Seroprevalence of Pandemic 2009 (H1N1) Influenza A Virus among Schoolchildren and Their Parents in Tokyo, Japan

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Since its emergence, the 2009 pandemic H1N1 virus has spread rapidly throughout the world. Previously, we reported that most individuals born after 1920 do not have cross-reactive virus-neutralizing antibodies against pandemic (H1N1) 2009 virus, indicating that they were immunologically naïve to the pandemic virus prior to its emergence. This finding provided us with an excellent opportunity for a seroepidemiological investigation of the transmission mode of the pandemic virus in the community. To gain insight into its transmission within communities, we performed a serosurvey for pandemic virus infection with schoolchildren at an elementary school in Tokyo, Japan, and their parents. We observed a high prevalence of neutralizing antibodies to the pandemic virus in the children at this school, although the percentage of children positive for the neutralizing antibodies varied among classrooms. While a much lower prevalence was observed among parents, seropositivity of the parents correlated with that of their schoolchildren. Moreover, many adults appeared to have experienced asymptomatic infection with the pandemic virus. These data suggest that the pandemic virus was readily transmitted among schoolchildren in elementary schools and that it was also transmitted from schoolchildren to their parents.

In the spring of 2009, the swine-origin H1N1 influenza A virus emerged in Mexico and spread around the world within a few months, resulting in the first influenza pandemic of the 21st century, as declared by the World Health Organization on 11 June 2009 (3, 4, 24). In Japan, this H1N1 virus was first detected on 9 May 2009 and eventually spread throughout the country (18). In Tokyo, Japan, the first wave of this pandemic started in mid-August 2009 and peaked in late October. The number of infected patients then gradually declined, with the wave ending in early 2010 (Fig. 1). Pandemic vaccines were introduced on 19 October 2009. These were targeted to medical workers first, followed by individuals with underlying diseases, pregnant females, and then schoolchildren. Previously, we reported that only low levels of cross-reactive virus-neutralizing antibodies against the pandemic (H1N1) 2009 virus were found in individuals born after 1920, with a few exceptions (16), indicating that most individuals were immunologically naïve to the pandemic H1N1 virus prior to its emergence. The Centers for Disease Control and Prevention (6) also reported a similar low prevalence of antibodies cross-reactive with the 2009 pandemic virus in people born after 1945, although they found a higher prevalence of antibodies in those born before 1949. Despite this finding, the low levels of antibodies cross-reactive with the pandemic H1N1 virus detected in people born before 1920 provided us with an excellent opportunity for a seroepidemiological investigation of the transmission mode of the pandemic virus in the community.

Schoolchildren and children attending day care centers are principal amplifiers of seasonal influenza viruses in the community and introduce viruses into households (11, 12, 17, 22). Here, we selected schoolchildren at an elementary school in Tokyo, Japan, and their parents as a model community to understand the transmission of the virus during a pandemic. To this end, we tested for the presence of neutralizing antibodies to the 2009 pandemic virus in this study population.

MATERIALS AND METHODS

Cells and virus. Madin-Darby canine kidney (MDCK) cells were maintained in Eagle’s minimal essential medium (MEM) containing 5% newborn calf serum at 37°C in 5% CO2. Pandemic H1N1 virus A/Osaka/364/09 was isolated from a patient in August 2009.
Sample collection. A total of 212 serum samples were collected. Sera were collected at the school on 21 November 2009 and 30 January 2010 from volunteer schoolchildren (6 to 12 years old; group 1) at an elementary school in Tokyo, Japan (the total number of pupils in this school was 225 in 7 classes of 6 grades), with the informed consent of their parents and from their parents (31 to 53 years old; group 2). Sera were collected from other adult volunteers (31 to 53 years old; group 3), who had no connection to this elementary school, at the University of Tokyo on 22 December 2009 and 23 to 30 March 2010. These adult volunteers were students and staff at the University of Tokyo (Table 1). Individuals who were vaccinated with a 2009 pandemic H1N1 vaccine were excluded from this study.

Prior to venipuncture, adult volunteers were interviewed to receive information on their vaccination history and history of recent influenza-like illnesses. The parents of the schoolchildren participating in the study provided this information for the schoolchildren. Our research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science, University of Tokyo (approval number 21-38-1117).

