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Running title: *Cross-reactive immunity post pandemic H1N1*

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ABSTRACT

2009 H1N1 pandemic influenza (A(H1N1)pdm09) virus infected large numbers of people worldwide. Recent studies suggest infection with A(H1N1)pdm09 virus elicited cross-reactive anti-hemagglutinin (HA) memory B cell response to conserved regions of HA. However, the breadth and magnitude of cross-reactive immunity in children and adults following A(H1N1)pdm09 infection are unknown. Methods: We investigated serum anti-HA immunity to a number of group-1 and -2 viruses in children and adults using hemagglutination inhibition (HAI), enzyme-linked immunosorbent assay and virus neutralization assay. Results: Applying hemagglutination inhibition (HAI) titres≥40 against A(H1N1)pdm09 as threshold of sero-positivity, we observed significantly higher levels of anti-HA antibodies to a number of virus subtypes, including those neutralizing H5N1, in subjects with HAI titre≥40 than those with HAI<40. Adults demonstrated broader and stronger cross-reactive anti-HA antibodies than children, including cross-reactive anti-HA1 and -HA2 antibodies. By comparison, individuals with serologic evidence of recent exposure to seasonal H1N1 or H3N2 did not show such broad cross-reactive immunity. Conclusion: Our results suggest individuals exposed to A(H1N1)pdm09 virus developed a broad and age-associated cross-reactive anti-HA immunity which may have important implications for future vaccination strategies to enable protection against a broader range of influenza viruses.

Key words: *influenza virus; pandemic H1N1 infection; serum antibody, cross-reactive immunity; anti-hemagglutinin antibody; virus neutralization.*

1. INTRODUCTION

2009 pandemic H1N1 virus caused infection in large numbers of people worldwide. Recent studies suggest infection with A(H1N1)pdm09 virus elicited cross-reactive anti-hemagglutinin (HA) memory B cell response to conserved regions of HA (1-4) which has the potential to provide protection against different virus subtypes . It remains unclear whether and to what extent in terms of breadth and magnitude, humans infected with A(H1N1)pdm09 virus developed cross-reactive immunity, and whether this develops in both children and adults. Understanding the cross-reactive immunity following A(H1N1)pdm09 infection may provide important information on future vaccination strategies against influenza.

Anti-HA antibodies play a key role in protection. HA consists of two domains: a globular head, composed of part of HA1, and a stalk structure, composed of HA2 and a small portion of HA1 (5). The globular head contains the variable region and is the major target for neutralizing antibodies that inhibit virus binding and are traditionally detected by hemagglutination inhibition assay (HAI). The stalk domain is more conserved. Recent studies have suggested that antibodies targeting stalk region also have neutralizing activity and contribute to cross-protective immunity (3, 6-8). There are 18 different subtypes of HA, clustered into group1 and group 2 based on the molecular relatedness of HA (9, 10).

In this study, we analyzed the magnitude and breadth of cross-reactive anti-HA immunity in children and adults following A(H1N1)pdm09 pandemic.

2. METHODS

2.1. Patients and samples. Peripheral blood samples from children and adults from the UK (aged 2-35) undergoing elective adenotonsillectomy due to upper airway obstruction were collected between April 2010 and March 2012, as part of a study on memory B cell responses to influenza virus following A(H1N1)pdm09 infection (4). Subjects were excluded

if immunocompromised or previously vaccinated against influenza. The study was approved by Liverpool Pediatric Research Ethics Committee(08/H1002/97) and written informed consent was obtained.

