

Viral Load in Patients Infected With Pandemic H1N1 2009 Influenza A Virus

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Viral shedding profile of infections caused by the pandemic H1N1 2009 influenza A virus has not been reported. The aim of this study was to determine the viral load in different body sites. Viral loads of pandemic H1N1 virus in respiratory specimens, stool, urine, and serum were determined by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Respiratory specimens from patients with seasonal influenza were used as historical controls. Initial pre-treatment viral load were compared between these two groups. Serial respiratory specimens from patients with pandemic H1N1 virus infection were obtained for analysis of viral dynamics. Twenty-two pandemic H1N1 cases and 44 seasonal influenza historical controls were included. The mean initial viral load before oseltamivir therapy was 1.84×10^8 copies/ml for pandemic H1N1 virus compared with 3.28×10^8 copies/ml in seasonal influenza historical controls ($P = 0.085$). Among patients with pandemic H1N1 virus infection, peak viral load occurred on the day of onset of symptoms, and declined gradually afterwards, with no virus being detectable in respiratory specimens by RT-PCR 8 days and by culture 5 days after the onset of symptoms respectively, except in one patient. Pandemic H1N1 virus was detected in stool and in urine from 4/9 and 1/14 patients, respectively. Viral culture was also positive from the stool sample with the highest viral load. Younger age was associated with prolonged shedding in the respiratory tract and higher viral load in the stool. Data from this quantitative analysis of viral shedding may have implications for formulating

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KEY WORDS: pandemic; urine; stool; serial

INTRODUCTION

The pandemic H1N1 2009 influenza virus has disseminated globally after being identified in Mexico and the United States in April 2009 [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009]. Due to sustained and widespread human-to-human transmission, the World Health Organization raised the pandemic alert level from phase 5 to 6 on 11 June 2009. Unlike seasonal influenza, people below 60 years of age were infected preferentially worldwide [World Health Organization, 2009], with unusually high rate of severe respiratory disease and mortality among young patients in Mexico [Chowell et al., 2009]. This phenomenon may be attributed to the lack of pre-existing cross-reactive antibody against pandemic H1N1 virus in this age group [Centers for Disease Control and Prevention, 2009]. On

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the contrary, most if not all of these individuals have pre-existing antibody against the prevailing seasonal influenza virus. Apart from cross reactive antibody level, viral load profile from different body sites is also important in predicting disease severity and transmissibility in viral infections [Hayden et al., 1998; Chu et al., 2004; Hung et al., 2004; Cheng et al., 2004a; de Jong et al., 2006; Lowen et al., 2009]. The correlation between the virological profile and clinical characteristics of pandemic H1N1 virus infection would provide important knowledge for epidemiological control and clinical management in terms of antiviral therapy and infection control measures.

MATERIALS AND METHODS

Patients and Specimen Collection

Clinical specimens of respiratory secretion taken by nasopharyngeal aspirate or nasopharyngeal-throat swab, serum, urine, and stool were collected from patients with clinical suspicion of pandemic H1N1 virus infection in Hong Kong. Serial sampling of respiratory specimens was conducted during hospitalization or upon outpatient follow-up. Archived respiratory specimens from patients with seasonal influenza A virus infection from year 2007 to 2009 were selected as historical controls. Patients' records were reviewed to determine the demographic and clinical information.

Quantitative PCR and Viral Culture

Total nucleic acid extraction was performed by using NucliSens easyMAG instrument (bioMerieux, Boxtel, Netherlands) according to the manufacturer's instruction. Briefly, 250 μ l of clinical specimen was added to 2 ml of lysis buffer and incubated for 10 min at room temperature. The lysed sample was then transferred to the well of a plastic vessel with 100 μ l of silica. It was followed by automatic magnetic separation. Nucleic acid was recovered in 55 μ l elution buffer [Chan et al., 2008b].

The diagnosis of pandemic H1N1 virus was performed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using primers targeting hemagglutinin gene of pandemic H1N1 virus, while the quantitation of both pandemic H1N1 virus and seasonal influenza A virus was performed using quantitative RT-PCR targeting influenza A virus M gene, as described previously [Chan et al., 2008a; Lau et al., 2009]. Briefly, 2 μ l eluted RNA of Influenza A virus was used for cDNA by Invitrogen Superscript II Kit with random primer as described, and then, cDNA was amplified in Lightcycler instrument with a FastStart DNA Master SYBR Green I Mix reagent kit (Roche Diagnostics GmbH, Mannheim, Germany). In a typical reaction, 2 μ l cDNA was amplified in a 20 μ l of LC-PCR master mix containing 1 \times Fast-Start DNA master SYBR green I mix, 4.0 mM MgCl₂, 0.5 μ M of each primer. To determine the specificity of the assay, all PCR products were subjected to melting curve analysis (65–95°C; 0.1°C per second) at the end of the assay.

