postvaccination sera and plasmablast-derived polyclonal antibodies from adult C5, adult C8, and adult C9 had substantially reduced or absent neutralizing activities against clade 6B viruses isolated from 2014 to 2016, which harbored the characteristic change of K163Q at the hemagglutinin Sa antigenic site. Human monoclonal antibody T3-3A neutralized the pdm09 virus [14], targeted the K163 residue of hemagglutinin, and lost activities against clade 6B viruses as well. The neutralizing activities of antibodies from other vaccinated subjects and control monoclonal antibody 2-12C [14] were not affected by the residue changes of clade 6B viruses. Plasmablast-derived polyclonal antibodies from an enterovirus 71-infected patient did not react with influenza viruses.

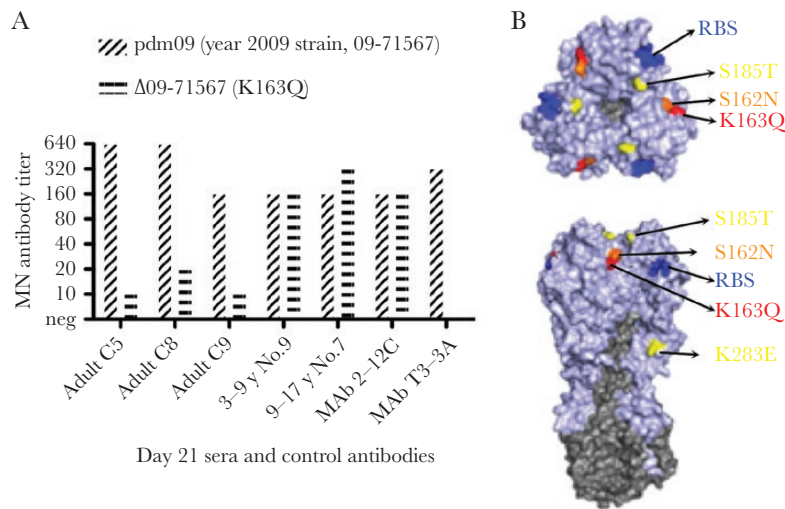


Figure 3. A, Postvaccination sera from adult C5, adult C8, and adult C9 had greatly reduced neutralizing activities against the HA1 K163Q escape variant of H1N1 pdm09 virus 09-71567. Their serological responses are focused on the Sa antigenic site. Human monoclonal antibody T3-3A was a pdm09-neutralizing antibody derived from another vaccinated donor [14], targeted the K163 residue of hemagglutinin, and did not neutralize the K163Q variant either. The neutralizing activities of postvaccination sera from other vaccinated subjects and control monoclonal antibody 2-12C [14] were not affected by K163Q substitution of the pdm09 virus. B, Characteristic residue substitutions of the HA of clade 6B viruses. The top and side surface views of HA trimer of H1N1 A/California/07/2009 (PDB 3LZG) are shown. The HA1 region is colored light blue, and the HA2 region is colored gray. Abbreviation: RBS, receptor-binding site.

Plasmablast-derived antibodies allow for the assessment of vaccine-induced antibody responses without interference from the preexisting cross-reactive antibodies in the serum [17]. We isolated peripheral plasmablast B cells from adult subjects on day 7 after vaccination and cultured them *ex vivo* to collect plasmablast-derived polyclonal antibodies. Functional activity was detected in PPAb, as determined by microneutralization assay. Furthermore, PPAb derived from vaccinated adults C5, C8, and C9 failed to neutralize clade 6B viruses isolated in 2014–2016, whereas they reacted well with an earlier isolate of pdm09 viruses (Figure 2C). By contrast, control antibodies from other vaccinated subjects showed comparable neutralizing activities against clade 6B and an earlier isolate of pdm09 viruses. These results indicate that the plasmablast response strongly correlated with the reactivity of postvaccination sera from adult individuals and their failure to neutralize clade 6B viruses.