2.2. Influenza HA antigens. Purified recombinant HA0 (whole-length HA) of pH1N1(A/California/04/2009), seasonal H1N1 (sH1N1) (A/H1N1/Brisbane/59/2007), seasonal H3N2 (sH3N2) (A/Brisbane/10/2007), avian H5N1 (aH5N1) (A/Vietnam/1203/2004), H2N2 (A/Singapore/1/57), aH7N3 (A/Canada/RV444/04), aH7N7 (A/Netherland/219/03) and aH7N9 (A/Anhui/1/2013) viruses were obtained from BEI Resources (Manassas,VA). The recombinant HAs of pH1N1, sH1N1 and H7N9 viruses contain a C-terminal histidine tag and were produced in High Five™ insect cells using a baculovirus expression vector system (11). The HAs were purified from cell culture supernatant by Immobilized-metal affinity chromatography (IMAC) and contain a trimerizing (foldon) domain (11). The recombinant HAs of sH3N2 and aH5N1, H2N2, H7N3, H7N7 viruses were full length glycosylated HA that were produced in Sf9 insect cells using a baculovirus expression vector system, and membrane-extracted from infected cells and purified under native conditions by affinity chromatography that preserve their biological activity and tertiary structure. The purified HA forms trimers (12). Recombinant HA1 and HA2 subunit proteins (BEI Resources and SinoBiologicals) were his-tagged and produced using a baculovirus expression vector system and was purified by immobilized metal affinity chromatography. The proteins were re-folded into a soluble form and were shown to react to specific anti-HA1/HA2 antibodies by Western blotting. HA1 proteins were derived from the same subtype viruses as above for HA0. HA2 proteins were derived from pH1N1(A/California/04/2009) and aH5N1 (A/Vietnam/1203/2004).

2.3. Hemagglutination inhibition (HAI) assay. HAI assays were performed following standard methods (13) at Health Protection Agency (London, UK). The virus strains included

A(H1N1)pdm09 (A/England/195/2009, the prototype UK isolate antigenically and genetically related to A/California/4/2009), A/H1N1/Brisbane/59/2007 and A/H3N2/Brisbane/10/2007. The starting dilution for HAI assay was 1:10 and the threshold titre for positivity was ≥40 (four times the minimum level of detection). A similar threshold was used previously and shown to be a specific marker of recent infection (13, 14). The HAI assay detects HA-specific antibody titres to individual subtypes of influenza virus in serum samples. It is generally thought to measure the neutralizing antibody level targeting the variable region (globular head) of HA, and is specific to the virus subtype (eg. pH1N1) to be analyzed.

2.4. Measurement of HA-specific antibodies by ELISA. Anti-HA IgG antibodies were analyzed as previously described (4). Briefly, ELISA plates were coated with recombinant HA0, HA1 or HA2 proteins. Following blocking, serum samples were incubated at optimized dilutions. Alkaline phosphatase-conjugated anti-human IgG was added followed by addition of p-nitrophenyl phosphate, and optical density measured. As there was no single universal reference serum standard for measurement of different antibodies, individual reference sera containing relatively high antibody titers to different HA were used as individual standards. Human convalescent sera from a subject with confirmed A(H1N1)pdm09 infection and another with confirmed aH5N1 infection (BEI Resources) were used as reference standards for anti-pH1N1 and -aH5N1 antibodies respectively. Sandoglobulin (Sandoz, UK) which contains relatively high antibody titres to HA of other seasonal viruses was used as a reference standard for antibodies to other subtypes. Following a standard ELISA procedure with the use of serial dilutions of individual reference standards, the antibody units were assigned to each standard as the reciprocal of the dilution giving an $OD_{405} = 1$. Once the antibody the antibody units were assigned for the standards, they were used on each ELISA plate to create a standard curve for analysis of all samples (15). The specificity of the ELISAs

measuring anti-HA antibodies was confirmed by antigen-specific inhibition assays following methods as described previously (16) by adsorption with individual HA and subunits HA1 or HA2. These ELISA assays measure the antibody titers to HA0 (whole HA molecule), HA1 (mainly the head of HA), or HA2 (main part of HA stalk) respectively, and the antibody titers detected represent all the antibodies binding to the coating HA antigen thus may include both neutralizing and non-neutralizing antibodies.

2.5. Influenza pseudotype virus neutralization assay. Lentivirus pseudotypes expressing HA from different influenza strains can be used as a safer surrogate of the whole virus to measure the neutralizing antibody titers to HA in serum samples. The assay was shown to detect neutralizing antibodies to both the head and stalk of influenza HA (8). The construction of lentiviral pseudotypes with HA envelope glycoproteins derived from avian H5N1 (A/Vietnam/1194/2004 and Anhui/1/2005) and A/Italy/1082/1999 H7N1 viruses were described previously (17, 18). Virus neutralization assay was performed as described previously (4, 19). Serum samples were 2-fold serially diluted and mixed with each pseudotype virus at a 1:1 (vol/vol) ratio. After incubation at 37°C for 1 h, $1x10^4$ HEK293T cells were added to each well of a white 96-well flat-bottomed tissue culture plate. Firefly RLU values were determined 48 h later by luminometry using a Bright-Glo assay system (Promega, UK).

