We report shedding duration of 2009 pandemic influenza A (pH1N1) virus from a school-associated outbreak in Pennsylvania during May through June 2009. Outbreak-associated students or household contacts with influenza-like illness (ILI) onset within 7 days of interview were recruited. Nasopharyngeal specimens, collected every 48 hours until 2 consecutive nonpositive tests, underwent real-time reverse transcriptase polymerase chain reaction (rRT-PCR) and culture for pH1N1 virus. Culture-positive specimens underwent virus titrations. Twenty-six (median age, 8 years) rRT-PCR–positive persons, for pH1N1 virus, were included in analysis. Median shedding duration from fever onset by rRT-PCR was 6 days (range, 1–13) and 5 days (range, 1–7) by culture. Following fever resolution virus was isolated for a median of 2 days (range, 0–5). Highest and lowest virus titers detected, 2 and 5 days following fever onset, were 3.2 and 1.2 log10 TCID50/mL respectively. Overall, shedding duration in children and adults were similar to seasonal influenza viruses.

BACKGROUND

In May 2009, the World Health Organization and the United States declared a public health emergency following the rapid emergence and spread of pandemic influenza A (pH1N1) virus. The pH1N1 virus is characterized by a unique assortment of gene segments not previously identified among human or swine influenza viruses [1].

The Centers for Disease Control and Prevention (CDC) currently recommends that people with influenza-like illness (ILI), defined as fever and cough and/or sore throat, remain at home for 24 hours after fever has resolved while off of antipyretic medications in order to reduce the spread of influenza [2]. The duration of viral shedding is likely an important determinant of infectivity and transmissibility and should inform infection prevention and control measures. However, at the time this investigation was undertaken little was known about the viral shedding duration of the pH1N1 virus and any similarities or differences in the shedding duration with seasonal influenza viruses, in relation to fever onset and resolution, especially among children. As part of an investigation of an outbreak of pH1N1 illness at an elementary school in southeastern Pennsylvania during May through June 2009, we assessed the shedding duration of the virus among students and their household members utilizing classical virological methods (cell culture and TCID50 determination) as well as the CDC real-time RT-PCR diagnostic influenza assay cleared for use under Emergency Use Authorization by the Food and Drug Administration.
METHODS

Elementary School Outbreak
On 11 May 2009, a K-4 elementary school with 456 students in a semi-rural area of southeastern Pennsylvania reported a 3-fold increase (from 4% to 12%) in absenteeism to the Pennsylvania Department of Health (PADOH). Calls to ill students’ homes showed that a large proportion was absent due to respiratory illness, and 1 day later an ill student was found to be positive for the pH1N1 virus by real-time reverse transcriptase polymerase chain reaction (rRT-PCR). The high absenteeism and positive laboratory test resulted in the first school dismissal in Pennsylvania, which lasted 7 days beginning on 14 May, and an investigation was initiated.

Enrollment
To identify persons with ILI, a telephone survey was conducted among elementary school students and their household members during 16–21 May. A standard questionnaire collected information on clinical symptoms, onset and resolution dates, history of seasonal influenza (2008–2009) vaccination, and antiviral treatment history. Persons whose ILI onset date was in the 7 days before the telephone interview were asked to participate in the investigation of duration of viral shedding. Informed consent for participation in the investigation was obtained for all participants. For children under 18 years of age, parental consent was obtained. Persons consenting to provide laboratory specimens for pH1N1 testing were enrolled in the investigation. This investigation was part of the emergency public health practice response to the pandemic and was reviewed by appropriate CDC authorities and deemed not to be research in accordance with the federal human subjects protection regulations at 45 Code of Federal Regulations 46.101c and 46.102d and CDC’s Guidelines for Defining Public Health Research and Public Health Non-Research.

Sample Collection
Among those reporting ILI from the telephone survey and consenting to provide specimens for rRT-PCR, attempts were made to sequentially collect nasopharyngeal (NP) swab specimens every 48 hours beginning with the day of enrollment until 2 negative rRT-PCR results were obtained, or an indeterminate followed by a negative rRT-PCR result was obtained. During each sample collection patients were reassessed for symptoms and antiviral use. Nasopharyngeal specimens were obtained using sterile applicators, placed in 3.0 mL of Remel MicroTest M4RT liquid viral transport media (Lenexa, Kansas), refrigerated at 5°C, and transported to the Pennsylvania State Department of Health Bureau of Laboratories (BOL) within 12 hours of collection and tested by rRT-PCR within 24 hours.