For quantitative assay, a reference standard was prepared using pCRII-TOPO vector (Invitrogen, San Diego, CA) containing the corresponding target viral sequences. A series of 5 log₁₀ dilution equivalent to 1 \times 10¹ to 1 \times 10⁶ copies per reaction were prepared to generate calibration curves and run in parallel with the test samples. If the specimen result was outside the upper limit of the expected range, the extract of the sample was repeated with suitable dilution. The detection limit of this assay was 900 copies of RNA per milliliter. For viral culture, Madin–Darby canine kidney (MDCK) cell monolayer in culture tubes were inoculated with 200 μ l of clinical samples as described previously [Yuen et al., 1998]. They were examined daily for cytopathic effect (CPE), and direct immunofluorescence test with specific antibody against influenza A virus nucleoprotein was done on fixed cell smears when CPE appeared or at the end of the incubation period.

Data Analysis

Comparison was made between patients with pandemic H1N1 virus and seasonal influenza virus infection regarding their demographics, underlying diseases, presenting symptoms, total white blood cell counts, absolute lymphocyte counts, and initial pre-treatment viral load in respiratory specimens on the day of diagnosis. Among patients with pandemic H1N1 virus infection, the same parameters was compared between those with longer duration (\geq 5 days) and shorter duration (\leq 4 days) of viral shedding, as defined by the time from onset of symptoms to the last positive sample by RT-PCR.

For underlying disease, chronic immunosuppressive states included autoimmune diseases, malignancies, diabetes mellitus, and solid organ transplants. Chronic pulmonary diseases included asthma and chronic obstructive pulmonary disease. Statistical analysis was performed by Fisher's exact test for categorical variables and by Mann–Whitney *U*-test for continuous variables. Univariate linear regression analysis was employed to determine correlation between age and duration of viral shedding, and between age and fecal viral load. A two-tailed *P*-value $<$ 0.05 was considered significant.

RESULTS

Twenty-two patients diagnosed with pandemic H1N1 virus infection from the period of 30 April 2009 to 3 June 2009 were included in this study. Their respiratory specimens were positive for both influenza A virus M gene and pandemic H1N1 virus H1 gene by RT-PCR. A total of 114 respiratory specimens were collected from the day of diagnosis to 11 days after diagnosis. Three or more serial respiratory specimens were obtained from 21 of 22 patients. Within the initial 7 days of diagnosis, respiratory specimens were collected from 93 out of 154 patient days (60.4%). The reason for refusal was due to either the discomfort associated with the procedure or improvement in symptoms. Stool, urine, and serum

specimens were available from 9, 14, and 13 patients, respectively. All patients with pandemic H1N1 virus infection received oseltamivir, except a 17-year-old girl. Oseltamivir therapy was initiated on the same day (66.7%), 1 day (28.6%), and 2 days (4.8%) after diagnosis. Forty-four patients with seasonal influenza A virus infection at the time of admission in year 2007–2009 were selected as historical controls randomly. None of them received any antiviral therapy. Coughing, vomiting, diarrhea, and duration of symptoms before diagnosis were significantly different between patients with pandemic H1N1 virus and seasonal influenza virus infection (Table I). Demographics, underlying diseases, and initial pre-treatment viral loads were not significantly different between the two groups.

For both pandemic H1N1 cases and seasonal influenza historical controls, respiratory specimens collected on the day of onset of symptoms (day 0) had the highest mean viral load (Fig. 1). There was a tendency for a lower initial viral load in the pandemic H1N1 cases. Though these were not serial samples from the same patient, there was an obvious decreasing trend of the initial viral load from 7 or 8 log 10 on the day of onset of symptoms to 6 or 7 log 10 on the third day after onset of symptoms. For seasonal influenza virus infection, this decreasing trend reversed from day 4 after onset of symptoms. Since no pandemic H1N1 cases were diagnosed on the 4th, 5th, 6th, and 8th day after onset of symptoms, direct comparison between the two groups were not possible for those days.