2.6. Statistical analysis. Differences in antibody titers between different groups were analyzed by Students *t* test. Association between two factors was analyzed by Pearson's correlation. A p value of $p<0.05$ was considered statistically significant.

3. RESULTS

3.1. HAI positivity to pH1N1, sH1N1 and sH3N2 viruses and correlation with anti-HA antibodies. A total of 134 subjects including 78 children (aged 2-16) and 56 adults (17-35)

were studied. The prevalence of HAI positivity to pH1N1, sH1N1 and sH3N2 viruses based on HAI titers ≥40 were respectively 56.4%, 19.2% and 73.1% in children, and 48.2%, 12.5% and 84.0% in adults. No significant difference was found in HAI positivity between children and adults. There were positive correlations between anti-HA0 or -HA1 antibody titers and HAI titers to pH1N1, sH1N1 and sH3N2 viruses respectively in both children and adults. (Figure 1).

3.2. Infection with A(H1N1)pdm09 elicited cross-reactive antibodies in children and adults.

To determine the breadth of cross-reactive immunity following A(H1N1)pdm09 infection, serum IgG antibodies to HA0 and HA1 of a number of group -1 and -2 virus subtypes were compared between subjects with and without serologic evidence of infection. As shown in Figure 2a, in children, GMT of anti-HA0 antibody titers to pH1N1, sH1N1, H2N2 and aH5N1 in those with HAI≥40 were higher than in those with HAI<40 ($p<0.01$), and this difference remained significant when subjects with sH1N1 HAI≥40 or those with sH3N2 HAI≥40 were excluded from the analysis. However, there was no difference in anti-HA0 titers to sH3N2, aH7N3, aH7N7 and aH7N9 between the two groups. By contrast, in adults, anti-HA0 titers to pH1N1, sH1N1, aH5N1, sH3N2, H2N2, aH7N3, aH7N7 and aH7N9 strains were all higher in those with pH1N1 HAI≥40 than in those with HAI<40 (Figure 2b, $p<0.001$). Again, this difference remained significant when subjects with sH1N1 HAI ≥40 were excluded.

When anti-HA0 antibody titers were compared between adults and children, it was shown that among subjects with pH1N1 HAI≥40, GMT of antibodies to sH1N1, sH3N2, H2N2, aH5N1, aH7N3, aH7N7 and aH7N9 in adults were all higher than in children, despite that anti-HA0 antibody titers to pH1N1 were comparable (Figure 2c).

Antibody titers to HA1 domain of pH1N1, sH1N1, sH3N2, aH5N1, H2N2, aH7N7, aH7N9 strains were analyzed and compared between individuals with HAI≥40 and HAI<40 against pH1N1. Overall, anti-HA1 antibody titers to aH5N1, H2N2, aH7N7, aH7N9 were low in both children and adults, and no difference was found between subjects with HAI≥40 and those with HAI<40. However, higher anti-HA1 titers to sH1N1 as well as pH1N1 were found in children with HAI≥40 than in those with HAI<40 (Figure 3a). Also, higher anti-HA1 titers to sH1N1 and sH3N2 as well as pH1N1 were observed in adults with HAI≥40 than in those with HAI<40 (Figure 3b).

Anti-HA2 antibody titers to pH1N1 and aH5N1 were also analyzed and shown to be higher in subjects with pH1N1 HAI≥40 than in those with HAI<40 in both children and adults (Figure 3c+d).

3.3. Seasonal H1N1 and H3N2 elicited less cross-reactive antibodies than A(H1N1)pdm09 virus. To establish whether exposure to previous seasonal viruses also elicited cross-reactive antibodies, anti-HA0 antibody titres were compared between subjects with HAI≥40 and HAI<40 against sH1N1 and sH3N2 respectively. There was no significant difference between children with sH1N1 HAI ≥40 and sH1N1 HAI<40 in anti-HA0 titers to pH1N1, H2N2, aH5N1 sH3N2, and H7 strains, when only subjects with pH1N1 HAI <40 were considered (data not shown). However, when antibodies were analysed from subjects with both pH1N1 and sH1N1 HAI≥40, antibody titers to sH1N1, H2N2 and aH5N1 were higher than in those with pH1N1 HAI≥40 but sH1N1 HAI<40 (Figure 4a).