Virus neutralization assay. Virus neutralization assays were performed by using the methodology outlined in the WHO Manual on Animal Influenza Diagnosis and Surveillance (23) with the following modifications. Briefly, sera were treated with receptor-destroying enzyme (RDE; RDEII; Denka Seiken Co., Ltd., Tokyo, Japan) to remove nonspecific inhibitors of influenza virus and heat inactivated for 30 min at 56°C. Virus (100 50% tissue culture infectious doses [TCID50s]) was incubated with 2-fold serial dilutions of RDE-treated sera for 30 min at 35°C, and the mixtures were added to confluent MDCK cells on 96-well microplates to determine the neutralizing activity.

Statistical analysis. All statistical analyses were performed using JMP software, version 8.0.2 (SAS Institute Inc., Cary, NC). All tests were 2-tailed; statistical significance was set at a $P$ value of $<0.05$. Serological titers were log transformed. Welch's $t$ test was performed to determine the level of significance between means of serological titers. Fisher’s exact test or Pearson’s test was used to compare the prevalence of seropositivity between two groups.

RESULTS

Comparison of virus neutralization titers among children and adults. To determine the overall infection rate in the test

| Group | Population                           | Age range (avg) | 21 Nov 2009 | 22 Dec 2009 | 30 Jan 2010 | 23–30 Mar 2010 | Total  |
|-------|--------------------------------------|-----------------|-------------|-------------|-------------|----------------|--------|
| 1     | Schoolchildren in an elementary school | 6–12 (9.3)      | 60          |             | 19 (4)      |                | 79 (4) |
| 2     | Parents of children in group 1        | 31–53 (42.0)    |             | 69          |             | 28 (6)         | 97 (6) |
| 3     | Other adults                          | 31–53 (40.3)    |             |             | 4 (0)       | 32 (1)         | 36 (1) |
| Total |                                     |                 |             |             |             |                | 212 (11) |
had neutralizing antibody titers of 862.

Symptoms during the 2009 pandemic, the majority (both schoolchildren and adults) who experienced influenza from the other time points. Of the 35 individuals (including those from this collection time were analyzed separately from those reported on 21 November (the 47th week) 2009 (Fig. 1), the data number of H1N1 pandemic virus patients was still being re-

ciated with the elementary school (Fig. 2A). Since a substantial
dren (6 to 12 years old) and adults (31 to 53 years old) asso-

populations, we first compared the seropositivity between chil-

dren (6 to 12 years old) and adults (31 to 53 years old) asso-

associated with the elementary school (Fig. 2A). Since a substantial

number of H1N1 pandemic virus patients was still being re-

ported on 21 November (the 47th week) 2009 (Fig. 1), the data

from this collection time were analyzed separately from those

from the other time points. Of the 35 individuals (including

both schoolchildren and adults) who experienced influenza

symptoms during the 2009 pandemic, the majority (n = 33)

had neutralizing antibody titers of ≥32. Using this criterion, we

analyzed our previous data set (16), in which seropositivity

against the pandemic 2009 virus was tested by using sera

obtained prior to the pandemic, and found that 4.55% of

individuals in the cohorts ages 31 to 53 years (i.e., those cor-

responding to groups 2 and 3) had pandemic 2009 virus cross-

reacting antibodies. We therefore decided to use a neutraliza-

tion antibody titer of 32 as the cutoff value. Although we used

A/California/4/2009 for our previous analyses (16) and A/Osa-

ka/364/09 for this analysis, these two viruses differ by only three

amino acids and are antigenically highly similar. For the serum

samples collected on 21 November 2009, the children pos-

sessed neutralizing antibodies to the pandemic virus at a sig-

ificantly higher rate than the adult population (for 21 Novem-

ber 2009 samples, 46.7% for children versus 5.8% for parents;

P < 0.001 by Fisher’s exact test). Among the seropositive

children, 75.0% experienced influenza symptoms. In contrast,

only 5.8% of parents were seropositive and only one individual

was symptomatic (Fig. 3).

Similar data were obtained with the samples collected at the

elementary school on 30 January 2010. At this time, 89.5% (68.4% with symptoms and 21.1% without symptoms; Fig. 3) of

the children had neutralizing antibodies against the pandemic

H1N1 virus. In contrast, 25.0% of the parents were seroposi-

tive, which was significantly different from group 1 (P < 0.001

by Fisher’s exact test) (Fig. 2A).