Viral load in respiratory specimens in pandemic H1N1 cases correlated negatively with time after onset of symptom ($r^2 = 0.1603$, $P = 0.003$), as shown in Figure 2. The median duration of viral shedding after

onset of symptoms was 4 days, but two patients had viral shedding for up to 7 days after onset of symptoms. Among the 21 patients who received oseltamivir, 19 patients had the last positive RT-PCR respiratory specimen while still receiving oseltamivir, with a median duration of viral shedding 4 days after onset of symptoms. The remaining two patients had the last positive RT-PCR specimen 1 day after last dose of oseltamivir, with viral shedding up to 7 days after onset of symptoms. Among the 14 patients with viral shedding ≤ 4 days, 10 patients had negative RT-PCR on day 5 after onset of symptoms. Four patients, who did not have respiratory specimen obtained on day 5 after onset of symptoms, had a low viral load at $<10^5$ copies/ml on day 3 or 4 after onset of symptoms. Younger age correlated with longer period of viral shedding after onset of symptoms ($r^2 = 0.210$, $P = 0.032$). Apart from age, there were no significant differences between patients with shorter and longer duration of viral shedding with regard to presenting symptoms, underlying diseases, blood tests, and initial pre-treatment viral loads (Table II).

Viral culture of the initial respiratory specimen was positive in 21 out of 22 patients with pandemic H1N1 virus infection, ranged from 1 to 5 days after onset of symptoms, except in one patient. Serial viral culture was performed in 12 patients, 7 of whom had further positive culture, from 1 to 5 days after onset of symptoms.

Pandemic H1N1 virus was detected by RT-PCR in the stool from four out of nine patients aged 1, 4, 17, and 20 years old. The positive stool specimens were obtained 4–7 days after onset of symptoms. The mean viral load in stool was 1.44×10^4 copies/ml (range 4.70×10^3 to

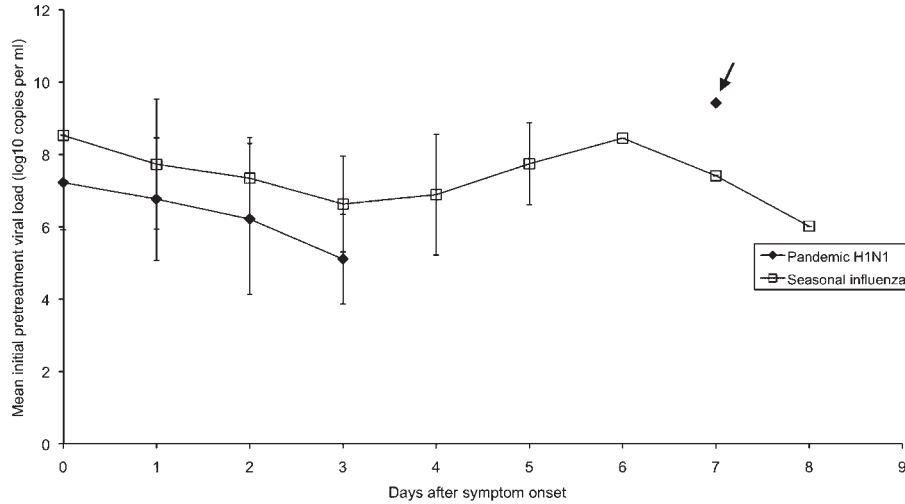
TABLE I. Comparison Between Pandemic H1N1 and Seasonal Influenza Infection

	Pandemic H1N1 (n = 22)	Seasonal influenza A (n = 44)	P-value
Demographics			
Age in years, mean (median)	21 (21)	22 (19)	0.787 ^b
Male/female ratio	10:12	25:19	0.440 ^a
Underlying diseases			
Chronic immunosuppressive states	1 (4.5)	8 (18.1)	0.253 ^a
Chronic pulmonary diseases	1 (4.5)	3 (6.8)	1.000 ^a
Presenting symptoms			
Days of symptoms before diagnosis, mean (median)	1.9 (2)	2.7 (2)	0.034 ^b
Fever	21 (95.4)	43 (97.7)	1.000 ^a
Cough	14 (63.6)	40 (90.9)	0.014 ^a
Sputum production	4 (18.1)	18 (40.9)	0.096 ^a
Runny nose	9 (40.9)	28 (63.6)	0.114 ^a
Sore throat	10 (45.4)	21 (47.7)	1.000 ^a
Vomiting	0 (0)	11 (25.0)	0.012 ^a
Diarrhoea	0 (0)	10 (22.7)	0.024 ^a
Blood test			
Total white blood cell ($\times 10^9/L$), mean (SD)	5.80 (1.81)	7 (3.37)	0.267 ^b
Lymphocyte ($\times 10^9/L$), mean (SD)	1.10 (0.63)	1.00 (1.28)	0.653 ^b
Viral load			
Initial pre-treatment viral load on diagnosis in log 10 copies/ml, mean (SD)	6.40 (1.82)	7.28 (1.29)	0.085 ^b