Similarly, there was no significant difference between subjects with sH3N2 HAI ≥40 and sH3N2 HAI<40 in anti-HA0 titers to pH1N1, sH1N1, H2N2 and aH5N1 when only subjects with pH1N1 HAI<40 were analyzed. Also, no difference was found between subjects with both pH1N1 and sH3N2 HAI≥40 and those with pH1N1 HAI≥40 but sH3N2 HAI<40 in

antibody titers to sH1N1, H2N2 and aH5N1 (data not shown). However, there was a difference in anti-HA0 titers to aH7N3, aH7N7 and aH7N9 between adults with sH3N2 HAI≥40 and sH3N2 HAI<40, although the anti-HA0 titers were generally low (Figure 4b).

3.4. Cross-reactive neutralizing antibodies following A(H1N1)pdm09infection. To determine whether cross-reactive antibodies detected have virus-neutralizing activity, serum samples were assayed by virus neutralization against pseudotype aH5N1 and aH7N1 viruses. The virus neutralization titers were shown to correlate with anti-HA titers to aH5N1 (Vietnam/1194/2004), aH5N1(Anhui/01/2005) and aH7N7 strains ($r=0.68$, 0.78, 0.50 respectively, $p<0.01$). Neutralizing antibodies to the two aH5N1 strains were shown to be higher in children with pH1N1 HAI≥40 than in those with pH1N1 HAI<40, although no difference in that to aH7N1 strains (Figure 4c). However, in adults, those with pH1N1 HAI≥40 had higher neutralization titers to the aH5N1 strains as well as H7N1 strain, than those with pH1N1 HAI<40 (Figure 4d).

4. DISCUSSION

We analyzed the breadth and magnitude of cross-reactive anti-HA immunity to a number of group-1 and -2 viruses following A(H1N1)pdm09 infection. We demonstrated that anti-HA0 antibody titers to sH1N1, sH2N2 and aH5N1 (group 1) were higher in both children and adults who had serological evidence of infection with A(H1N1)pdm09 (HAI≥40) than those who had HAI<40. Furthermore, in adults, anti-HA0 antibody titers to sH3N2, aH7N3, H7N7, H7N9 (group 2) were also higher in those with pH1N1 HAI≥40 than in those with HAI<40. This suggests that individuals infected with A(H1N1)pdm09 virus developed cross-reactive anti-HA immunity against different virus subtypes. However, the breadth of the cross-reactivity appeared to be correlated with age, eg. cross-reactivity more limited to group-1 viruses in children and more broader reactivity to both group-1 and group-2 viruses in adults.

We also showed that levels of cross-reactive anti-HA0 antibodies were higher in adults than in children. This age-associated increase in both the breadth and magnitude of cross-reactive anti-HA0 antibodies was consistent with the hypothesis that such cross-reactive immunity is a result of priming from previous exposure to seasonal viruses and mediated by preexisting memory B cells binding to conserved epitopes and significantly enhanced by A(H1N1)pdm09 infection (3, 7). Of note, the adults included in this study were aged 16-35 years, therefore they fell into the age group that would have been exposed to sH1N1, but not the 1918 H1N1-like virus that induced lasting immunity to protect older adults in the 2009 pandemic.

It is believed that the improved cross-reactivity post A(H1N1)pdm09 infection is due to exposure to dramatically altered HA (as supposed to gradually changing due to drift) – which may have implications for development of universal influenza vaccines(20). Our results here showed cross-reactive anti-HA0 antibodies to a broad range of virus subtypes following A(H1N1)pdm09 infection would support this. Furthermore, the finding of cross-reactive anti-HA1 antibodies to sH1N1 and sH3N2 would support also the presence of some cross-reactive epitopes within the globular head region of HA. Although anti-HA1 titer to aH5N1 was not shown to increase, a marked increase in anti-HA2 titers was observed in both children and adults with serologic evidence of A(H1N1)pdm09 infection. These results support that cross-reactive anti-HA antibodies are mainly against the conserved stalk region that is predominantly HA2. In contrast to the broad cross-reactive immunity observed following A(H1N1)pdm09 infection, we did not observe significant enhancement of cross-reactivity in individuals with serologic evidence of recent exposure to sH1N1 or sH3N2 viruses. Nevertheless, higher antibody titers (to sH1N1, H2N2, aH5N1) in subjects with both pH1N1 and sH1N1 HAI≥40 than in those with pH1N1 HAI≥40 but sH1N1<40 suggests previous sH1N1 infection may contribute to the priming of cross-reactive immunity. However, we did

not observe this phenomenon in subjects with both pH1N1 and sH3N2 HAI≥40 compared to those with pH1N1 HAI≥40 but sH3N2<40, which suggests previous sH3N2 infection may not contribute significantly to the cross-reactive immunity to the group 1 strains.

