IgM, IgG, and IgA Antibody Responses to Influenza A(H1N1)pdm09 Hemagglutinin in Infected Persons during the First Wave of the 2009 Pandemic in the United States

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The novel influenza A(H1N1)pdm09 virus caused an influenza pandemic in 2009. IgM, IgG, and IgA antibody responses to A(H1N1)pdm09 hemagglutinin (HA) following A(H1N1)pdm09 virus infection were analyzed to understand antibody isotype responses. Age-matched control sera collected from U.S. residents in 2007 and 2008 were used to establish baseline levels of cross-reactive antibodies. IgM responses often used as indicators of primary virus infection were mainly detected in young patient groups (≤5 years and 6 to 15 years old), not in older age groups, despite the genetic and antigenic differences between the HA of A(H1N1)pdm09 virus and pre-2009 seasonal H1N1 viruses. IgG and IgA responses to A(H1N1)pdm09 HA were detected in all age groups of infected persons. In persons 17 to 80 years old, paired acute- and convalescent-phase serum samples demonstrated ≥4-fold increases in the IgG and IgA responses to A(H1N1)pdm09 HA in 80% and 67% of A(H1N1)pdm09 virus-infected persons, respectively. The IgG antibody response to A(H1N1)pdm09 HA was cross-reactive with HAs from H1, H3, H5, and H13 subtypes, suggesting that infections with subtypes other than A(H1N1)pdm09 might result in false positives by enzyme-linked immunosorbent assay (ELISA). Lower sensitivity compared to hemagglutination inhibition and microneutralization assays and the detection of cross-reactive antibodies against homologous and heterologous subtype are major drawbacks for the application of ELISA in influenza serologic studies.

Influenza A(H1N1)pdm09 virus emerged in humans in North America in the spring of 2009 and quickly spread worldwide to cause the first influenza pandemic in >40 years (1). By 1 August, 2010, >214 countries and overseas territories or communities had reported laboratory-confirmed cases of A(H1N1)pdm09 virus infection (2). The A(H1N1)pdm09 virus was a triple-reassortant virus containing genes originating from avian, human, and swine influenza viruses, with a hemagglutinin (HA) gene from the classical swine influenza virus lineage which is genetically and antigenically distinct from genes of former seasonal H1N1 viruses (3).

The enzyme-linked immunosorbent assay (ELISA) has been used to detect influenza virus-specific antibody responses following influenza infection and vaccination (4–6). To detect HA-specific antibody responses, purified HA from virions and various constructs of recombinant HA, including the ectodomain of HA or the globular head domain HA1, have been used (7–9). Previous studies have demonstrated rises in HA-specific serum IgM (86 to 94%), IgG (100%), and IgA (76 to 96%) antibodies following primary influenza virus infection in children and adults (4, 10). Less frequent rises in IgM (5%), IgG (68%), and IgA (74%) were seen among persons experiencing secondary infections (4).

At the onset of the 2009 influenza pandemic, we investigated the potential use of detection of HA-specific IgM for identifying A(H1N1)pdm09 virus infections, in light of the antigenic differences between the HA of this virus and the HA of recent seasonal H1N1 viruses that had circulated in the human population. To address this question, we measured A(H1N1)pdm09 HA (pH1 HA)-specific IgM, IgG, and IgA antibodies by ELISA in persons infected with A(H1N1)pdm09 virus during the first wave of the 2009 pandemic in the United States and in unexposed persons of similar ages.

MATERIALS AND METHODS

Expression and purification of recombinant HA. Based on H3 numbering, cDNA corresponding to the HA ectodomains of A/Texas/05/09 [A(H1N1)pdm09] (GenBank accession number GQ457487 [HA1:11-329 and HA2:1-176]), A/Brisbane/59/07 (H1N1) (GenBank accession number CY030232 [HA1:11-329 and HA2:1-176]), A/Wisconsin/67/05 (H3N2) (GenBank accession number EU103823.1 [HA1:1-329 and HA2:1-175]), A/Vietnam/1203/04 (H5N1) (GenBank accession number EF541404.1 [HA1:11-329 and HA2:1-175]), and A/Portland/Delaware/68/04 (H3N9) (GenBank accession number CY005931.1 [HA1:11-329 and HA2:1-175]) were cloned into the baculovirus transfer vector pAcGp67-A (BD Bioscience, CA). To obtain uncleaved HA0 of A/Vietnam/1203/04 (H5N1), the original protease cleavage site QRERRRKGR was changed to QRETRG. All recombinant HAs (HA) have amino acid residues ADP at the N terminus and a C-terminal tag with a thrombin cleavage site RERRKKGR. Each recombinant HA was expressed and purified from the supernatant of recombinant baculovirus-infected Sf9 cells. Amino acid residues ADP at the N terminus and a C-terminal tag with a thrombin cleavage site RERRKKGR was changed to QRETRG. All recombinant HAs (HA) have amino acid residues ADP at the N terminus and a C-terminal tag with a thrombin cleavage site RERRKKGR.
tion, and receptor-binding activity were confirmed as previously described (12–14).