DISCUSSION

The influenza season in 2014 was the first since the 2009 pandemic when H1N1 pdm09 viruses predominated; it was characterized by higher rates of medically attended cases and hospitalization among adults compared with earlier years [8, 10]. Linderman et al. investigated the baseline serological titer from healthy donors and found that the circulating H1N1 virus that harbors the HA K163Q substitution was associated with reduced HI titers in 40% of middle-aged adults [22]. Petrie et al. subsequently reported that adults with reduced serological titer to the K163Q H1N1 virus before the 2013–2014 influenza season were more susceptible to clade 6B virus infection than those with higher serological titer [23].

These cross-sectional baseline serological data led to an interpretation that an insufficient level of protective antibodies was linked to increased susceptibility to clade 6B viruses during the influenza season in certain individuals. Similarly, we noted that before the vaccination, a higher percentage of adults were seronegative to the clade 6B virus than children and adolescents (28% vs 13%). We further showed that after receiving the 2013–2014 inactivated vaccine, 73% of subjects (100% of children and adolescents and 43% of adults) who were seronegative at baseline could become seropositive for the clade 6B virus.

We demonstrated that a subset of adult individuals generated robust antibody response to the pdm09 antigen (A/California/07/2009-like) of the 2013–2014 inactivated vaccine, but these antibodies failed to react against the clade 6B variant with the HA K163Q change. These adults were born between 1976 and 1988 (average age \pm SD [range], 31 ± 7 [24–37] years). Two other studies in the United States showed that a proportion of middle-aged adults developed substantially reduced HI titers against the clade 6B virus compared with the A/California/07/2009-like virus after receiving inactivated influenza vaccines containing the A/California/07/2009-like HA component [24, 25]. It was speculated that the imprinting exposure to A/USSR/90/1977-like viruses in childhood may shape the specificity of B-cell response to pdm09 HA and lead to the development of cross-reactive antibodies to a shared dominant epitope, that is, K163, which lies in the conserved space between the HAs of the A/USSR/90/1977-like and A/California/07/2009-like viruses in certain adults [9, 22–24]. Although an in-depth analysis of B-cell clonal signature correlation with the serological response of strong and poor responders to the clade 6B

virus is lacking in the present study, several studies have demonstrated the B-cell clonal signature for human antibody response to the pdm09 HA [9, 26, 27]. Krause et al. identified a panel of pdm09 HA-neutralizing antibody clones from an adult after the 2009 pandemic, which showed convergent antibody rearrangements of IGHV3-7/J6 and recognized the Sa antigenic site of HA [27]. Previously, we characterized the plasmablast B-cell repertoire of an individual who was born in the 1970s and had poor serological titers against the clade 6B virus after receiving the 2011–2012 influenza vaccine [9]. The results showed that his neutralizing antibody response to the pdm09 vaccine antigen involved a dominant set of IGHV3-7 and IGHV3-15 gene usage. In addition, these antibody clonal lineages failed to neutralize the clade 6B virus and focused on a subregion of the HA (K163), which lies in a patch of conserved surface common to the 1977 and 2009 HAs [9].

Increasing evidence implies that the focus of neutralizing antibody responses on immunodominant antigenic epitopes may drive the selection of viral escape variants of circulating viruses [5, 9, 28, 29]. For example, the substitution K163Q at the focused region has been frequently detected in clade 6B pdm09 viruses circulating worldwide. However, it remains unclear if this immune pressure in a fraction of individuals is the primary force driving antigenic evolution at the population level. The antigenic evolution of seasonal influenza viruses is a major contributing factor to disease burden, as it may allow for the spread of variants and reinfection of previously immunized and infected individuals [1, 30]. The virus surface glycoprotein HA is the primary target of the host immune response, and evolutionary selection pressure drives it to acquire mutations to escape immune recognition without eliminating its receptor binding ability. The antigenic change of pdm09 HA was selected in a ferret challenge model or with the ferret antisera under conditions of antibody pressure, and the majority of antigenic mutants possess single substitutions at residues 156–158 in the antigenic site of HA1 [28, 31]. These residues have been conserved in the H1N1 pdm09 reference and recent clade 6B viruses, which also explains that the antigenic drift of emerging clade 6B viruses was not observed with ferret antisera [10, 11, 28]. These studies demonstrated that the pattern of antigenic relatedness in circulating isolates characterized by ferret antisera might not always predict the spectrum of human antibody immunity. The lost or reduced reactivity of human serum toward recent clade 6B pdm09 viruses prompted the update of the H1N1 antigen component in the vaccine [28]. Our results further highlight the need to incorporate human serology in influenza surveillance and vaccine strain selection [28, 29].