Whether cross-reactive anti-HA antibodies offer protection against other virus subtypes with pandemic potential (eg. aH5N1 and aH7N9) is, of course, of interest. Although anti-HA antibody levels to aH7N3 and aH7N9 were generally low, antibody titers to H2N2, aH5N1 and aH7N7 strains were shown to be enhanced following A(H1N1)pdm09 infection (figure 1a+b). Furthermore, the virus neutralization titers appeared to correlate with anti-HA antibody titers, suggesting that these cross-reactive antibodies may offer some protection against these virus subtypes. Neutralization titers against 2 different strains of aH5N1 virus (Vietnam/1194/2004-clade1 and Anhui/1/2005-clade 2.3) were markedly enhanced in samples from subjects with serologic evidence of A(H1N1)pdm09 infection. Given that aH5N1 is highly pathogenic and a potential cause of a future pandemic, enhanced immunity to this virus following A(H1N1)pdm09 infection in children and adults could have important implications. Although cross-reactive antibodies to aH5N1 did not target the HA1 domain, the prominent enhancement of anti-HA2 antibody to aH5N1, likely targeting the conserved stalk region, is consistent with recent studies showing a protective role for such antibodies (7, 21). Whether such cross-reactive anti-HA antibodies elicited following pH1N1 infection would be sufficient to protect against a new infection with these viruses remains to be seen. However, vaccines to enhance such cross-reactive immunity may provide a promising vaccination strategy.

The substantially higher anti-HA0 titers in adults to H7 HA of aH7N7 (A/Netherlands219/03) compared with other H7 HA is of interest. It may be related to the outbreaks of aH7N7 in humans and/or poultry between 2003 and 2009 in some European countries including Netherland and UK, and analysis in Netherland suggested much higher levels of aH7N7

transmission to humans than previously thought and person to person transmission may have occurred on a large scale (22).

Significant enhancement of anti-HA1 titers to sH1N1 virus was shown in subjects with serological evidence of pH1N1 infection. As antibodies targeting receptor binding site of HA are considered crucial in preventing new infections, such additional cross-reactive immunity targeting HA circular head region (mainly HA1) of sH1N1 may help explain the apparent disappearance of sH1N1 following A(H1N1)pdm09 pandemic (7, 21). Although adults with pH1N1 HAI≥40 had enhanced anti-HA1 titers to sH3N2, there was no significant enhancement in anti-HA1 titers in children.

In summary, we demonstrated serological evidence of an age-associated cross-reactive anti-influenza HA immunity to a number of group-1 and group-2 influenza viruses in children and adults following pandemic H1N1 infection, which was not seen in individuals with serologic evidence of recent exposure to sH1N1 or sH3N2 viruses. Our results lend support to the efforts to develop "universal vaccines" that target the cross-reactive regions of HA for broad immunity against multiple subtypes of influenza virus (23). Examples of such vaccination strategy may include the use of a vaccine virus vector (eg. non-replicating adenovirus vector) expressing the conserved stalk-domain of HA to induce broader immunity. However, the more limited breadth of cross-reactive anti-HA immunity following A(H1N1)pdm09 infection in children compared to adults, suggests that such vaccination strategy to induce broad immunity in children may need more efficient priming of cross-reactive memory B cells, and strategies such as prime-and-boost schemes may be required (24) .