Sources of serum samples. The serum panel from unexposed persons (3 to 79 years old) was a subset of banked serum specimens selected from different age groups of the 2007 to 2008 National Health and Nutrition Examination Survey (NHANES) collection in the United States (15). Use of the serum samples in the present study was approved by the National Center for Health Statistics Research Ethics Review Board. The serum panel was selected to investigate the age-specific anti-HA antibody response and was not representative of the total 2007 to 2008 NHANES serum panel or the U.S. population.

Sera from 69 A(H1N1)pdm09 virus-infected persons were collected during the first wave of the pandemic (April to July 2009) in the United States. All cases were seropositive either by the demonstration of seroconversion in the microneutralization (MN) and/or hemagglutination inhibition (HI) assays or by the demonstration of achieving an MN titer of ≥40 and an HI titer of ≥20 in a convalescent-phase serum sample (16). Many individuals were also real-time reverse transcription (RT)-PCR-positive for A(H1N1)pdm09, but not all were tested. The collection of these sera was part of the CDC public health emergency response to the pandemic and was considered a nonresearch activity that did not require CDC Institutional Review Board review.

To identify likely primary H1N1 infection among the A(H1N1)pdm09 virus-infected persons, sera from 19 individuals ≤5 years of age were tested by HI assay using two former seasonal H1N1 viruses (A/Brisbane/59/2007 [sH1 HA] and A/New Caledonia/20/1999) (data not shown). Thirteen individuals who had sera with an HI titer of ≤10 to both former seasonal H1N1 viruses were designated primary infection with A(H1N1)pdm09 virus. Thirty-nine A(H1N1)pdm09 virus-infected persons from 17 to 80 years old were defined as nonprimary infection persons based on the age of the case and the assumption that these individuals would have had prior exposure to seasonal H1N1 viruses. Paired acute- and convalescent-phase sera from 30 out of 39 nonprimary infection persons were collected 1 to 7 days and 15 to 52 days after symptom onset, respectively.

ELISA. Purified, trimeric HA was captured via the C-terminal 6-histidine tag on HisGrab nickel-coated plates (Thermo Fisher Scientific, IL) at 200 ng/well. Standard curves were prepared using purified human IgM, IgG, and IgA antibodies (Thermo Fisher Scientific, IL). Phosphate-buffered saline (PBS) containing 0.1% NaN₃ and 0.01% bovine serum albumin (BSA) was used to prepare immunoglobulin (Ig) standards. Each dilution of the appropriate standard series was used to coat wells of each plate at the same time the other wells were coated with HA. Plates were incubated at 4°C overnight. All sera were diluted in antibody diluent buffer (PBS containing 5% [vol/vol] nonfat dry milk [LabScientific, Inc., NJ] and 0.05% [vol/vol] Tween 20 [Sigma, MO] at appropriate dilutions). The WHO international A(H1N1)pdm09 antibody standard, IS/09/194 (National Institute for Biological Standards and Control [NIBSC], Hertfordshire, United Kingdom) (17), was included in each plate as a positive control. The diluted sera were added to each well in duplicate followed by incubation at room temperature for 1 h. The plates were washed with wash buffer (PBS containing 0.05% [vol/vol] Tween 20), and excess horseradish peroxidase-labeled goat anti-human IgM, anti-IgG, or anti-IgA (Kirkegaard & Perry Laboratories [KPL], MD) was added to each well. SureBlue TMB (3,3′,5,5′-tetramethylbenzidine) microwell peroxidase substrate (KPL, MD) was added to each well, and after 5 min the reaction was stopped with TMB stop solution (KPL, MD). Plates were read at 450 nm with a SPECTRAmax plate spectrophotometer (Molecular Devices, CA).

The anti-HA IgM, IgG, and IgA concentrations were calculated according to the corresponding standard curve using SoftMax Pro v. 5 software (Molecular Devices, CA). To reduce intra- and interassay variability, the concentration of each test serum was divided by the concentration of the IS/09/194-positive antibody control. This test-to-positive-control ratio (T/P) reflects the concentration of anti-HA Ig in each serum sample. For acute (≤7 days after symptom onset) and convalescent (15 to 52 days after symptom onset) serum samples, the fold rise of the immunoglobulin concentration was determined. In order to obtain relevant fold rise data, acute T/P values lower than 0.04 were arbitrarily set at 0.04.

