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Full Length Research paper

Quantification of viral load in clinical specimens collected from different body sites of patients infected with influenza viruses

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Viral shedding profile of infections caused by the novel influenza A (pH1N1) virus has not been extensively studied. In the present study we aimed to compare the influenza viral load in clinical specimens collected from different body sites of patients to analyze the best specimen for detecting viral load and predicting disease severity. The respiratory specimens (throat and nasal swabs), urine and serum were collected from patients on first day of their hospital visit within 48 h of onset of influenza like illness (ILI) and screened for influenza positivity in respiratory specimens by real-time RT-PCR. A total of 10 pandemic H1N1 and 15 seasonal influenza positive cases were included in this study and viral load was estimated in all the types of specimens by real-time RT-PCR. Our findings revealed that the nasal swab had the highest mean viral load of 21.406 x 10^4 followed by throat swab (12.777 x 10^4), urine (0.026 x 10^4), serum (0.0007 x 10^4). These findings confirm that nasal secretions are the best specimen, followed by throat swab, urine and serum. The importance of this study is to show the viral shedding profile in different specimen types and to suggest alternatives to respiratory specimens for the diagnosis of influenza.

Key words: Influenza virus, clinical specimen, viral load, real-time RT-PCR, viremia.

INTRODUCTION

The pandemic H1N1 (pH1N1) influenza virus has swept across the entire globe in a short duration of time. The health authorities registered a stupendous increase in the number of affected cases that WHO had to declare it a pandemic within months of its inception (Khanna et al., 2009a; WHO, 2009a). The virus was unique as it was a product of genetic reassortment, deriving its genomic segments from avian, swine and human influenza virus strains (Khanna et al., 2009b; MacKenzie, 2009). A cocktail of different genomic segments has conferred a survival advantage to this lethal virus (Matsuzaki et al., 2003). A meticulous analysis traced its evolutionary roots in North American swine, Eurasian swine, North American avian and human influenza lineage (Garten et al., 2009). In the wake of alarming situation, CDC recommended screening of the passengers at the airports, social distancing and use of oseltamivir for the treatment of pandemic H1N1 infection (CDC, 2009a; Glass et al., 2006). Drugs for prophylaxis are not recommended, as in many countries namely, Japan, Denmark, Hong Kong and India, oseltamivir resistant cases have been reported in a cohort of immunocompromised patients (WHO, 2009b). Studies have shown that the pandemic H1N1 virus is mainly affecting younger people, the most productive group of the population (Vaillant et al., 2009). Unlike seasonal influenza, pandemic H1N1 preferentially infected people below 60 years of age preferentially

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worldwide, with unusually high rate of severe respiratory disease and mortality among young patients in Mexico (Chowell et al., 2009). The influenza virus establishes itself in the respiratory tract where it replicates and increase in titers and disseminates itself in the community (Munster et al., 2009). After acquiring the infection, the person remains infectious from 1 day prior to the onset of symptoms to 5 to 7 days of infection (CDC, 2009a; Khanna et al., 2008).The correlation between the virological profile and clinical characteristics of pandemic H1N1 virus infection is of paramount importance as an integral part of pandemic preparedness for disseminating the measures to check its spread from the infected to the healthy person. The viral load profile from different body sites could be important in predicting the disease severity and transmissibility in viral infections. The present study was designed to measure the influenza viral load present in different types of clinical specimens, namely: nasal swab, throat swab, urine and serum, collected from same patients with suspected cases of influenza virus infection on first day of their hospital visit and to understand the viral transmission from person to person in the community and to suggest alternatives to respiratory specimens for the diagnosis of influenza.