Interestingly, none of the seropositive parents (25.0%) from

the January serosurvey (Fig. 3) recalled experiencing influ-

enza-like symptoms from April 2009 to the time of serum

collection, suggesting that most of them may have been asymp-

tomatically infected with the pandemic virus. Other unvacci-

nated adults who had no connection to the elementary school

exhibited 8.6% seropositivity, which was not significantly dif-

ferent from that of group 2 (P = 0.0771 by Fisher’s exact test).

None of the group 3 seropositive adults recalled experiencing

influenza symptoms from April 2009 to the time of serum

collection (Fig. 3). There were no significant differences be-

tween groups 2 and 3 with respect to age distribution and

seasonal influenza vaccination history, with the exception of

gender (Table 2). Therefore, these data suggest a lower prev-

ance of infection in adults than in children and a high inci-

dence of asymptomatic infection among adults.

Comparison of seropositivity among classrooms of an ele-

mentary school. Next, we analyzed the rates of infection of

schoolchildren in each classroom of the elementary school.

The number of volunteers in each classroom varied, and the

seropositivity rates also differed across classrooms (18.2% to

100%) (Fig. 4). The seropositivity rate was the lowest in class-

rooms A and G. It is possible that those who were diagnosed to

be influenza A virus positive with a rapid diagnosis kit (note

that in Japan most patients with respiratory illness at clinics are

tested for influenza virus infection with a rapid diagnosis kit)

may have opted not to participate in our study. However, in all

classes, there was no significant difference in the rate of stu-

dents in the classroom who had symptoms (i.e., the population

shown in dark gray in the far left column of Fig. 4) and the rate

of volunteers who were seropositive with symptoms (i.e., the

population shown in red in the second column from the right

of Fig. 4), except for class A, suggesting that our volunteer

schoolchildren were representative of each class, except for

class A. Consistent with this seropositivity, classrooms B, C, D,
E, and F, but not A and G, were closed due to increased numbers of children with upper respiratory illnesses in either October or November 2009 (Fig. 1).

Association between adult seropositivity to the pandemic virus and seropositive children. We then looked to see if there was an association of seropositivity between the schoolchildren and their parents or between adults and their children. First, we focused on the children. Among the 44 seropositive children, there were 14 (31.8%) seropositive parents and 30 (68.2%) seronegative parents. Similarly, among the 31 seronegative children, 10 (32.3%) had seropositive parents and the remainder (67.7%) had seronegative parents (Table 3). In contrast, among the 9 seropositive parents, 8 (88.9%) had seropositive children, whereas among the 82 seronegative parents, 51 (62.2%) had seropositive children, demonstrating a significant association between adult seropositivity to the pandemic virus and seropositive children (Table 3).

Seasonal influenza vaccine efficacy for pandemic 2009 influenza. A total of 57 individuals in this study received seasonal influenza vaccines. To evaluate the efficacy of trivalent seasonal influenza vaccines against pandemic 2009 influenza, we compared the rates of seropositivity to the pandemic 2009 virus between those who received and those who did not receive seasonal influenza vaccines in 2009 and found no significant differences (Fig. 5), consistent with recent reports (7, 8, 13, 19, 20).

DISCUSSION

Children are known to spread seasonal influenza, and schools provide a ready environment for this spread to the community (11, 12, 17, 22). In fact, several outbreaks of pandemic (H1N1) 2009 in schools were reported worldwide (1, 2, 5, 14, 15, 21). However, little is known about the impact of this dissemination in a pandemic situation. Here, we showed for the 2009 pandemic H1N1 virus infection that the infection rate was significantly higher in children than in adults, that asymptomatic infections were prevalent in adults, and that the infection rate in adults was higher in those with seropositive children in their family than in those with seronegative children. A report by the Ministry of Health, Labor, and Welfare in Japan indicated that children ranging from 5 to 14 years old were infected with the 2009 pandemic virus earlier than adults (Fig. 6). On the basis of these findings, we conclude that schoolchildren played an important role in dispersal of the 2009 pandemic virus, by sustaining infections among their classmates and spreading the virus to their parents.

The rates of seropositivity to the pandemic virus were significantly different between children and their parents (89.5% versus 35.7% on 30 January 2010). Although previous studies showed that some adults may possess cross-reactive antibodies to the seasonal
H1N1 virus (6, 16), the above-described difference in seropositivity cannot be explained on the basis of preexisting cross-reactive antibodies. These data therefore indicated a high level of susceptibility of children to the 2009 pandemic virus.

