Clinical - epidemiological profile and diagnosis of Influenza A H1N1 cases by real time RT-PCR at a tertiary care institute of India: the war is not over yet

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ABSTRACT

Background: Influenza A virus is a common human pathogen that has caused serious respiratory illness and death over the past century. In April 2009, WHO declared pandemic influenza A H1N1 public health emergency of international concern. India is reeling under the worst H1N1 influenza outbreak with over 18,000 affected cases and over 1000 deaths by the year 2015. **Methods:** The present study was conducted to find the clinical and epidemiological profile of H1N1 influenza A cases and a real time RT-PCR was standardized and evaluated for the detection of H1N1 influenza A virus in suspected cases admitted in a tertiary care hospital of northern India. **Results:** Of the total 184 clinical samples tested, 48(26.0%) samples were found to be positive for influenza A H1N1 virus by real time RT-PCR. The highest percentage of cases was in the age group of 40-55 years followed by the 20-40 years. The main clinical symptoms were fever(95.8%), breathlessness(77.0%) , cough(68.7%) and sore throat (56.2%).The mortality rate of cases admitted with H1N1 infection was 52.0%. **Conclusion**: The mutational behavior of H1N1 has been a major future challenge in the part of pharmacotherapy. Rapid and sensitive diagnostic methods like real time RT-PCR increase the capability to detect, understand and assess new viruses for pandemic risk and to track their international spread.

Key words: Influenza A H1N1, Real Time RT-PCR.

Introduction

Influenza virus is a common human pathogen that has caused serious respiratory illness and death over the past century. It always had potential to cause widespread pandemics whenever a new type of Influenza strain appeared in the human population [1].A new strain of Influenza A (H1N1) virus was first identified in humans in Mexico and the United States (US) in 2009 and has since spread worldwide (WHO, 2009). The World Health Organization (WHO) declared pandemic alert stage 6 on 11 June 2009, indicating an ongoing influenza pandemic. India confirmed its first case in May 2009, and is reeling under the worst outbreak with over 18,000 affected cases and more than 1000 deaths due to H1N1 flu by the end of February 2015 [2].The H1N1 virus strain has been found to be closely related to the swine flu virus, but with a genetic composition that is quite different from the earlier known isolates. This novel strain is antigenically distinct from seasonal influenza A virus and possesses previously unrecognized molecular determinants that could be responsible for rapid human to human transmission[3].To limit community or hospital transmission, as well as to initiate antiviral therapy in time as recommended by

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the WHO, the rapid detection of the virus in suspected cases remains crucial [4].Several methods are available to diagnose influenza virus infection including rapid tests for the viral antigen, immunofluorescence (IF) and virus RNA isolation by PCR. Real-time RT PCR is the latest gold standard for diagnosis of the novel pandemic swine 2009-H1N1 virus. Real-time RT-PCR assays for influenza virus using TaqMan-based probes have been recommended by WHO for the diagnosis of the currently circulating pandemic swine 2009-H1N1 virus. In the clinical diagnosis of influenza, nucleic acid testing by RT-PCR has widely replaced traditional virus culture due to shorter turnaround times and increased sensitivity [5].As there are very limited studies in the literature depicting epidemiological profile of H1N1 influenza A in Indian subcontinent, the study aims at finding the various epidemiological trends, clinical profile and rapid detection of pandemic 2009 H1N1 virus using Taqman based real time RT-PCR assay from respiratory specimens at a tertiary care hospital in northern India.

Materials and Methods

Nasal, nasopharyngeal and throat swabs were collected from 184 patients suspected of being infected with novel 2009 H1N1 virus from various hospitals of district Amritsar between the periods January 2015 to May 2016. Most of the clinical specimens were collected from patients presented with high fever (>38°C), running nose, sore throat and having close contact with laboratory confirmed case and a recent travel history to place with sustained human to human transmission of the virus. All the samples were transported in viral transport media and accompanied with duly filled proforma indicating demographic characteristics, clinical symptoms, co-morbidities, antiviral treatment, outcome, duration of stay in hospital *etc*. The samples were handled in BSL-2 facility and were subjected to real time RT-PCR at the Viral research and diagnostic Laboratory, Government Medical College, Amritsar in accordance with the protocol as recommended by WHO [6].