Hemagglutination inhibition assay. An HI assay using the A(H1N1)pdm09 virus was performed as described previously (18) with updates as described by the WHO (19). Briefly, all serum samples were treated with receptor-destroying enzyme (RDE) (Denka Seiken) overnight and heated at 56°C for 30 min. Sera were adsorbed with turkey red blood cells (RBCs) when nonspecific agglutinins were observed (≥20). Serial 2-fold dilutions of sera were prepared in PBS starting from 1:10. The HI assay was performed in a V-shaped microtiter plate using 0.5% turkey RBCs. The titer was determined by the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

Statistics. Data were graphed using Microsoft Excel and GraphPad Prism 5 software (GraphPad Software, Inc., CA). Student t and Fisher exact tests were performed using GraphPad Prism 5 software and SAS 9.2 software. Differences were considered statistically significant at P values of <0.05.

RESULTS

HA-specific IgM, IgG, and IgA antibody responses in influenza A(H1N1)pdm09 cases and unexposed subjects. To understand baseline levels of antibody to pH1 HA, the age-specific levels of pH1 HA cross-reactive antibody present in sera collected from U.S. residents prior to the pandemic (unexposed persons) were determined. In this study, the ratio of the Ig concentration of the test sample (T) to the Ig concentration of a positive serum pool (IS/09/194) (P) was less variable than the absolute Ig concentration determined via a standard curve (data not shown). Therefore, the T/P ratio was used as a measurement of Ig concentration. One hundred thirty unexposed persons (3 to 79 years old) were separated into three age groups (3 to 5 years [n = 19], 6 to 15 years [n = 25], and 16 to 79 years [n = 86]). The mean pH1 HA-specific IgM T/P ratios were 0.61, 0.60, and 0.87, respectively, for the three age groups (Fig. 1A). No significant difference in IgM concentration was observed between any two age groups (P > 0.1).

In contrast, the mean IgG and IgA T/P ratios increased as age increased in unexposed persons. For pH1 HA-specific IgG, the mean T/P ratios were 0.01, 0.05, and 0.15 for the age groups 3 to 5, 6 to 15, and 16 to 79 years, respectively (Fig. 1B). The ratio between any two age groups was significantly different (P < 0.01). For pH1 HA-specific IgA, the mean T/P ratios were 0.11, 0.19, and 0.31 for the same age groups, respectively (Fig. 1C). The IgA concentration was significantly different between the youngest (3 to 5 years) and oldest (16 to 79 years) age groups (P < 0.01), but not between the 3- to 5-year and 6- to 15-year (P = 0.12) or between the 6- to 15-year and 16- to 79-year (P = 0.08) age groups. For each Ig isotype, HA-specific antibody concentrations varied over a wide range, especially in the 16- to 79-year age group, as shown by the error bars indicating the standard deviations of the mean T/P ratios (Fig. 1). These results suggest that concentrations of IgG and IgA antibodies that are cross-reactive with pH1 HA increased with age in unexposed persons.

Next, we measured pH1 HA-specific IgM, IgG, and IgA antibody responses in 69 convalescent-phase sera from laboratory-confirmed A(H1N1)pdm09 virus-infected persons in 3 age groups (≤5 years [n = 19], 6 to 15 years [n = 11], and >15 years [n = 39]) and compared them with levels of age-specific baseline antibody present in unexposed persons. The mean IgM T/P ratios were 3.06, 3.30, and 1.19 for the 0- to 5-year, 6- to 15-year, and >15-year age groups of A(H1N1)pdm09 virus-infected persons, respectively.
Antibody titers of test sample to the concentration of the positive control (IS/09/194) in ELISA. A(H1N1)pdm09-infected persons were divided into three age groups, virus-infected persons all in the United States. Immunoglobulin concentrations in convalescent-phase sera are expressed as the ratio of the concentration of the test sample to the concentration of the positive control, IS/09/194 (T/P ratio), that achieved at least 90% specificity for all age groups. The T/P ratio thresholds were 2.0, 0.4, and 0.6 for the IgM, IgG, and IgA responses, respectively. For each group, the mean Ig concentration ± the standard deviation is shown. The P values between study groups are shown. (A) IgM antibody responses. (B) IgG antibody responses. (C) IgA antibody responses.

(FIG 1A). The mean IgM T/P ratios in A(H1N1)pdm09 virus-infected persons of ages ≤5 years and 6 to 15 years were significantly higher than those in the age-matched unexposed groups (P < 0.01) (FIG. 1A). In contrast, for A(H1N1)pdm09 virus-infected persons older than 15 years, there were no significant differences in mean IgM concentrations between A(H1N1)pdm09 virus-infected and age-matched unexposed individuals (P = 0.14). Furthermore, using a threshold IgM T/P of 2.0, which gave a specificity of 90% for sera from unexposed persons (Table 1), 10 of 19 (53%) A(H1N1)pdm09 virus-infected persons ≤5 years old and 4 of 11 (36%) infected persons 6 to 15 years old had detectable IgM responses. On the other hand, as few as 4 of 39 (10%) of the A(H1N1)pdm09 virus-infected persons >15 years old had detectable IgM responses (Table 1). The mean IgG T/P ratios were 1.03, 1.46, and 1.14 and the mean IgA T/P ratios were 0.67, 0.98, and 1.76 in the ≤5-, 6- to 15-, and >15-year-old age groups, respectively (FIG. 1B and C). For both IgG and IgA responses, mean T/P ratios in infected individuals were significantly higher than those in the age-matched unexposed group (P < 0.01).