In our elementary school study, the percentage of seropositive children varied among classrooms. Most of the Japanese elementary school system is designed such that children belong to a class and each class has its own room.

| Class   | Sero-positivity (no. of individuals) | P value |
|---------|-------------------------------------|---------|
| Class A (1st grade) | ![Diagram](https://via.placeholder.com/150) | 0.0295* |
| Class B (2nd grade) | ![Diagram](https://via.placeholder.com/150) | 0.2233 |
| Class C (3rd grade) | ![Diagram](https://via.placeholder.com/150) | 1.000 |
| Class D (4th grade) | ![Diagram](https://via.placeholder.com/150) | 0.5186 |
| Class E (5th grade) | ![Diagram](https://via.placeholder.com/150) | 1.000 |
| Class F (5th grade) | ![Diagram](https://via.placeholder.com/150) | 0.6855 |
| Class G (6th grade) | ![Diagram](https://via.placeholder.com/150) | 1.000 |

**FIG. 4.** Seropositivity to the pandemic H1N1 virus among children in different classrooms. The virus neutralization activity in sera was tested as described in the Materials and Methods. For those who provided sera on several occasions at different time points, the latest serological data points were used. *, in Japan, children who are found to be influenza virus positive are forbidden to attend school. Before these children can return to school, a medical certificate from a physician confirming their recovery must be provided. We used this information to estimate the influenza morbidity in classrooms. *, P < 0.05 by Fisher’s exact test indicates significant difference.
Therefore, schoolchildren spend most of their time in only one classroom while in school. In Tokyo, class closure was implemented when over 10% of children in a given class showed pandemic influenza-like symptoms. Indeed, school officials closed classrooms B, C, D, E, and F, all of which had a high seropositivity rate (57.1%), due to increasing numbers of children with pandemic virus infection, whereas classrooms A and G, which had low seropositivity rates (22.2%), were not closed during the pandemic. Therefore, close contacts in a classroom likely promote efficient virus transmission within schools. Consistent with our finding, a previous study indicated that students tend to be assortative, interacting mainly with peers of the same age or in the same class, and in so doing may form the local transmission backbone of a pandemic (10).

Interestingly, we found that most of the adults who were seropositive for the 2009 pandemic virus did not recall experiencing influenza symptoms after its emergence. The fact that only one immunization with a pandemic vaccine elicited substantial antibody responses in the adult population (25) may support the notion that while neutralizing antibodies are absent, primed immune systems yield rapid cross-protective responses to the pandemic H1N1 virus, resulting in asymptomatic infections. Whether these asymptomatically infected individuals played a role in spreading the virus remains unknown. Further epidemiological and virological studies of asymptomatic influenza patients may help further our understanding of the spread of pandemic and seasonal viruses.

**TABLE 3. Relationship between seropositivity to the pandemic virus between children and their parents**

| Population | No. (%) of individuals |
|------------|------------------------|
| Children who were: | |
| Positive with a seropositive parent(s) | 14 (31.8) |
| Positive with a seronegative parent(s) | 30 (68.2) |
| Negative with a seropositive parent(s) | 10 (23.2) |
| Negative with a seronegative parent(s) | 21 (46.7) |
| Parents who were: | |
| Positive with a seropositive child(ren) | 8 (88.9)* |
| Positive with a seronegative child(ren) | 1 (11.1) |
| Negative with a seropositive child(ren) | 51 (62.2) |
| Negative with a seronegative child(ren) | 31 (37.8) |

* P < 0.05 by Fisher’s exact test indicates a significant difference.

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**FIG. 5.** Comparison of seropositivity between volunteers who were vaccinated and those who were not vaccinated with a trivalent seasonal influenza vaccine in 2009. *P < 0.05* by Pearson’s test indicates a significant difference.

**FIG. 6.** Estimated number of patients who consulted a physician for influenza-like illness. Weekly summary of patients showing influenza-like illness in Japan from 3 August 2009 to 13 December 2009 reported by the Ministry of Health, Labor, and Welfare (http://www.mhlw.go.jp/bunya/kenkou/kekka.html). These numbers were estimated from the data collected at sentinel clinics in Japan. y.o., years old.
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