RNA Extraction

The samples were extracted using the QIAamp viral RNA extraction kit (Qiagen, Hilden, GERMANY) according to the manufacturer's specifications. Viral RNA was extracted from 140 µl of clinical specimens and eluted in 60µl of QIAamp elution buffer. The eluted RNA was used immediately or stored at -80°C.

Real-Time RT-PCR Assay

PCR parameters of the real time RT-PCR H1N1 Panel were optimized using Invitrogen SuperScript III Platinum One-Step quantitative RT-PCR kits (Life Technologies, Carlsbad, USA). All real time RT-PCRs were performed at a total reaction volume of 25 µl. All primers and probes (Applied Biosystem) were provided by National Institute of Virology, Pune. The Influenza A assay was designed for universal detection of the matrix (M) gene of all influenza A viruses. The Influenza A H1N1 assay was designed to specifically detect the Haemagglitinin (HA) gene of influenza A (H1N1). The RNase P assay detects the human RNase P gene and is used with human clinical specimens to measure the quality of the specimens as well as to indicate that nucleic acid was extracted adequately from the clinical specimen.

Table 1: The primer probe sequences and concentrations

Primers and Probes	Sequence (5'>3')	Working Concentration
InfA Forward	GAC CRA TCC TGT CAC CTC TGA C	40 µM
InfA Reverse	AGG GCA TTY TGG ACA AAK CGT CTA	40 µM
InfA Probe	TGC AGT CCT CGC TCA CTG GGC ACG	10 µM
InfA(H1N1) Forward	GCA CGG TCA GCA CTT ATY CTR AG	40 µM
InfA(H1N1) Reverse	GTG RGC TGG GTT TTC ATT TGG TC	40 µM
InfA(H1N1) Probe	CYA CTG CAA GCC CAT ACA CAC AAG CAG GCA	10 µM
Rnase P Forward	AGA TTT GGA CCT GCG AGC G	40 µM

RnaseP Reverse	GAG CGG CTG TCT CCA CAA GT	40 µM
RnaseP Probe	TTC TGA CCT GAA GGC TCT GCG CG	10 µM

All analytical performance data and clinical specimen data were collected using a Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, USA) (Figure 1)