To further understand IgM responses in A(H1N1)pdm09 virus-infected persons, 13 probable primary H1N1-infected persons were identified among the ≤5-year-old age group, as described in Materials and Methods. IgM responses were significantly higher in these individuals than in those among the nonprimary infection cases (P < 0.01) (FIG. 2). No such differences were observed between primary H1N1 and nonprimary infection cases for IgG and IgA responses (P = 0.92 and P = 0.10, respectively) (FIG. 2). These results suggest that IgM responses were seen largely in

![TABLE 1 Sensitivities and specificities of ELISA to detect anti-A(H1N1)pdm09 HA IgM, IgG, and IgA antibodies in infected and unexposed persons](http://cvi.asm.org/)

| Isotype | Sensitivity (%) for age group: | Sensitivity (%) for: | Specificity (%) for age group: |
|---------|-------------------------------|---------------------|-------------------------------|
|         | ≤5 yr (n = 19) | 6–15 yr (n = 11) | 16–80 yr (n = 39) | Primary infection (n = 13) | Nonprimary infection (n = 39) | 3–5 yr (n = 19) | 6–15 yr (n = 20) | 16–79 yr (n = 86) |
| IgM     | 53%              | 36%                | 10%               | 62%                       | 10%               | 95%              | 100%             | 90%               |
| IgG     | 79%              | 91%                | 72%               | 85%                       | 72%               | 100%             | 100%             | 93%               |
| IgA     | 53%              | 55%                | 82%               | 46%                       | 82%               | 100%             | 96%              | 91%               |

*Assay sensitivities were based on antibody responses obtained in convalescent-phase sera from A(H1N1)pdm09-infected persons. All had MN antibody titers of ≥40 and HI antibody titers of ≥20 (Veguilla et al. [24]). Sera were grouped by age or by primary or nonprimary infections status determined as described in Materials and Methods. Individuals experiencing a primary infection were ≤5 years old, and those with nonprimary infections were >16 years old. Assay specificities were determined using sera from healthy age-matched individuals collected in 2007 and 2008 prior to the emergence of the A(H1N1)pdm09 virus. Seropositivity was based on an immunoglobulin concentration ratio of test serum sample to the concentration of the positive control, IS/09/194 (T/P ratio), that achieved at least 90% specificity for all age groups. The T/P ratio thresholds were 2.0, 0.4, and 0.6 for the IgM, IgG, and IgA responses, respectively.

The sensitivity was determined in 3 age groups (≤5 years, 6–15 years, and 16–80 years).

Statistically significant at a P value of <0.05.
A(H1N1)pdm09 virus-infected persons experiencing the H1N1 subtype for the first time.

**Evaluation of acute- and convalescent-phase serum samples via ELISA with pH1 HA and seasonal H1N1 A/Brisbane/59/2007 HA.** As most of the human population has had prior exposure to influenza viruses, paired human sera are generally required to differentiate recent infection from residual antibodies resulting from prior infection or vaccination, by detecting a rise in antibody titer in a convalescent-phase serum sample compared to an acute-phase sample. We analyzed 30 paired sera (acute, ≤7 days; convalescent, 15 to 52 days) from A(H1N1)pdm09 virus-infected persons with ages ranging from 17 to 80 years who were presumed to have prior exposure to former seasonal H1N1 viruses and therefore were considered to have mounted a nonprimary infection in response to the A(H1N1)pdm09 virus. To understand better the cross-reactive antibody responses to the HAs used in ELISA, we used pH1 and sH1 HAs. Using the criterion of a demonstration of a ≥4-fold rise in antibody concentration as an indication of a recent infection, an IgM response against pH1 or sH1 HAs was detected in only 3% of nonprimary infections (Table 2). On the other hand, a ≥4-fold rise in IgG antibody concentrations was seen in 80% and 67% of A(H1N1)pdm09 virus-infected persons, respectively, when IgG and IgA were measured using pH1 HA.

The proportion with IgG and IgA responses increased to 90% and 77%, respectively, if a less stringent 3-fold rise in antibody concentration was used (Table 2). However, IgG responses were particularly cross-reactive in that 33% of A(H1N1)pdm09 virus-infected persons also exhibited a ≥4-fold rise in antibody concentration detected by sH1 HA and 43% exhibited a ≥3-fold rise to sH1 HA (Table 2). A similar trend was observed in IgA responses (Table 2). These results demonstrated that IgG and IgA, but not IgM, antibody responses were detected in the majority of persons experiencing an A(H1N1)pdm09 virus infection as a nonprimary H1N1 infection.