MATERIALS AND METHODS

Specimen collection

Clinical specimens from suspected cases of swine influenza patients were collected for the surveillance studies of influenza virus from different hospitals, clinics and emergency care centers during the 2009 to 2010 pandemic periods. A total of 15 seasonal influenza (10 H3N2 and 5 H1N1) and 10 pandemic H1N1-2009 positive specimens were included in the study. The blood and urine samples, apart from respiratory specimens, were also collected from the patients on the first day of their visit at the hospital within 48 h of onset of influenza like illness (ILI), which consisted of fever (≥ 38°C) with more than 2 of the following symptoms: cough, sore throat, head- ache, fatigue, coryza, myalgia and chills/rigor. The collected specimens were transported to the laboratory in suitable transport medium within 24 h of collection, maintaining the temperature at 4^0C. The patient's clinical case-sheets were thoroughly reviewed for the necessary clinical and demographic details. Written consent forms were taken before specimen collection from patients or their guardians.

Nucleic acid isolation and RT-PCR

Total nucleic acid extraction was performed by using QIAamp Viral RNA Isolation kit (Qiagen) according to the manufacturer's instructions. The samples were tested as per the CDC/WHO protocol for the presence of human influenza A virus. The quantitative analysis of viral load in all type of clinical specimens from influenza positive patients, on first day of their hospital visit, were performed by quantitative real-time PCR (RT-PCR) using primers and probes targeting hemagglutinin gene of both seasonal (H1N1 and H3N2) and pandemic (H1N1-2009) virus (Novel swine-origin influenza A (H1N1) virus investigation team, 2009; Ninove et al., 2010). The genetic amplification was performed in a one-step RT-PCR set-up in a 25 µl final volume. Briefly, 5 µl of RNA extract, 0.5 µl of each 10 µM primer solution, 0.5 µl of 10 µM probe solution, and the Superscript III Platinum one step qRT PCR System (Invitrogen, Cergy Pontoise, France) was used in each 25 µl reaction. The reactions were conducted in BioRad IQ5 cycler with the following thermal profile: 50°C for 30 min, 95°C for 2 min and 45 cycles at 95°C for 15 s and 55°C for 45 s (CDC, 2009b).

Assessment of viral load

Viral load assessment was done by preparing a reference standard using pGEM-T Easy vector (Invitrogen, San Diego, CA) containing the corresponding target viral sequences of both pandemic H1N1 and seasonal influenza viruses. A series of seven dilutions equivalent to 1×10^1 to 1×10^7 copies per reaction for all three strains were prepared to generate calibration curves and run in parallel with the test samples. The experiments were conducted in duplicate to generate a graph of mean Ct value vs. Log_{10} copy number. The detection limit of the assay for pandemic H1N1, seasonal H3N2 and seasonal H1N1 was around 100 copies of RNA per reaction.

Statistical data analysis

One sample t test using the Prism software (version 5) was performed for statistical analysis of the data. A two-tailed $p<0.05$ was considered significant.

RESULTS

Virus shedding

For determination of viral load and the real-time RT-PCR assay reproducibility, the pGEM T Easy vector harboring the viral target sequences (pandemic H1N1, H3N2 and seasonal H1N1) were diluted serially (10^1 to 10^7 copies per reaction) to create a standard curve. It was observed that the linear detection range of real-time RT-PCR was 100 copies to 10^7 copies of target nucleic acid covering a wide 10^5 fold range for pandemic H1N1. Almost similar linear detection range was also observed for the seasonal viruses. A total of 15 seasonal influenza (10 H3N2 and 5 H1N1) and 10 pandemic H1N1-2009 positive specimens were included in the study. All specimens were subjected to qRT-PCR for analysis of viral load. The seasonal influenza A/pandemic H1N1-2009 viruses (HA gene) were detected in all the respiratory specimens, while their presence was detected in few specimens in serum and urine (Figure 1). Almost all the specimens collected from different body sites showed presence of seasonal or pandemic H1N1 viruses however the interesting feature was that the pandemic H1N1 virus was not detected in any serum samples. There was a direct correlation between the viral load and the clinical signs and symptoms as shown in Table 1. The infected patients had both respiratory (for example fever, cough with expectoration, coryza) and systemic symptoms (for example tachycardia, tachypnea and abdominal complaints). The real-time RT-PCR viral load analysis as shown in Figure 2 revealed that the nasal swab was the best site of specimen collection with the mean viral load