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FIGURE LEGENDS

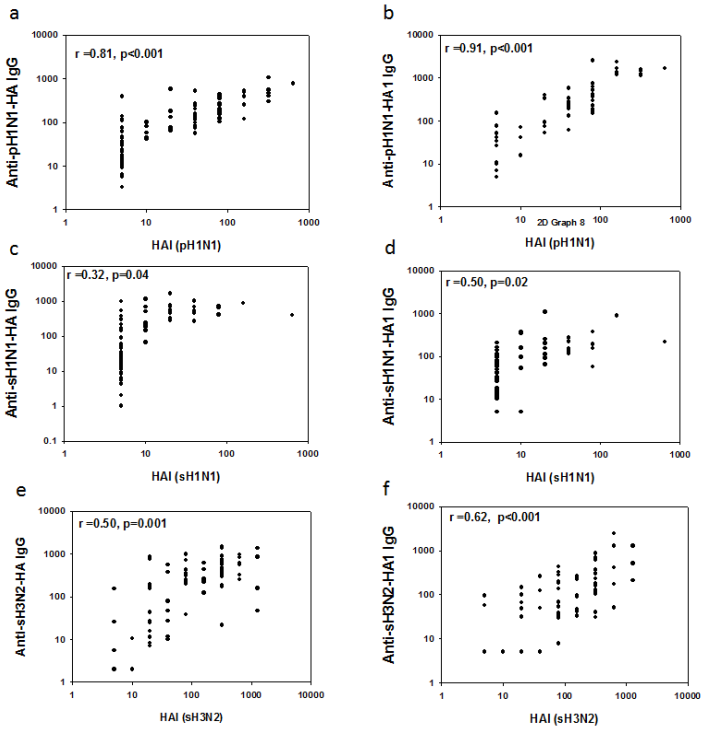
Figure 1. Correlation between serum anti-HA0 (a,c,e) or –HA1 (b,d,f) antibody titers and HAI titers to pH1N1, sH1N1 and sH3N2 viruses in both children and adults.

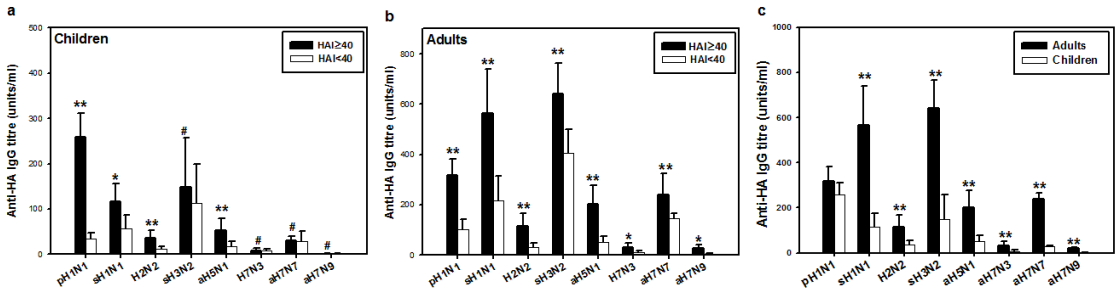
Figure 2. Comparison of anti-HA0 antibody titers (GMT+95%CI) between subjects with serologic evidence of infection (pH1N1 HAI≥40) and those without (pH1N1 HAI<40) in children (a) and adults (b). *p<0.05, **p<0.01 compared with HAI<40. Comparison of magnitude of cross-reactive anti-HA0 antibodies between adults and children with pH1N1 HAI≥40. **p<0.01 compared to children (c).

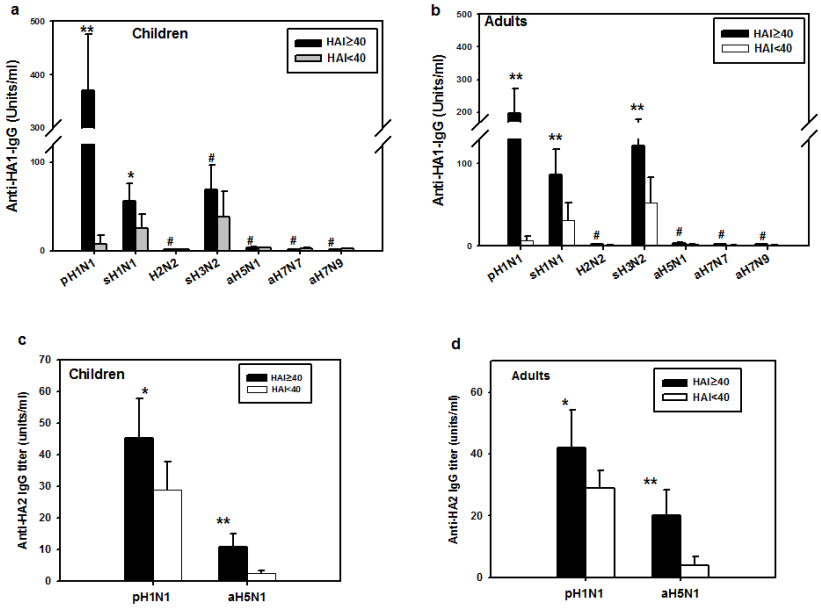
Figure 3. Comparison of HA1 (a+b) and HA2 (c+d) antibody titers (GMT+95%CI) between subjects with serologic evidence of infection (pH1N1 HAI≥40) and those without (pH1N1 HAI<40) in children (a,c) and adults (b,d). *p<0.05, **p<0.01 compared with HAI<40.