**Heterosubtypic antibody response detected by IgG ELISA.** To characterize cross-subtype antibody reactivity of the IgG response in ELISA, we selected paired sera from 6 individuals (ages ranging from 4 to 80 years) with a ≥4-fold rise in HI and MN antibody titer to A(H1N1)pdm09 virus and performed ELISA using HAs from A(H1N1)pdm09, A/Brisbane/59/2007 (H1N1), A/Wisconsin/67/2005 (H3N2), A/Vietnam/1203/2004 (H5N1), and A/shorebird/Delaware/68/2004 (H3N9) viruses. All six sera showed the highest fold rise for pH1 HA-specific IgG compared to the other HAs. Nevertheless, ≥4-fold rises in IgG concentrations were observed with some other HAs, in particular with H5 HA and to a lesser extent with H3 HA (Table 3). These results indicate that although the most robust ELISA IgG responses were detected using the homologous pH1 HA subtype, cross-reactivity is readily detected, at least when the ectodomain HA is used as the antigen.

**HI titer to A(H1N1)pdm09 virus and pH1 HA-specific IgG concentration.** To better understand the relationship between the antibody detected by ELISA and that detected by the HI assay, the correlation coefficient (Pearson’s r) between the A(H1N1)pdm09 virus HI titer and the pH1 HA-specific IgG concentration ratio in the convalescent-phase sera from 69 A(H1N1)pdm09 virus-infected persons was calculated. There was a positive association between the pH1 HA-specific IgG antibody response and the HI titer to A(H1N1)pdm09 virus (r = 0.62) (Fig. 3). Low correlation coefficient values were obtained for HI and IgM/IgA (r = 0.11/0.15) (data not shown). Furthermore, HI titers against A(H1N1)pdm09 virus were poorly correlated with sH1-specific IgM, IgG, and IgA responses (all r < 0.29) (data not shown).

**DISCUSSION**

The A(H1N1)pdm09 pandemic highlighted the public health need for simpler and more rapid serologic assays to detect influ-
estimated the frequency of IgM responses in a primary A(H1N1)pdm09 virus infection. This may have occurred if (i) prior exposure to seasonal H1N1 virus had occurred but no antibodies to contemporary seasonal viruses were detected by the HI assay, (ii) collection of the serum samples did not coincide with the peak IgM response, and (iii) there was a sampling bias due to the small number of primary infection cases evaluated in this study.

Recently, Qiu et al. investigated antibody responses in 131 A(H1N1)pdm09-infected persons ranging in age from 0 to 55 years and reported an anti-influenza A IgM response in 84.7% of cases (23). The IgM antibody response was not age related and in the majority of cases occurred in individuals who by virtue of their age had prior H1N1 infection. In addition, IgM antibody was detected as early as 2 days after symptom onset, with only a modest rise in mean geometric mean titers through day 10 after symptom onset (23). One reason for the discrepancies between the studies is that Qiu et al. used a commercial ELISA kit containing seasonal (late 1990s) influenza A(H1N1) and A(H3N2) vaccine antigens and as such likely detected cross-reactive IgM antibody recognizing HA as well as other viral proteins.

IgM detection may be affected by the presence of rheumatoid factor (RF) and/or excess IgG antibody; removal of either IgG or RF has been used to overcome these issues (24). In our studies with paired acute- and convalescent-phase serum samples, IgM concentration did not increase following removal of >99% of IgG in the serum by protein G adsorption (data not shown). Therefore, we believe that detection of pH1 HA-specific IgM antibody in this study was not hindered by the presence of RF and IgG antibody.

Influenza A(H1N1)pdm09 virus infection was not primary for most of the U.S. population who had previous exposure to former seasonal H1N1 infection, even though A(H1N1)pdm09 HA was significantly different from seasonal H1N1 HA, with only 50% amino acid identity in HA1 antigenic sites (3, 25). Further investigations are necessary to evaluate HA-specific IgM detection for other novel HA subtype virus infections, such as H7N9 and H5N1.

Similar to patterns observed with neutralizing antibodies cross-reactive with A(H1N1)pdm09 (26, 27), we found an increase in IgG and IgA cross-reactive antibodies with increasing age of the serum donor for samples collected prior to the emergence of the A(H1N1)pdm09 virus (Fig. 1B and C). These results highlight the need to evaluate age-specific baseline levels of antibody in unexposed human populations, when evaluating the antibody response to a novel influenza virus with any assay, in order to understand age-specific cross-reactive antibodies that might complicate interpretation of seropositivity and inferred infection status.

In this study, antibody responses to influenza HA protein were detected by ELISA using correctly folded and functional HA trimers. Recombinant HA’s bound to nickel-coated plates are expected to resemble the presentation of HA on the virion surface, exposing subtype-specific epitopes on the HA globular domain and potentially restricting antibody access to conserved epitopes on the HA stalk region. This approach reduced but did not eliminate cross-reactions between HA subtypes in ELISA (Table 3 and data not shown).