Values are given as no. (%) unless otherwise specified. n, number; SD, standard deviation.

^aBy Fisher's exact test.

^bBy Mann–Whitney u-test.



	Number of patients									
Seasonal influenza	1	6	20	6	6	2	1	1	1	
Pandemic H1N1	2	8	6	5	0	0	0	1	0	

Fig. 1. Distribution of mean initial (pre-treatment) viral load in respiratory specimens in relation to days after onset of symptoms. Error bar indicates one standard deviation. The arrow indicates the viral load of pandemic H1N1 virus in the 4-year-old boy with exceptionally high viral load (see text).

7.19×10^6 copies/ml), with a significant correlation between higher viral load and younger age ($r^2 = 0.555$, $P = 0.021$). Viral culture was positive in the stool specimen with the highest viral load. One out of 14 patients had detectable pandemic H1N1 virus in urine by RT-PCR 7 days after onset of symptoms, but was negative by viral culture. None of the available sera, which were collected from days 1 to 10 after onset of symptoms, had detectable viruses by RT-PCR.

A 4-year-old boy with pandemic H1N1 virus infection was unique in several aspects. Firstly, he was the only patient with detectable viral shedding in respiratory specimen, stool, and urine. Secondly, the viral load was highest among all patients. The initial pre-treatment viral load in his respiratory specimen, stool, and urine was 2.68×10^9 , 7.19×10^6 , and 7.24×10^4 copies/ml, respectively. His specimens with high viral load were all collected 7 days after onset of symptoms, whereas for

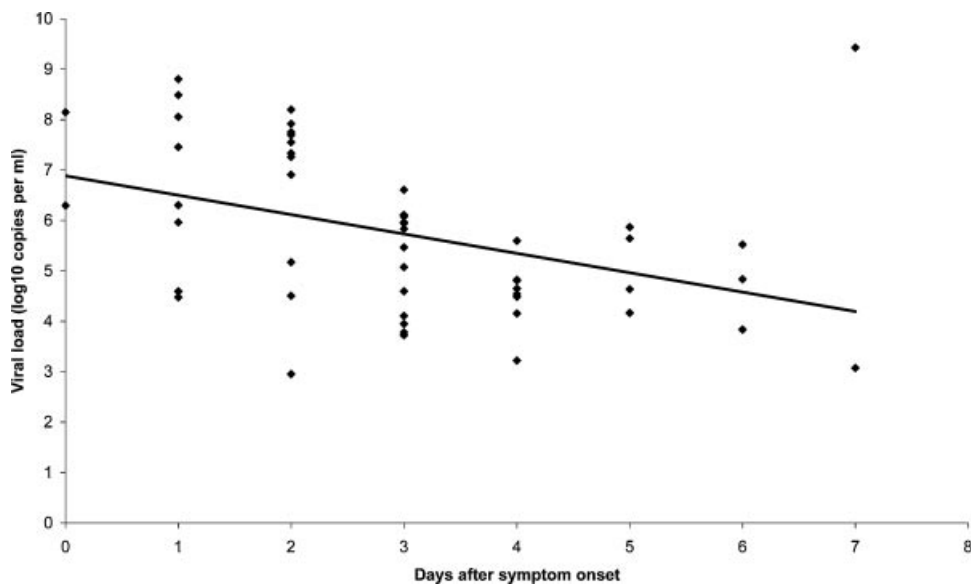


Fig. 2. Correlation of viral load in respiratory specimens with the duration after onset of symptoms. ($r^2 = 0.1603$, $P = 0.003$).