Children tend to have poorer immune responses to inactivated influenza vaccine than adults, and a second vaccine dose substantially improves immune response [32]. We showed that young children mount a high antibody response to H1N1

pdm09, H3N2, and type B viruses at day 21 after the second dose of vaccination. In addition, young children had significantly greater fold increases in antibody titers than adolescents and adults. These results are consistent with the data reported in other vaccine studies [15, 33]. Children with preexisting antibodies to influenza antigens from previous infection are more likely to generate a protective immune response after vaccination [34]. Although similar postvaccination seropositivity rates against the H1N1 pdm09 and H3N2 viruses were detected in children and adults in the study, a higher HI antibody titer may be required for the protection of children against influenza A viruses [35].

There are limitations to this study. First, only antibody response was measured before and after vaccination. In the absence of neutralizing antibody when new influenza variants emerged or diminished antibody level, cellular immunity is critical in mitigating virus shedding and disease severity [36]. The cross-reactive T-cell response is directed primarily against conserved internal proteins of influenza virus. However, the magnitude of influenza-specific T-cell response is not determined, and its role in protection against the clade 6B variant is unclear in our subjects. Second, elderly subjects, especially those >65 years old, were not included in the study. This population is at the highest risk for influenza complications and also has poor response to the inactivated influenza vaccination [37]. Nevertheless, recent studies have found that inactivated vaccination confers similar protective efficacy against H1N1 pdm09 influenza in the elderly population and other age groups [20, 38]. Third, human erythrocytes were used in the hemagglutination and HI assays in the study according to the WHO protocol [16]. It has been reported that human erythrocytes may yield lower hemagglutination titer than turkey and chicken erythrocytes in the case of seasonal influenza strains, and the HI titers may vary depending on the species of erythrocytes [39]. We have tested a limited selection of viruses using human and turkey erythrocytes, and both erythrocytes resulted in comparable hemagglutination titers, and the HI titers using human erythrocytes were comparable to those using turkey erythrocytes (Supplementary Table 2).

In conclusion, this study demonstrated that vaccine-induced antibody response could be compromised by the antigenic evolution of H1N1 pdm09 viruses. Furthermore, our findings support that human serology could be a useful alternative to ferret antisera for the determination of antigenic distance between circulating and vaccine viruses.

Supplementary Data

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

Financial support. This work was supported by the Chang Gung Medical Research Program (CMRPG3G0922 and CORPG3J0111) and the Ministry of Science and Technology of Taiwan (MOST 107-2321-B-182A-003-, MOST 108-2321-B-182A-001-, MOST 107-2314-B-182-042-, and MOST 108-2314-B-182-035-).

Potential conflicts of interest. All authors declare no competing financial interests. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Author contributions. K.-Y.A.H. and T.-Z.L. were responsible for the study concept and design. K.-Y.A.H., Y.-C.H., C.-H.C., K.-C.T., and T.-Z.L. carried out the experiments and the statistical analyses. K.-Y.A.H. drafted the manuscript. All co-authors participated in discussions about the interpretation of the findings and critically reviewed the manuscript.