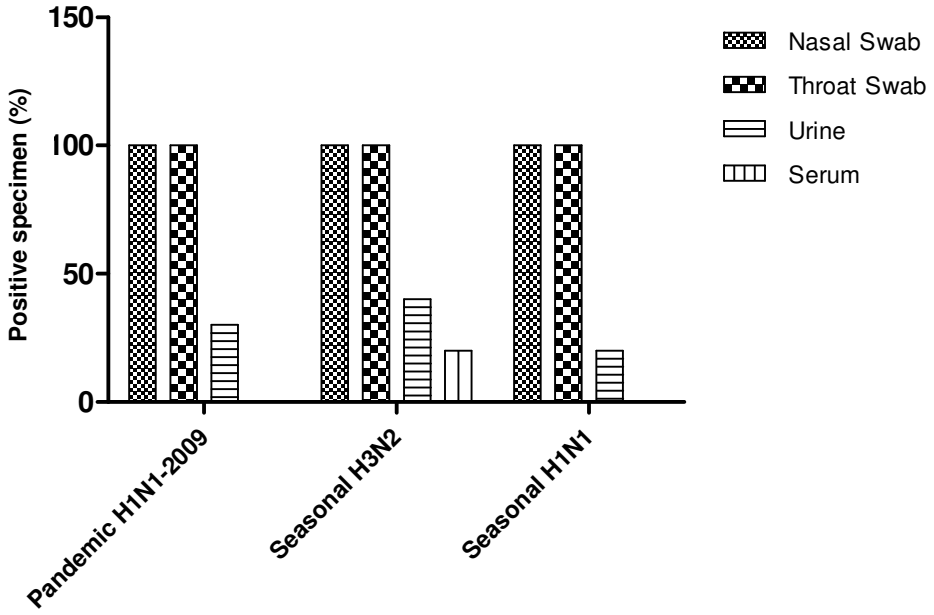

Figure 1. The real-time RT-PCR analysis showing percentage positivity of influenza A viruses in different types of clinical specimens collected from different body sites.

Table 1. Underlying medical conditions of patients infected with seasonal and pandemic influenza A (H1N1-2009) virus.

Presenting symptoms	Value (seasonal influenza A virus)	Value (pandemic H1N1-2009 virus)
Fever- no. (%)	15/15 (100)	10/10 (100)
Cough- no. (%)	14/15 (93.3)	9/10 (90)
Sputum production- no. (%)	4/15 (26.6)	1/10 (10)
Running nose- no. (%)	15/15 (100)	6/10 (60)
Sore throat- no. (%)	10/15 (66.6)	4/10 (40)
Vomiting- no. (%)	0/15 (0)	2/10 (20)
Diarrhea- no. (%)	0/15 (0)	0/10 (0)
Chest pain- no. (%)	2/15 (13.3)	2/10 (20)
Body pain- no. (%)	13/15 (86.6)	8/10 (80)
Pulse rate (/min); mean (median)	99.5 (96.40)	101.5 (98.0)
Respiratory rate (/min); mean (median)	30.50 (30.40)	33.6 (30)
Breathlessness- no. (%)	4/15 (26.6)	4/10 (40)
Blood test		
Total white blood cell (10^3/ µL), mean (SD)	7 (3.37)	8.363 (5.765)
Lymphocyte (X 10^3/ µL), mean (SD)	1.00 (1.28)	1.793 (1.073)

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

of 21.406 x 10^4 followed by throat swab (12.777 x 10^4), urine (0.026 x 10^4) and serum (0.0007 x 10^4).

The presence of viral load in urine and serum specimens indicated that they can serve as useful sites of collection for the diagnosis of influenza, because obtaining specimens from nasal and throat swabs is sometimes difficult in case of ventilated patients and young children. The statistical analysis showed the two tailed P<0.0001 by one sample t test for all the viral strains.