Real-time RT-PCR Analysis
Specimens were tested at the BOL using the CDC real-time RT-PCR assay for influenza A (H1N1) [3]. After completion of testing in Pennsylvania, specimens were frozen at –70°C and sent to the CDC Influenza Division for further testing. At CDC, specimens were retested by rRT-PCR for H1N1, and these results were used for subsequent analyses.

Real-time RT-PCR was considered to be positive for pH1N1 virus if the CDC assay detected all 4 of the following nucleic acid targets: universal influenza A (InfA), universal swine influenza A (SwInfA), swine influenza H1 (SWH1), and human RNase P (RP), within a cycle threshold (Ct) of ≤37. Results were negative if only RP was detected and indeterminate when RP and only one or 2 of the nucleic acid targets was detected. Human RNase P detection was required for a valid result.

Virus Cultures
Viral culture was performed on all specimens from participants in the investigation that were rRT-PCR–positive and on the earliest collected rRT-PCR–negative or indeterminate specimen from each participant. Specimens were inoculated into 25-cm² tissue culture flasks of Madin-Darby canine kidney cell cultures. Before inoculation the cell growth media was removed, cell monolayers were rinsed twice with phosphate buffered saline. Two hundred microliters of viral transport medium containing the NP specimen were inoculated into the flask. The inoculum was absorbed on cell culture monolayer for 30 minutes at 35°C–37°C. After absorption, 5 mL of cell maintenance media containing 2 µg/mL TPCK-trypsin was added to the monolayer. Cell cultures were incubated at 35°C–37°C and observed daily for cytopathic effect. Cell culture media were harvested either when cytopathic effect covered >75% of the monolayer or 8 days after inoculation when cytopathic effect was absent. To confirm the presence of influenza virus, the cell culture harvests were assessed for hemagglutinin activity with a .5% suspension of turkey red blood cells.

Virus Titration
The 50% tissue culture infectious dose (TCID₅₀) assay was modified from the previously described procedure [4]. Virus isolates were prediluted 1:5 without prior neutralization. Three-fold serial dilutions of the prediluted isolates were prepared on 96-well tissue culture plates in 6 replicates. One row on each plate contained media only to serve as a negative control. A second row contained 200 TCID₅₀ of influenza virus A/California/4/2009 to serve as an infection control. MDCK cells (4 × 10⁵) were added per well. The immunostaining procedures were performed without changes. The analysis of colorimetric data was modified as follows: The cut-off value was calculated based on the average optical density (OD) value derived from the negative control wells plus 3 standard deviations. The TCID₅₀/mL of each isolate was calculated [5] taking into account predilution, 3-fold serial dilution and sample size.
RESULTS

Patient Information
A total of 36 persons who met enrollment criteria agreed to participate in the investigation. Among these persons, 26 (72%) tested positive for pH1N1 infection by rRT-PCR and were included in the shedding duration analysis. The median age of the 26 persons was 8 years (range, 2–45 years). The majority of patients (85%) were children aged 2–14 years; 61% were male (Table 1). Half the patients were students at the elementary school. Persons enrolled in the shedding survey were otherwise healthy and did not report any underlying medical conditions.

Fever and cough were reported by all participants per enrollment criteria. Other symptoms reported during the illness were coryza (62%), sore throat (39%), muscle aches (31%), diarrhea (12%), and vomiting (8%). The median duration of illness (resolution of all symptoms) was 11 days (range, 4–16 days). The durations of fever, cough, and sore throat are shown in Table 1.

Table 1. Demographic and Clinical Characteristics of 26 Patients with Confirmed Pandemic Influenza A H1N1 Virus Infection

<table>
<thead>
<tr>
<th>Age</th>
<th>n = 26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (range)</td>
<td>8 (2–45) yr</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
</tr>
<tr>
<td>&lt;5 yr</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>5–9 yr</td>
<td>13 (50%)</td>
</tr>
<tr>
<td>10–18 yr</td>
<td>6 (23%)</td>
</tr>
<tr>
<td>19–50 yr</td>
<td>4 (15%)</td>
</tr>
<tr>
<td>Female</td>
<td>10 (39%)</td>
</tr>
</tbody>
</table>

Clinical data

| Fever duration, median (range) | 4 (1–13) days |
| Cough duration, median (range) | 9 (1–17) days |
| Sore throat duration, median (range) | 4 (1–12) days |
| Seasonal flu vaccination | 9 (60%) |
| Oseltamivir treatment | 3 (12%) |

**Table 2**

Numbers of rRT-PCR results, or an indeterminate result followed by a negative result. The other 8 persons provided a total of 11 specimens but withdrew from the investigation before either 2 negative rRT-PCR results or an indeterminate result followed by a negative result. Ninety-eight specimens from the 26 persons enrolled in the investigation were first tested by rRT-PCR at BOL and 85 specimens (mean, 3.5 samples per patient) were retested at the CDC laboratory (13 specimens were not cultured, titrated, or tested by real-time RT-PCR as these specimens were negative for influenza upon testing by the BOL).