References

1. Iuliano AD, Roguski KM, Chang HH, et al; Global Seasonal Influenza-associated Mortality Collaborator Network. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. *Lancet* **2018**; 391:1285–300.
2. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond)* **1972**; 70:767–77.
3. Wiley DC, Wilson IA, Skehel JJ. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* **1981**; 289:373–8.
4. Koel BF, Mögling R, Chutinimitkul S, et al. Identification of amino acid substitutions supporting antigenic change of influenza A(H1N1)pdm09 viruses. *J Virol* **2015**; 89:3763–75.
5. Davis AKF, McCormick K, Gumina ME, et al. Sera from individuals with narrowly focused influenza virus antibodies rapidly select viral escape mutations in ovo. *J Virol*. **In press**.
6. Belongia EA, Kieke BA, Donahue JG, et al; Marshfield Influenza Study Group. Effectiveness of inactivated influenza vaccines varied substantially with antigenic match from the 2004–2005 season to the 2006–2007 season. *J Infect Dis* **2009**; 199:159–67.
7. Leung VK, Deng YM, Kaye M, et al. Annual report on influenza viruses received and tested by the Melbourne WHO Collaborating Centre for Reference and Research on Influenza in 2016. *Commun Dis Intell (2018)* **2019**; 43.
8. Hsieh YC, Tsao KC, Huang CT, et al. Clinical characteristics of patients with laboratory-confirmed influenza A(H1N1)pdm09 during the 2013/2014 and 2015/2016 clade 6B/6B.1/6B.2-predominant outbreaks. *Sci Rep* **2018**; 8:15636.
9. Huang KY, Rijal P, Schimanski L, et al. Focused antibody response to influenza linked to antigenic drift. *J Clin Invest* **2015**; 125:2631–45.
10. Epperson S, Blanton L, Kniss K, et al; Influenza Division, National Center for Immunization and Respiratory Diseases, CDC. Influenza activity - United States, 2013–14 season and composition of the 2014–15 influenza vaccines. *MMWR Morb Mortal Wkly Rep* **2014**; 63:483–90.
11. Pebody R, Warburton F, Ellis J, et al. Effectiveness of seasonal influenza vaccine for adults and children in preventing laboratory-confirmed influenza in primary care in the United Kingdom: 2015/16 end-of-season results. *Euro Surveill* **2016**; 21:38.
12. Grohskopf LA, Sokolow LZ, Broder KR, et al. Prevention and control of seasonal influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices—United States, 2018–19 influenza season. *MMWR Recomm Rep* **2018**; 67:1–20.
13. Shinjoh M, Sugaya N, Yamaguchi Y, et al; Keio Pediatric Influenza Research Group. Effectiveness of trivalent inactivated influenza vaccine in children estimated by a test-negative case-control design study based on influenza rapid diagnostic test results. *PLoS One* **2015**; 10:e0136539.
14. Shibata N, Kimura S, Hoshino T, et al. Effectiveness of influenza vaccination for children in Japan: four-year observational study using a large-scale claims database. *Vaccine* **2018**; 36:2809–15.
15. Ng S, Ip DK, Fang VJ, et al. The effect of age and recent influenza vaccination history on the immunogenicity and efficacy of 2009–10 seasonal trivalent inactivated influenza vaccination in children. *PLoS One* **2013**; 8:e59077.
16. World Health Organization. *Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza*. Geneva: World Health Organization; **2011**. Available at: https://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en/. Accessed 19 November 2019.
17. He XS, Sasaki S, Narvaez CF, et al. Plasmablast-derived polyclonal antibody response after influenza vaccination. *J Immunol Methods* **2011**; 365:67–75.
18. Broberg E, Melidou A, Prosenk K, Bragstad K, Hungnes O; WHO European Region and the European Influenza Surveillance Network members of the reporting countries. Predominance of influenza A(H1N1)pdm09 virus genetic subclade 6B.1 and influenza B/Victoria lineage viruses at the start of the 2015/16 influenza season in Europe. *Euro Surveill* **2016**; 21:13.