TABLE II. Comparison of Patients Categorized by Duration of Viral Shedding After Onset of Symptoms

	Duration of viral shedding ^a		P-value
	≤4 days (n = 14)	≥5 days (n = 8)	
Demographics			
Age in years, mean (median)	24.7 (21)	15.4 (19.5)	0.034 ^d
Male/female ratio	8:6	2:6	0.204 ^c
Underlying diseases			
Chronic immunosuppressive states	0 (0)	1 (12.5)	0.364 ^c
Chronic pulmonary diseases	1 (7.1)	0 (0)	1.000 ^c
Presenting symptoms			
Days of symptoms before diagnosis, mean (median)	1.42 (1)	2.75 (2.5)	0.070 ^d
Fever	13 (92.9)	8 (100)	1.000 ^c
Cough	9 (64.2)	5 (62.5)	1.000 ^c
Sputum production	3 (21.4)	1 (12.5)	1.000 ^c
Runny nose	4 (28.6)	5 (62.5)	0.187 ^c
Sore throat	5 (35.7)	5 (62.5)	0.377 ^c
Vomiting	0 (0)	0 (0)	1.000 ^c
Diarrhoea	0 (0)	0 (0)	1.000 ^c
Blood test			
Total white blood cell ($\times 10^9/L$), mean (SD)	6.16 (1.95)	5.18 (1.42)	0.308 ^d
Lymphocyte ($\times 10^9/L$), mean (SD)	0.99 (0.61) ^b	1.29 (0.67)	0.064 ^d
Viral load			
Initial pre-treatment viral load on diagnosis in log ₁₀ copies/ml, mean (SD)	6.38 (1.82)	6.51 (1.79)	0.976 ^d

Values are given as no. (%) unless otherwise specified.

n, number; SD, standard deviation.

^aDuration of viral shedding is defined as the time from onset of symptoms to the last positive sample by RT-PCR.

^bOnly 13 patients data are available.

^cBy Fisher's exact test.

^dBy Mann-Whitney test.

most of the other patients, pandemic H1N1 virus was not detectable.

DISCUSSION

Viral load data provide important information regarding virus–host interaction. Peak mean viral load occurred early during illness in both pandemic H1N1 virus and seasonal influenza virus infection, similar to studies in human volunteers [Hayden et al., 1998]. For patients who presented to hospital between days 0 and 3 after onset of symptoms, the initial pre-treatment viral load in pandemic H1N1 cases was lower than the seasonal influenza historical controls. This might be explained by differences in the severity of illness. Less upper respiratory tract and gastrointestinal symptoms were found in the pandemic H1N1 cases, since they were hospitalized despite mild symptoms during the containment phase of pandemic control in Hong Kong. Many cases were transferred directly from the airport to hospitals either because of their onset of symptoms during the flight or were found to have fever by body temperature screening upon arrival at the airport. Moreover, respiratory specimens were taken from every patient with suspected pandemic H1N1 virus infection independent of severity of symptoms or underlying diseases, whereas in non-pandemic period, only a minority of patients with seasonal influenza virus infection who attended hospital service had respiratory specimen collected for virological diagnosis. As a result,

these patients selected randomly as historical controls were likely to have more severe symptoms or with chronic underlying illnesses, which were associated with a higher initial pre-treatment viral load at diagnosis.

For both cases and controls, initial pre-treatment viral load was lower for patients who had longer duration of symptoms before hospitalization. However, this trend was not extended to patients who attended hospital 4 days after onset of symptoms. In the 4-year-old boy with pandemic H1N1 virus infection, his viral load was exceptionally high 7 days after onset of symptoms. The more prolonged symptomatic phase might be a reflection of poor host immunity, leading to a higher viral load. This observation would require verification by more patient data in the future.

As expected, viral load in pandemic H1N1 patients decreased gradually after onset of symptoms, as patient's immune system responded to infection [Hayden et al., 1998; Cheng et al., 2004b]. However, significant viral shedding, up to 6 log₁₀ copies/ml, was still present 6 days after onset of symptoms. In addition, younger age correlated with longer periods of viral shedding. This finding was similar to the previous studies in seasonal influenza virus infection, in which viral load was generally low or undetectable by day 5 of illness, but could persist for up to 21 days in children [World Health Organization Writing Group, 2006]. Viral load was found to be especially high in young children [Hall et al., 1979]. A more prolonged shedding could also occur in immunocompromised patients.