Of the 85 specimens, 52 (61%) were positive and 24 (28%) were negative for pH1N1 by rRT-PCR; 8 (9%) were indeterminate and 1 (1%) was invalid (RP not detected) (Figure 1). The 8 specimens from 7 patients that had an indeterminate rRT-PCR result were obtained at least 6 days after fever onset. The mean influenza A matrix gene (InfA) cycle threshold (Ct) values for these 8 specimens was 36.23 (SD ± 1.35), which was just below the limit of detection (37) for the assay. Five of the 7 persons who provided specimens following an indeterminate rRT-PCR result had no subsequent samples that were positive for pH1N1 virus. As the number of days since fever onset increased, the Ct value for the InfA as measured by rRT-PCR also increased (Spearman’s ρ = .6395; \( P < .01 \)) (see Figure 2). The InfA Ct values for specimens collected during febrile (28.1) and afebrile (31.8) periods were statistically significantly different (Mann-Whitney U test; \( P = .004 \)).

Overall, the median duration of detection of the pH1N1 virus by rRT-PCR was 6 days with a range of 1–13 days (Figure 3; Table 2). Younger children had longer viral shedding duration by rRT-PCR (Table 2) than adults but the difference was not statistically significantly different (tests for trend across ordered age groups, \( P = .6 \); K-sample test on the equality of medians, \( P = .3 \)).

The median duration of shedding by rRT-PCR among 3 persons who reported taking oseltamivir was 7 days (range, 3–10 days) as compared to 6 days (range, 1–13 days) for 23 persons who reported not taking any oseltamivir (K-sample test on the equality of medians, \( P = .3 \)).

Of the 85 specimens tested at CDC, 18 (21%) were collected while the patients were febrile and 67 (79%) while afebrile (Figure 1). The rRT-PCR result was positive for 17 (94%) of 18 specimens obtained from patients while febrile as compared to 35 (52%) of 67 specimens while afebrile (Fisher’s exact \( P = .002 \)). The rRT-PCR results were indeterminate for 7 specimens obtained from afebrile patients and one specimen from a febrile patient. Following fever resolution, the rRT-PCR result was positive for pH1N1 virus in 20 (76.9%) of the 26
persons enrolled in the analysis. The median duration of viral shedding, as detected by rRT-PCR, following fever resolution was 3 days (range, 0–10 days).

Virus Culture
A total of 27 (52%) of the 52 specimens that tested positive for pH1N1 virus by rRT-PCR yielded a viable virus by culture as confirmed by hemagglutinin (HA) titer measurement. None of the specimens that tested indeterminate or negative for the pH1N1 virus by rRT-PCR yielded a viable virus (Table 3). The mean influenza A matrix gene (InfA) cycle threshold (Ct) values for culture-positive (27.1) and culture-negative (33.1) specimens were statistically significantly different (Mann-Whitney U test; \( P < 0.001 \)).

The median duration of viral shedding by culture was 5 days (range, 1–7 days) following fever onset (Fig 3 and Table 2). The duration of viral shedding by culture for different age groups was not statistically significantly different (tests for trend across ordered age groups, \( P = .8 \); K-sample test on the equality of medians, \( P = .4 \)). Viral cultures were positive for 13 (72%) of 18 specimens obtained from febrile patients as compared to 14 (21%) of 67 specimens obtained from afebrile patients (Fisher’s exact \( P < .001 \)). Twelve (46%) of the 26 participants continued to shed culturable pH1N1 virus following fever resolution. The median duration of viral shedding by culture following fever resolution was 2 days (range, 0–5 days).

Virus Titrations
Of the 52 specimens that tested positive for pH1N1 virus by rRT-PCR, 8 specimens yielded detectable TCID\(_{50}\) values (Figure 4). Seven of these 8 specimens yielded pH1N1 virus by virus culture. The titers were 3.2 log\(_{10}\)TCID\(_{50}\)/mL at 2 days, 2.8 and 2.6 log\(_{10}\) TCID\(_{50}\)/mL at 3 days, 3.1, 2.7, and 2.1 log\(_{10}\) TCID\(_{50}\)/mL at 4 days, and 1.2 log\(_{10}\) TCID\(_{50}\)/mL at 5 days after fever onset.