Antibody binding sites have been identified throughout the HA sequence (28, 29), and it has been observed that the amino acid sequence of the HA2 region of the HA molecule is more conserved between subtypes than the amino acid sequence of the HA1 region (30, 31). The antigenic sites that are subject to antigenic drift are in the HA1 region (32–35). Some cross-subtype neutralizing epitopes in
HA2 have been characterized (36–40). One approach to reducing the cross-reactivity observed in the HA ELISA is to eliminate the more conserved HA stalk, primarily the HA2 region of the HA, and use the globular head domain HA1 as the antigen. Others have shown the feasibility of this approach for the detection of antibody responses to the A(H1N1)pdm09 virus (7, 41).

The detection of the binding antibodies to HA via ELISA or a similar assay is much simpler than detection via functional HI and MN assays. When appropriately paired serum samples are used, the demonstration of a significant rise in antibody titers indicates a recent infection or successful vaccination (4–6, 10). Although we demonstrated ELISA antibody responses against pH1 HA in persons infected with A(H1N1)pdm09 virus during the first wave of the pandemic, stringent threshold criteria were needed for optimal assay specificity based on sera from non-A(H1N1)pdm09-exposed persons. Now that A(H1N1)pdm09 viruses have circulated among humans for >4 years, interpretation of ELISA and other serological assays will need to allow for increased baseline levels of antibodies in the population due to extensive exposure to A(H1N1)pdm09 virus through infection or vaccination.

Overall detection of anti-HA IgM is an insensitive means of detecting infection even in primary infection cases (Fig. 2 and Table 1). When the specificity for the age-matched unexposed population was >90%, IgG and IgA ELISAs were both less sensitive (Table 1) than the MN and HI combination criteria reported previously (16). The major limitation for HA ELISA is the detection of antibodies that are cross-reactive with other HA strains and even other HA subtypes (Table 2, Table 3, and data not shown). This phenomenon was also observed in previous studies (42, 43). This limitation suggests that in the case of cocirculating influenza virus subtypes, an infection with one virus might result in an antibody response to the HAs of other virus subtypes in ELISA, making it difficult to determine which influenza virus caused the infection (Table 3). Indeed, we observed in this study that although rises in IgG were always highest against pH1 HA among paired sera from selected infected persons, 4-fold rises were also detected to other HAs, most notably sH1 and H5, suggesting that A(H1N1)pdm09 virus infection might yield false positives for antibody responses to novel subtypes like H5N1 in ELISA or other direct antibody binding assays. The presence of cross-reactive antibodies might also be associated with the phenomenon of “original antigenic sin” (OAS), in which reexposure to the same subtype influenza induces an HI antibody response to the original virus (44). It is possible that the OAS contributes to those cross-reactive IgM, IgG, and IgA antibodies to seasonal H1 HA (Table 2).

In summary, we detected IgM responses in just over half of the young children tested, including those who lacked evidence of HI antibodies to seasonal H1N1 viruses and were presumably experiencing their first H1N1 subtype infection. HA-specific IgG and IgA antibodies were detected in the serum samples from all age groups of A(H1N1)pdm09 virus-infected persons. However, unexposed individuals had a background level of reactivity to the pH1 HA that increased with age. The IgG antibody response to pH1 HA was cross-reactive with HAs from sH1, H5, and H13 subtypes, suggesting that infections with subtypes other than A(H1N1)pdm09 virus might result in false positives in ELISA. The development of simpler and more rapid serological assays for detection of recent influenza virus infection remains an urgent public health need. Well-defined age-matched control sera from non-exposed individuals are necessary to evaluate the specificity of any improved serological assay. The evaluation of the globular head domain HA1 as an antigen in ELISA and other antibody detection platforms is under way in our laboratory.

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We declare that we have no conflicts of interest.

REFERENCES

1. World Health Organization. 2009. World now at the start of 2009 influenza pandemic: statement to the press by WHO Director-General Dr Margaret Chan. World Health Organization, Geneva, Switzerland. http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html.

2. World Health Organization. 2010. Global alert and response (GAR). Pandemic (H1N1) 2009—update 2012. World Health Organization, Geneva, Switzerland. http://www.who.int/csr/don/2010_08_06/en/.

3. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailier P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD, Jr, Boxrud D, Sambol AB, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI, Cox NJ. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325:197–201. http://dx.doi.org/10.1126/science.1176225.

4. Burlington DB, Clements ML, Meiklejohn G, Phelan M, Murphy BR. 1983. Hemagglutinin-specific antibody responses in immunoglobulin G, A, and M isotypes as measured by enzyme-linked immunosorbent assay after primary or secondary infection of humans with influenza A virus. Infect. Immun. 41:540–545.

5. Karron RA, Talaat K, Luke C, Callahan K, Thumar B, Dolenzore S, McAuliffe J, Schappell E, Sugiouta A, Mills K, Chen G, Lamirande E, Coelingh K, Jin H, Murphy BR, Kemble G, Subbarao K. 2009. Evaluation of two live attenuated cold-adapted H5N1 influenza virus vaccines in healthy adults. Vaccine 27:4953–4960. http://dx.doi.org/10.1016/j.vaccine.2009.05.099.