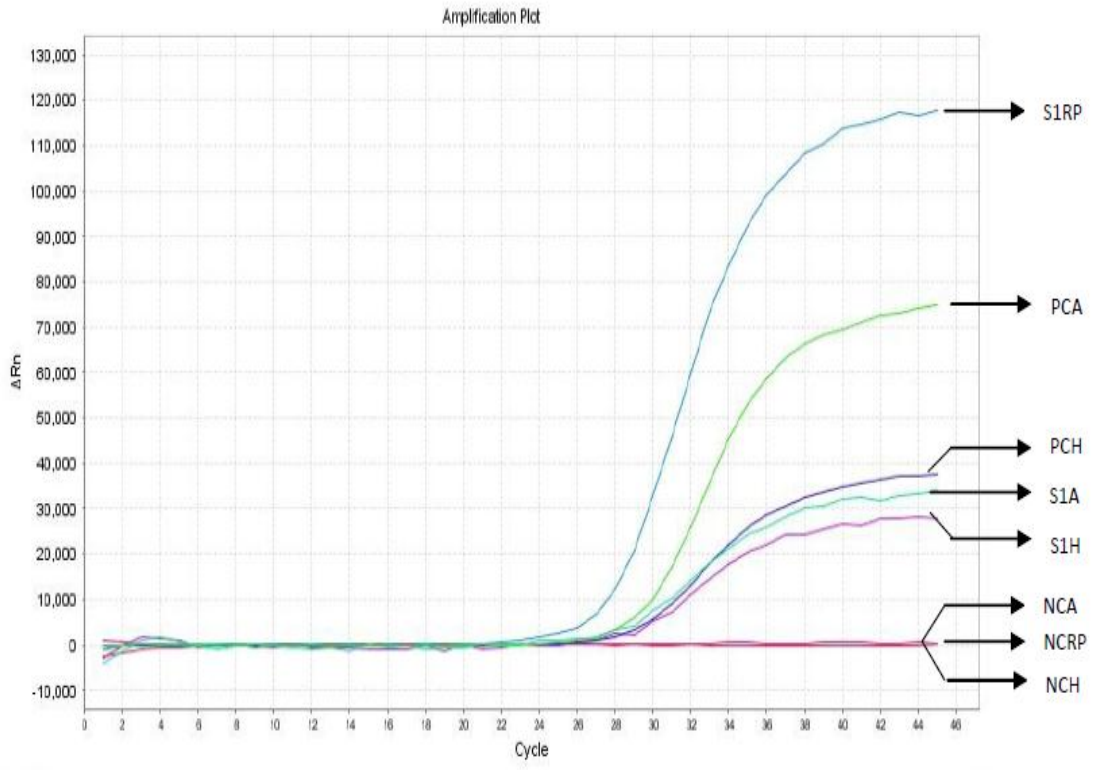

Figure 1: Amplification curve of real time RT-PCR

(NCA: Negative Control for Influenza A; **NCH**: Negative Control for Influenza A(H1N1); **NCRP**: Negative control for RNase P; **S1A**: Sample for Influenza A ; **S1H**: Sample for Influenza A(H1N1); **S1RP**: Sample for RNase P; **PCA**: Positive Control for Influenza A ; **PCH**: Positive Control for Influenza A(H1N1)

Results

From January 2015 to May 2016, a total of 184 patients were tested for H1N1 influenza and seasonal influenza A virus. Real time PCR analysis revealed that 107 (58.1%) samples were influenza A positive, and 48 (26.0%) samples confirmed for H1N1 influenza. As our viral research and diagnostic laboratory was standardizing the real time PCR for influenza viruses, few samples were also sent to state reference laboratory at PGIMER, Chandigarh for the validation of our results. All the confirmed cases were admitted in the Influenza A H1N1 isolation ward at Govt.Medical College & Hospital, Amritsar, of which 25(52.0%) succumbed to the disease. Monthwise analysis of the cases revealed a major peak during the winter season in this part of India, more than 90% cases of H1N1 influenza were detected in the months from December to February (Figure 2).

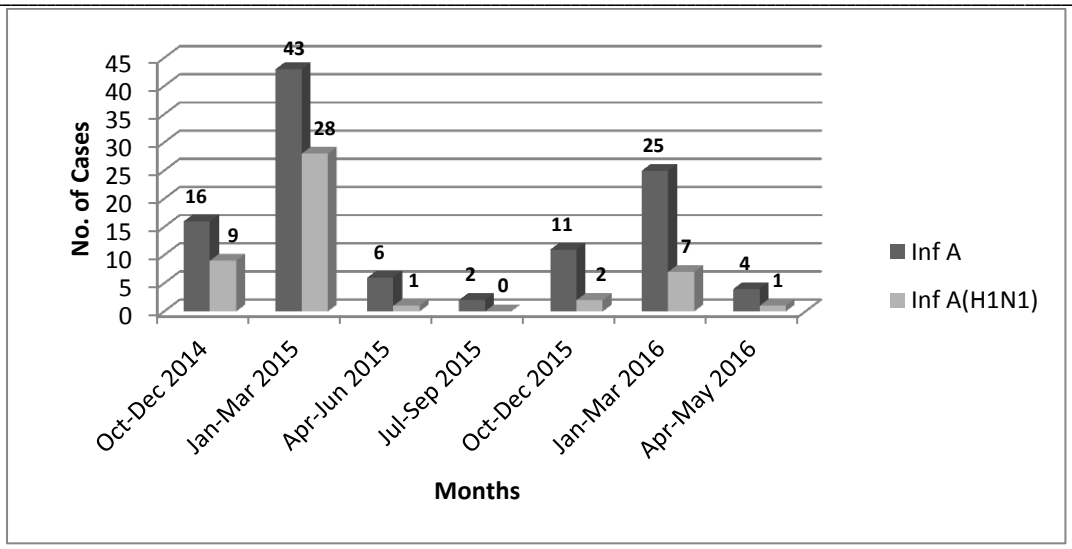

Figure 2: Trend of seasonal influenza A (n=107) and Influenza A H1N1 (n=48) cases