DISCUSSION
This was one of the first investigations conducted to examine shedding duration of the pH1N1 virus in relation to fever onset and resolution both by rRT-PCR and viral cultures primarily among schoolchildren. Patients with pH1N1 infection were found to shed the pH1N1 virus by rRT-PCR for up to 13 days after fever onset and to be culture-positive up to 7 days after fever onset. These findings are especially noteworthy because they come from nonhospitalized persons with no underlying co-morbidities who, with rare exceptions, did not receive antiviral treatment. The duration of viral shedding among children in this investigation might better reflect the typical shedding patterns...
for pandemic influenza virus [6] in the healthy child population, a group that is thought to play a key role in sustaining community outbreaks.

The median duration of viral shedding observed during the elementary school–associated pH1N1 virus outbreak in Pennsylvania is similar to the median 5-day duration reported by PCR and culture from studies of seasonal influenza in outbreak and experimental settings [6-9] as well as studies conducted during an outbreak of pH1N1 virus among young adults [10]. In Pennsylvania, there was some suggestion that younger children might have more prolonged shedding by rRT-PCR relative to older children and adults, a finding seen in some studies of seasonal influenza [8] and pH1N1 virus [11]. However, the differences in Pennsylvania between these groups were not statistically significantly different.

The highest proportion of specimens found to be positive for pH1N1 virus were collected within 4 days of fever onset. This finding is also compatible with studies of seasonal influenza and pH1N1 studies conducted after ours. Among adults with pH1N1, the highest proportion of nasopharyngeal specimens from which viable virus could be isolated were collected 1–3 days following fever onset [10, 11]. For seasonal influenza, viral shedding peaks during the first 1–3 days of illness [6].

In studies conducted during seasonal influenza outbreaks, the amount of virus that is shed is highest in the first 2 days of illness and then declines gradually [12], a finding that was also recently demonstrated in China for pH1N1 [11].

Virus titers of culture results could be obtained on only a few specimens in this investigation. Despite the small number, the
range of virus titers was similar to that seen with seasonal influenza [13, 14], and a typical pattern of decline was observed as the number of days from fever onset increased. The findings of this investigation indicate that virus shedding, and presumably infectiousness, is highest while ill individuals are febrile. However, the pH1N1 virus was commonly detectable after fever resolution, and could be found for up to 10 days by rRT-PCR and up to 5 days by culture after resolution of fever. This is also consistent with our general understanding of the shedding duration of seasonal influenza viruses [8].

CDC’s current guidelines recommend that persons with ILI not return to work or school until 24 hours after fever has resolved without the use of antipyretics [15]. These guidelines are designed to balance the need to minimize likelihood of transmission while minimizing the negative individual, parental, and societal consequences of prolonged absence from work or school and recognize that shedding may occur after fever resolution and that many people infected with influenza will not have a fever [16]. In this investigation, we also found that shedding was greatest during fever, but that lower amounts of virus are shed among outpatients for ~2 days after fever resolves. However, questions remain as the current investigation cannot determine the degree of infectiousness of persons who are still shedding virus after resolution of fever when viral titers are lower. It is likely that such terminal, low-level shedding is associated with a lower risk of transmission of pH1N1. However, because shedding is not completely resolved after fever, CDC recommendations indicate that patients should be reminded about their potential to spread influenza and to follow recommended hand and cough hygiene recommendations. Studies are needed to better understand the relationship between detectable shedding and infectiousness.

This investigation is subject to several limitations as this was a public health response and not a research study. The majority of the participants were children 2–14 years of age; therefore, the findings may not be applicable to infants, the elderly, persons with underlying medical conditions, those with nonfebrile illness or asymptomatic infection, or persons with more severe illness, such as hospitalized persons. A number of individuals chose not to participate through the collection of 2 negative specimens (or indeterminate and negative) and their shedding duration could not be fully assessed. And finally, virus titrations could only be determined for a small proportion of culture-positive specimens.

The shedding duration of pH1N1 virus reported here is similar to that found with other influenza viruses and can help inform public health guidelines for social distancing and treatment of persons with pH1N1 infection. Further examination of viral titers in clinical specimens of patients infected with the pH1N1 virus and epidemiologic studies to determine transmission probability in relation to symptom resolution are warranted. These data are needed to understand the infectivity of pH1N1 virus and to inform infection control policies for persons recovering from pH1N1 influenza.

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