19. Parida M, Dash PK, Kumar JS, et al. Emergence of influenza A (H1N1)pdm09 genogroup 6B and drug resistant virus, India, January to May 2015. *Euro Surveill* **2016**; 21:6–11.
20. Chambers C, Skowronski DM, Sabaiduc S, et al. Interim estimates of 2015/16 vaccine effectiveness against influenza A(H1N1)pdm09, Canada, February 2016. *Euro Surveill* **2016**; 21:30168.
21. Wrammert J, Koutsouanos D, Li GM, et al. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J Exp Med* **2011**; 208:181–93.
22. Linderman SL, Chambers BS, Zost SJ, et al. Potential antigenic explanation for atypical H1N1 infections among middle-aged adults during the 2013–2014 influenza season. *Proc Natl Acad Sci U S A* **2014**; 111:15798–803.
23. Petrie JG, Parkhouse K, Ohmit SE, et al. Antibodies against the current influenza A(H1N1) vaccine strain do not protect some individuals from infection with contemporary circulating influenza A(H1N1) virus strains. *J Infect Dis* **2016**; 214:1947–51.
24. Liu F, Zeng WP, Horner L, et al. Influence of immune priming and egg adaptation in the vaccine on antibody responses to circulating A(H1N1)pdm09 viruses after influenza vaccination in adults. *J Infect Dis* **2018**; 218:1571–81.
25. Barron MA, Frank DN, Claypool D, et al. Antibody responses to influenza A H1N1 vaccine compared to the circulating strain in influenza vaccine recipients during the 2013/2014 season in North America. *J Clin Virol* **2016**; 83:56–60.
26. Jackson KJ, Liu Y, Roskin KM, et al. Human responses to influenza vaccination show seroconversion signatures and convergent antibody rearrangements. *Cell Host Microbe* **2014**; 16:105–14.
27. Krause JC, Tsiabane T, Tumpey TM, et al. Epitope-specific human influenza antibody repertoires diversify by B cell intracloacal sequence divergence and interclonal convergence. *J Immunol* **2011**; 187:3704–11.
28. Liu STH, Behzadi MA, Sun W, et al. Antigenic sites in influenza H1 hemagglutinin display species-specific immunodominance. *J Clin Invest* **2018**; 128:4992–6.
29. Van Reeth K. The post-2009 influenza pandemic era: time to revisit antibody immunodominance. *J Clin Invest* **2018**; 128:4751–4.
30. Petrova VN, Russell CA. The evolution of seasonal influenza viruses. *Nat Rev Microbiol* **2018**; 16:47–60.
31. Guarnaccia T, Carolan LA, Maurer-Stroh S, et al. Antigenic drift of the pandemic 2009 A(H1N1) influenza virus in A ferret model. *PLoS Pathog* **2013**; 9:e1003354.
32. Gilca V, De Serres G, Hamelin ME, et al. Antibody persistence and response to 2010–2011 trivalent influenza vaccine one year after a single dose of 2009 A/S03-adjuvanted pandemic H1N1 vaccine in children. *Vaccine* **2011**; 30:35–41.
33. Zhu FC, Zhou W, Pan H, et al. Safety and immunogenicity of two subunit influenza vaccines in healthy children, adults and the elderly: a randomized controlled trial in China. *Vaccine* **2008**; 26:4579–84.
34. Hurwitz ES, Haber M, Chang A, et al. Studies of the 1996–1997 inactivated influenza vaccine among children attending day care: immunologic response, protection against infection, and clinical effectiveness. *J Infect Dis* **2000**; 182:1218–21.
35. Black S, Nicolay U, Vesikari T, et al. Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza vaccines in children. *Pediatr Infect Dis J* **2011**; 30:1081–5.
36. McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. *N Engl J Med* **1983**; 309:13–7.
37. Haq K, McElhaney JE. Immunosenescence: Influenza vaccination and the elderly. *Curr Opin Immunol* **2014**; 29:38–42.
38. Jackson ML, Chung JR, Jackson LA, et al. Influenza vaccine effectiveness in the United States during the 2015–2016 season. *N Engl J Med* **2017**; 377:534–43.
39. Trombetta CM, Olivieri C, Cox RJ, et al. Impact of erythrocyte species on assays for influenza serology. *J Prev Med Hyg* **2018**; 59:E1–7.