DISCUSSION

In less than a decade, the influenza has returned with a vengeance, changing its course from avian H5N1 that had a limited human penetration, to pandemic H1N1 virus being widely disseminated in humans (Guleria et al., 2009). It has been shown that the novel virus is able to

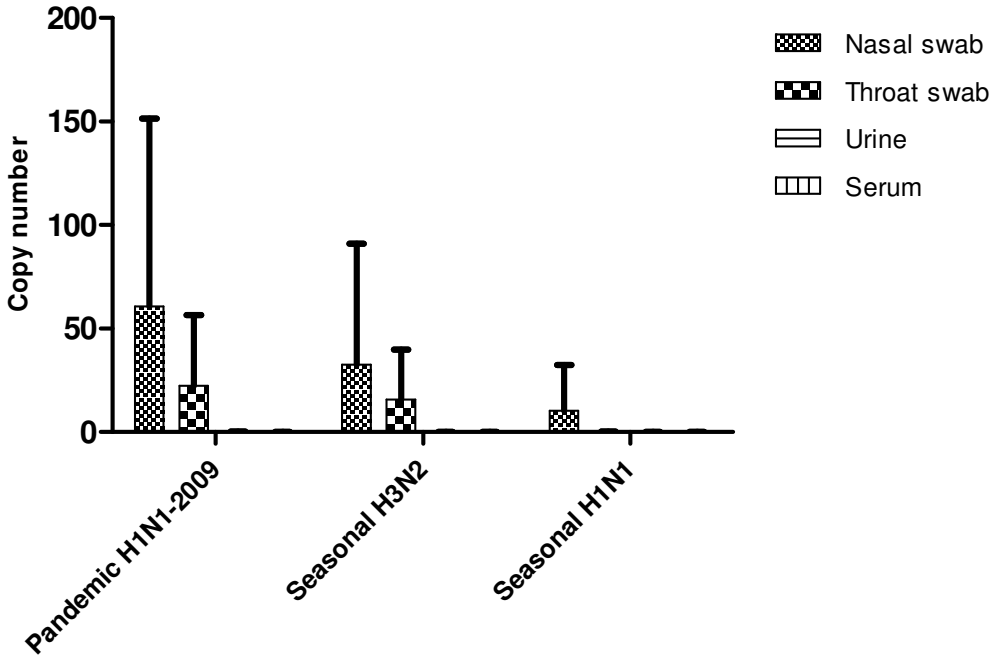

Figure 2. The real-time RT-PCR analysis showing different types of clinical specimens with the mean viral load of influenza viruses.

evade the immune response; leading to multi-organ failure in infected patients (Osterlund et al., 2009). In such cases, viral load at different sites and at different times can be an important predictor of disease severity and regression (Kelvin et al., 2010). Mean viral load at its peak were recorded in the early phases of illness in pandemic H1N1 virus infection, similar to studies in human volunteers (Hayden et al., 1998; Kelvin et al., 2010). Viremia, which has been observed in blood donors without symptoms, is recognized, although uncommon phenomenon in seasonal influenza (Likos et al., 2007; Zou, 2006). In influenza A H5N1 virus infection also, serum level has shown prognostic significance (De Jong et al., 2006). In our study, the viral load data provides important information to clinicians and researchers regarding virological profile in relation to the clinical specimen collected from different body sites and high-risk conditions associated with the disease. The site-specific collection of specimen might be helpful in formulating additional strategies for infection control. The viral load was detected in all the nasal and throat swabs while the urine and serum specimens showed variable virus detections (Figure 1). The presence of virus in urine suggests that urine analysis may be employed as a routine method to detect viral load especially in children, in whom virus transmission occurs through urine and feaces, and also in hospital admitted patients who are on ventilators. Viral load profile of influenza virus infection in this study provides an essential tool in designing management policies to successfully pay attention to crowd of patients with complaints of ILI in any pandemic situation. 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 (Hall et al., 1979).

Since prolonged shedding may occur despite oseltamivir therapy, routine infection control measures may also be enforced during, at risk period to prevent outbreaks in institutions. Thus our findings suggest that although the nasal and throat swabs are established methods of specimen collection, urine and serum may also be used as alternatives to respiratory specimens for influenza diagnosis.

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