Age wise analysis of the positive cases revealed that the maximum positivity (37.5%) for H1N1 influenza virus belonged to the age group of 40-55 years followed by 20-40 years (31.2%). However seasonal influenza A was predominant (50.3%) in age group of 20-40 years. Of the total patients screened 21(43.7%) were males and 27(56.3%) were females however no gender specificity of infection was observed. The common presenting symptoms in H1N1 positive cases were fever (95.8%) followed by breathlessness (77.0%), cough (68.7%), sore throat (56.2%), rhinitis (18.7%), haemoptysis(12.5%) and diarrhoea (4.1%)(Figure 3). Radiological findings of lungs showed bilateral opacities in 85.4% of cases. On analysis of complete haemogram it was found that 67.3% cases had normal total leukocyte count while 17.1% cases had neutrophilic leukocytosis and rest had leukemia. A total of 39 (81.2%) cases had associated co morbidities which included diabetes mellitus, hypertension, cardiovascular diseases, COPD, autoimmune diseases, dearranged liver or renal function tests. It was observed that mortality rate of cases who were admitted in H1N1 isolation ward was 25(52.0%). Maximum mortality (64.0%) was found in the younger age group (40-60 years) , followed by 28 % of deaths occurred in age group above 60 years. Most of the patients who died required intensive care and ventilatory support. Of the total (25) deaths, 19 (76.0%) occurred within 48 hours of admission, of which 3 were within 24 hours of admission.

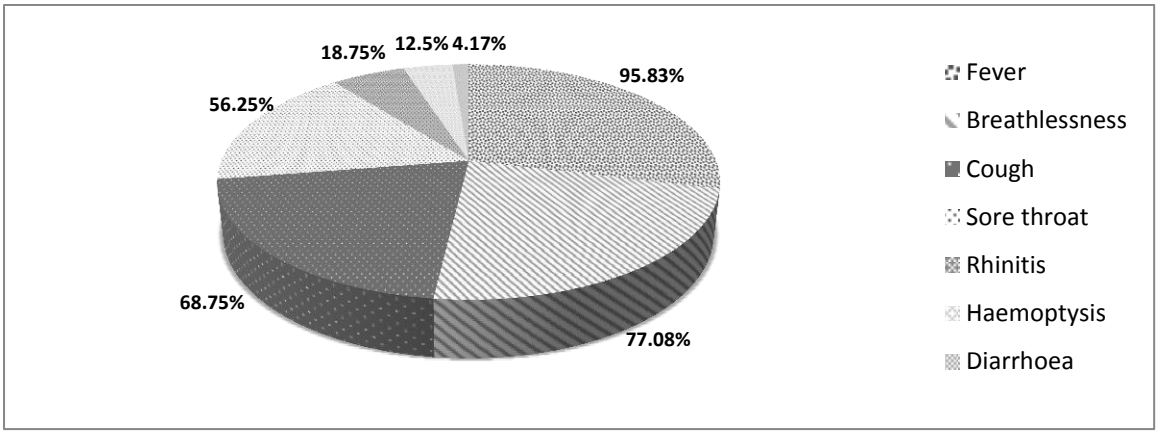

Figure 3: Various clinical presentations among laboratory confirmed cases (n=48) of H1N1 influenza