6. Talaat KR, Karron RA, Callahan KA, Luke CJ, Di Lorenzo SC, Chen GL, Lamirande EW, Jin H, Coelingh KL, Murphy BR, Kemble G, Subbarao K. 2009. A live attenuated H7N3 influenza virus vaccine is well tolerated and immunogenic in a phase I trial in healthy adults. Vaccine 27:3744–3753. http://dx.doi.org/10.1016/j.vaccine.2009.03.082.

7. Alvarez MM, Lopez-Pacheco F, Aguilar-Yanez JM, Portillo-Lara R, Mendoza-Ochoa GI, Garcia-Echaurren S, Freiden P, Schultz-Cherry S, Zertuche-Guerra MI, Bulnes-Arundis D, Salgado-Gallegos J, Elizondo-Montemayor L, Hernandez-Torre M. 2010. Specific recognition of influenza A(H1N1) 2009 antibodies in human serum: a simple virus-free ELISA method. PLoS One 5:e10176. http://dx.doi.org/10.1371/journal.pone.0010176.

8. Aranikale VA, Virkar RG, Tandale BV, Ingle NB. 2010. Utility of pandemic 2009 H1N1 influenza virus recombinant hemagglutinin protein-based enzyme-linked immunosorbent assay for surveillance. Clin. Vaccine Immunol. 17:1481–1483. http://dx.doi.org/10.1128/CVI.00223-10.

9. Murphy BR, Phelan MA, Nelson DL, Yarchoan R, Tierney EL, Alling DW, Chanock RM. 1981. Hemagglutinin-specific enzyme-linked immunosorbent assay for antibodies to influenza A and B viruses. J. Clin. Microbiol. 13:534–560.

10. Murphy BR, Nelson DL, Wright PF, Tierney EL, Phelan MA, Chanock
18. Kendal AP, Pereira MS, Skehel JJ. 1982. Concepts and procedures for laboratory-based influenza surveillance. Centers for Disease Control and Prevention, Atlanta, GA.

19. WHO Global Influenza Surveillance Network. 2011. Manual for the laboratory diagnosis and virological surveillance of influenza. WHO Press, Geneva, Switzerland.

20. Laurie KL, Huston P, Riley S, Katz JM, Willison DJ, Tam JS, Mounts AW, Hoschler K, Miller E, Vandemeulebroeke B, Van Kerkhove MD, Nicoll A. 2013. Influenza serological studies to inform public health action: best practices to optimise timing, quality and reporting. Influenza Other Respir. Viruses 7:211–224. http://dx.doi.org/10.1111/j.1750-2699.2012.00704.x

21. Erdman DD, Anderson LJ, Adams DR, Stewart JA, Markowitz LE, Bellini WJ. 1991. Evaluation of monoclonal antibody-based capture enzyme immunoassay for detection of specific antibodies to measles virus. J. Clin. Microbiol. 29:1496–1471.

22. Zambon M. 1998. Laboratory diagnosis of influenza. Blackwell Science, Oxford, United Kingdom.

23. Qiu C, Tian D, Wan Y, Zhang W, Zhu Y, Ye R, Song Z, Zhou M, Yuan S, Shi B, Wu M, Liu Y, Gu S, Wei J, Zhou Z, Zhang X, Zhang Z, Hu Y, Yuan Z, Xu J. 2011. Early adaptive humoral immune responses and virus clearance in humans recently infected with pandemic 2009 H1N1 influenza virus. PLoS One 6:e22603. http://dx.doi.org/10.1371/journal.pone.0022603

24. Salonen EM, Vaheri A, Suni J, Wager O. 1980. Rheumatoid factor in acute viral infections: interference with determination of IgM, IgG, and IgA antibodies in an enzyme immunoassay. J. Infect. Dis. 142:250–255. http://dx.doi.org/10.1093/infdis/iij035

25. Xu R, Eikert DC, Krause JC, Hai R, Crowe JE, Jr, Wilson IA. 2010. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. Science 328:357–360. http://dx.doi.org/10.1126/science.1186430.

26. Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, Liu F, Dong L, DeVos JR, Garriott PM, Brammer TL, Cox NJ, Tumpey TM, Katz JM. 2009. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. N. Engl. J. Med. 361:1934–1952. http://dx.doi.org/10.1056/NEJMoa0906453.

27. Reed C, Katz JM, Hancock K, Balish A, Fry AM, H1N1 Serosurvey Working Group. 2012. Prevalence of seropositivity to pandemic influenza A/H1N1 virus in the United States following the 2009 pandemic. PLoS One 7:e48187. http://dx.doi.org/10.1371/journal.pone.0048187

28. Zhao R, Cui S, Guo L, Wu C, Gonzalez R, Paranhos-Bacela G, Vernet G, Wang J, Hung T. 2011. Identification of a highly conserved H1 subtype-specific epitope with diagnostic potential in the hemagglutinin protein of influenza A virus. PLoS One 6:e23374. http://dx.doi.org/10.1371/journal.pone.0023374.