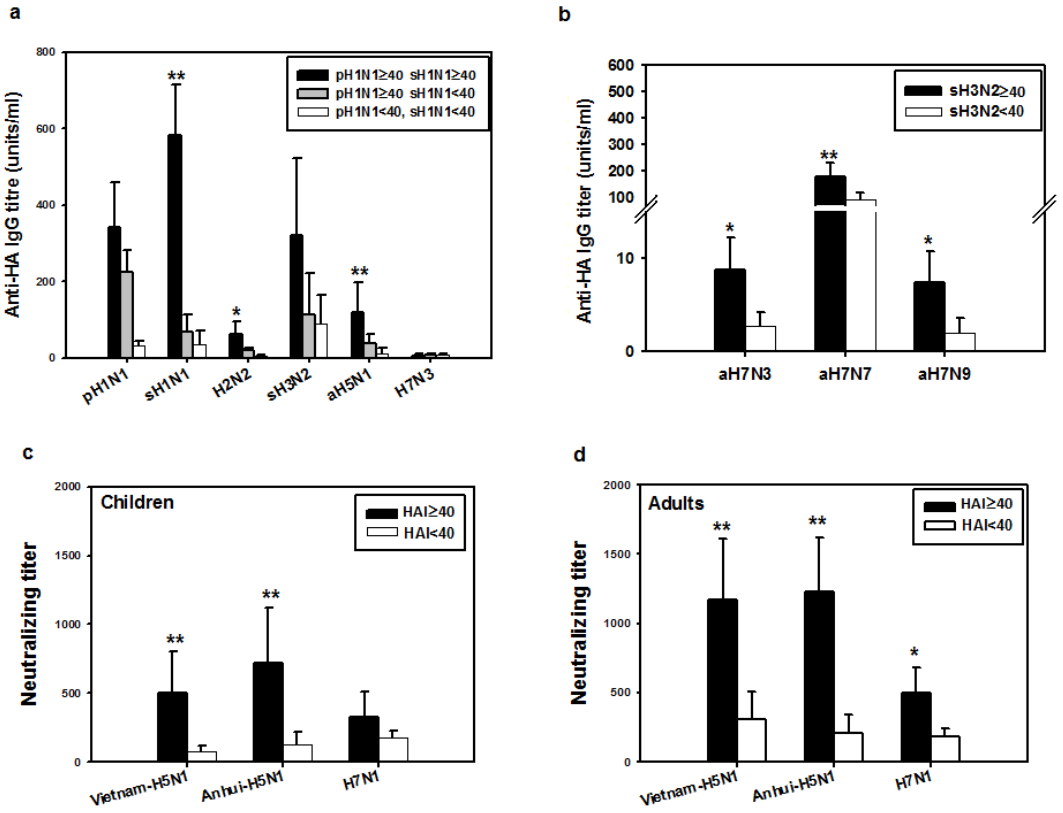
Figure 4. Comparisons of anti-HA0 antibody titers between children with both pH1N1 and sH1N1 HAI≥40 and those with pH1N1 HAI≥40 but sH1N1 HAI<40 (a), between adults with sH3N2 HAI≥40 and those with sH3N2 HAI<40 (b); and comparison of neutralizing antibody

titers against aH5N1 and H7N1 strains between subjects with pH1N1 HAI≥40 and those with pH1N1 HAI<40 in children (c) and adults (d). *p<0.05, **p<0.01.









Highlights

- We analyse the breadth and magnitude of cross-reactive anti-influenza HA immunity in humans after pandemic H1N1 infection.
- Pandemic H1N1 induced a broad cross-reactive response to group-1 and -2 viruses, including avian H5 and H7 viruses.
- The breadth and magnitude of the cross-reactive immunity is age-dependent.
- The results support efforts to develop “universal vaccines” targeting cross-reactive regions of HA for broad immunity.