Discussion

In its strongest resurgence since the pandemic of 2009, the influenza type A virus known as H1N1, has broken out in different parts of India with deaths surpassing 1000 mark and the number of affected cases exceeding 18,000 by the end of February 2015 [7].The outbreak of the H1N1 virus is the deadliest in India since 2010. As many parts of the country have stopped reporting individual cases, particularly the milder illnesses, the case count is significantly lower than the number of cases that have occurred acually. The present study aims at finding the various epidemiological trends, clinical profile and Taqman based real time RT-PCR assay for rapid detection of H1N1 influenza A virus from respiratory specimens of suspected cases admitted in tertiary care hospital of northern India. Age wise analysis of the positive cases revealed that maximum positivity for H1N1 influenza belonged to the age group of 40-55 years (37.5%) followed by 20-40 years(31.2%) followed by elderly patients (> 55 years), revealed that patients of younger age group were more vulnerable to H1N1 influenza A infection while the seasonal influenza virus seen more common in patients of 20-40 years. The probable reason of less infection in elderly population could be due to the cross preventive effect of antibodies developed against the circulationg seasonal influenza A viruses. Other studies done by various authors also revealed similar findings .Study done by Vijaydeep *et al* in 2012 clearly reflects the high prevelance, morbidity and mortality due to H1N1 virus among younger population [8].Another study conducted at New Zealand concluded that H1N1 influenza A predominantly affected young with relative sparing of the older population[9].According to another study done in Queensland, a large number of cases were reported in the 10-19 years age group (28%), followed by the 20-29 years age group (26.0%) In our study no gender specificity of infection was observed as percent infection among genders were similar.The most common symptoms with which patients presented were fever (95.8%), breathlessness(77.0%), sorethroat (68.7%) and cough (56.2%) (Figure 3).[10]. In a study done in mainland china, fever (81.0%), cough (40%)and sore throat (35.0%) were found to be the most common symptoms (Li *et.al,* 2009). Fever was also reported to be the most common (80.1%) symptom followed by sorethroat (61.3%) , breathlessness (58.3%) and cough (49.3%) in a study done by Domadia *et al*. Another study conducted at Chandigarh also described fever (87.2%) as the most common symptom followed by cough (49.7%), sorethroat (27%) and breathlessness (23.9%) [11].Various other authors in their studies done in Chile and Japan reported similar clinical pattern of H1N1 influenza [12].The clinical presentation varies from asymptomatic cases to primary viral pneumonia resulting in respiratory failure, acute respiratory distress, multiple organ failure and death as seen by other authors in their findings [13].Most but not all the hospitalized patients had underlying conditions such as cardiovascular diseases, respiratory diseases like asthma and COPD, autoimmune disorders and other comorbidities include diabetes, hypertension etc. Our study also revealed that more than 60% deaths occurred in the age group 18-40 years, with 26.3% deaths reported in the age group of 40-60 years and 10.5% in patients >60 years. Vijaydeep *et al* reported 57% total mortality was observed in patients less than 40 years of age. In the present study, we also demonstrated that the real time RT-PCR assay specifically targets the haemagglutinin gene of the H1N1 influenza A virus. The primers and probes recommended by WHO is a realiable identification method used for the detection of H1N1 virus in the clinical samples taken from suspected patients. Unlike traditional RT-PCR, the real time RT PCR assay not only reduces the risk of contamination but also reduces turnaround time to 1-2 hours. In a study conducted by Kumar B and others, 48.40% of the samples tested positive by real time RT PCR assay while the traditional PCR detected only 42.46% thereby showing the superior sensitivity of real time RT PCR assay [14].In our study we detected 26.0% of cases found to be positive for H1N1 influenza A using real time RT-PCR assay. In India, influenza virus had been generally ignored in public health and in healthcare settings. Etiology specific diagnosis requires laboratory tests that are not widely available in here. Therefore what we know about epidemiology and clinical features are mostly from research studies only. In tropical countries like India, year around circulation of influenza strains have been reported. In the present study, most of the cases of both seasonal as well as H1N1 influenza A virus infection peaks during the cold winter season (December to February). We also observed that co circulation of both pandemic and seasonal influenza during the winter season which is in contrast to reports from other regions in India and worldwide where pandemic influenza had completely replaced seasonal influenza strains. Mukherjee and associates in 2010 also reported in their study that in northern India with very cold winter season, peaks of infection was observed during winter season (December to Feburary) and during rainy season.[15,16] From the present study we have also seen that the prevalence of H1N1 influenza has

decreased, since only 8 cases were reported in year 2016 as compared to 2015 when 40 patients were found to be infected with H1N1 influenza A (Figure 2). The flu season seems to be dying down but the war is yet not over. Lessons must be learnt from the previous influenza pandemics and it is still important to get vaccinated against the H1N1 flu and be prepared as activity as well as virulence might increase again in coming season. Although patients in this study comprised cases from Amritsar and nearby districts, the findings of this study need to be carefully reviewed and cannot be generalized to large population. We also restricted our study to one hospital; many cases of influenza A H1N1 admitted in our hospitals may have been missed. Moreover not being a community based study we may not be able to calculate the exact measures of epidemiology. There may be small number of cases that may have been missed out, although every attempt was taken to include all the cases, but this figure would not have been significant. This study provides a hospital based epidemiological information, but a community based wider studies are required to arrive at a more precise and accurate understanding of influenza A H1N1.Inspite of low pathogenesis, overall the H1N1 influenza A confirmed the ease with which infection can be spread and facilitated by community networks and gatherings. This further necessitate the need of active influenza surveillance and easy access to sensitive and rapid diagnostic methods, cheap vaccines and antiviral drugs in developing countries for better control and prevention of future pandemics.

Acknowledgements

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