29. Khurana S, Cheerawala W, Castellino F, Manisiewicz J, King LR, Honor- konja MA, Rock MT, Gubareva L, Giudice G, Rappuoli R, Golding H. 2010. Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. Sci. Transl. Med. 2:15ra15. http://dx.doi.org/10.1126/scitranslmed.3006624.

30. Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y, Nakajima K. 1991. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology 192:472–485. http://dx.doi.org/10.1016/0042-6822(91)90588-3.

31. Krystal M, Elliott RM, Benz EW, Jr, Young JF, Palese P. 1982. Evolution of influenza A and B viruses: conservation of structural features in the hemagglutinin genes. Proc. Natl. Acad. Sci. U. S. A. 79:4890–4894. http://dx.doi.org/10.1073/pnas.79.15.4800.

32. Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31:417–427. http://dx.doi.org/10.1016/0022-8477(82)90135-0.

33. Poole AM, Balfour HH, Jr, McHale EP, Balfour RB, Jr, Rappuoli R, Golding H. 2010. Structure of the uncleaved human H1 hemagglutinin from the ex- cited 1918 influenza virus. Science 303:1866–1870. http://dx.doi.org/10.1126/science.1099337.

34. Yang H, Carney P, Stevens J. 2010. Structure and receptor binding properties of a pandemic H1N1 virus hemagglutinin. PLoS Curr. 2:RRN1152.

35. Yang H, Carney PJ, Chang JC, Villanueva JM, Stevens J. 2013. Structural analysis of the hemagglutinin from the recent 2013 H7N9 influenza virus. J. Virol. 87:12343–12346. http://dx.doi.org/10.1128/JVI.01854-13.

36. Yang H, Chang JC, Guo Z, Carney PJ, Shore DA, Donis RO, Cox NJ, Villanueva JM, Klomov AI, Stevens J. 2014. Structural stability of influenza A/H1N1 pdm09 virus hemagglutinins. J. Virol. 88:4828–4838. http://dx.doi.org/10.1128/JVI.02278-13.

37. Centers for Disease Control and Prevention. 2014. National health and nutrition examination survey. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov/nchs/chnhes/search/chnhes007_08.aspx.

38. Veguilla V, Hancock K, Schiffer J, Gargiullo P, Lu X, Aranio D, Branch A, Dong L, Holiday C, Liu F, Stewart-Clark E, Sun H, Tsg B, Wang D, Whaley M, Bai Y, Cronin L, Browning D, Bababneh H, Noland H, Thomas L, Foster L, Quinn CP, Soroko SD, Katz JM. 2011. Sensitivity and specificity of serologic assays for detection of human infection with 2009 pandemic H1N1 virus in U.S. populations. J. Clin. Microbiol. 49:2210–2215. http://dx.doi.org/10.1128/JCM.00229-11.

39. Wood JM, Major D, Heath A, Newman RW, Hoschler K, Stephenson I, Clark T, Katz T, Jambon MC. 2012. Reproducibility of serology assays for pandemic influenza H1N1: collaborative study to evaluate a candidate WHO International Standard. Vaccine 30:210–217. http://dx.doi.org/10.1016/j.vaccine.2011.11.019.

40. Kendall AP, Skehel JJ. 1982. Concepts and procedures for laboratory-based influenza surveillance. Centers for Disease Control and Prevention, Atlanta, GA.

41. WHO Global Influenza Surveillance Network. 2011. Manual for the laboratory diagnosis and virological surveillance of influenza. WHO Press, Geneva, Switzerland.

42. Xu R, Eikert DC, Krause JC, Hai R, Crowe JE, Jr, Wilson IA. 2010. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. Science 328:357–360. http://dx.doi.org/10.1126/science.1186430.

43. Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, Liu F, Dong L, DeVos JR, Garriott PM, Brammer TL, Cox NJ, Tumpey TM, Katz JM. 2009. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. N. Engl. J. Med. 361:1934–1952. http://dx.doi.org/10.1056/NEJMoa0906453.

44. Reed C, Katz JM, Hancock K, Balish A, Fry AM, H1N1 Serosurvey Working Group. 2012. Prevalence of seropositivity to pandemic influenza A/H1N1 virus in the United States following the 2009 pandemic. PLoS One 7:e48187. http://dx.doi.org/10.1371/journal.pone.0048187.

45. Zhao R, Cui S, Guo L, Wu C, Gonzalez R, Paranhos-Bacela G, Vernet G, Wang J, Hung T. 2011. Identification of a highly conserved H1 subtype-specific epitope with diagnostic potential in the hemagglutinin protein of influenza A virus. PLoS One 6:e23374. http://dx.doi.org/10.1371/journal.pone.0023374.