Antiviral therapy was also proven to reduce viral shedding for seasonal influenza [Hayden et al., 1999; Boivin et al., 2003; Ward et al., 2004]. However, since most of the patients in this series received oseltamivir, the effect of antiviral therapy for this novel strain could not be determined. Further studies involving patients without oseltamivir therapy would be necessary to assess the impact of antiviral treatment on viral shedding and clinical outcomes.

Pandemic H1N1 virus was detected in the stool of four patients, with positive viral culture from the specimen with the highest viral load. Previous studies showed that human influenza A H5N1 virus could be detected in stool [Buchy et al., 2007], and this virus was further demonstrated in the biopsy of the small and large intestine of fatal cases [Uiprasertkul et al., 2005; Zhang et al., 2009]. Other respiratory viruses that have been found in stool include SARS coronavirus [Hung et al., 2004], respiratory syncytial virus [von Linstow et al., 2006], adenovirus [Wilhelmi et al., 2003] and bocavirus [Lau et al., 2007]. Seasonal influenza virus detection by RT-PCR in stool has only been reported in very young children between the age of 5 weeks and 9 months, but positive viral culture has not been reported [Wootton et al., 2006]. The finding that fecal shedding occurred in patients with pandemic H1N1 virus infection highlights the importance of contact precaution when handling stool. This is especially important for younger children as they have higher viral loads in stool.

Despite its presence in the gastrointestinal tract, none of the cases developed significant gastrointestinal symptoms. This might be related to the small sample size and the relatively mild infection. Vomiting and diarrhea were common among pandemic H1N1 cases in USA, accounting for 25% of patients [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009]. The rate was similar to seasonal influenza virus and influenza A H5N1 virus infection [Wang et al., 2003; Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Virus, 2008]. The cause of gastrointestinal manifestation remains unknown, and further studies are required to understand the mechanism.

Urinary shedding of pandemic H1N1 virus occurred in a previously healthy 4-year-old boy with an apparent symptomatic phase of 7 days before diagnosis, accompanied by a persistently high viral load in both nasopharyngeal aspirate and feces 7 days after onset of symptoms. Although influenza virus can be cultured routinely on kidney cell lines, very few clinical manifestations were attributed to the urinary tract. In Tehran, 27 out of 33 patients with hemorrhagic cystitis during the 1975 influenza season had either virus isolated from throat swabs or a fourfold rise in hemagglutination inhibition antibody titer to influenza A/Tehran/5/75 virus [Khakpour and Nik-Akhtar, 1977]. In that series, influenza virus was isolated from urine in three patients. Other respiratory viruses that have been detected in urine include adenovirus [Hatakeyama

et al., 2006], SARS coronavirus [Lau et al., 2005] and bocavirus [Poza et al., 2007].

Pandemic H1N1 virus was not detected in any serum samples, which might be related to the small sample size. Viremia, which has been observed in blood donors without symptoms, is a recognized although uncommon phenomenon in seasonal influenza [Zou, 2006; Likos et al., 2007]. In influenza A H5N1 virus infection, serum level had prognostic significance [de Jong et al., 2006].

This study has several limitations. The viral load in different types of respiratory specimens might be affected by the method of collection. Nasopharyngeal aspirate is considered as the gold standard in obtaining nasopharyngeal epithelial cells. However, nasopharyngeal swab, nasopharyngeal flocculated swab and nose-throat swabs have been suggested as alternatives because of high sensitivity [Frayha et al., 1989; Lambert et al., 2008; Chan et al., 2008a]. For patients with influenza A virus infection, the quantity of virus was not significantly different between nasopharyngeal aspirate and nasopharyngeal flocculated swab [Chan et al., 2008a]. The analysis of serial viral changes might also be affected by the absence of specimens on consecutive days. Since obtaining respiratory specimens was associated with discomfort, many patients refused daily specimen collection once their symptoms improved.

Virological profile of pandemic H1N1 virus infection, as determined by the extent of viral shedding and changes in viral load in this study, provides essential information in designing management policies. Fecal and urinary shedding of pandemic H1N1 virus, especially in children, may contribute to inadvertent human-to-human transmission despite emphasis on droplet and contact precautions for respiratory secretions. Since prolonged shedding may occur despite oseltamivir therapy, routine infection control measures should also be enforced during at risk period to prevent outbreaks in institutions.

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