DOI: 10.18231/2394-5478.2019.0005

Recent trends of pandemic 2009 H1N1 virus infection in Guwahati, Assam: A prospective study

Syed Tanwir Alam[1,*], Gitika Rajbongshi[2], Debajit Rabha[3], Ajanta Sharma[4]

[1]Assistant Professor, [2]Research Scientist, [4]Professor, Dept. of Microbiology, Gauhati Medical College, Guwahati, Assam, India

***Corresponding Author: Syed Tanwir Alam**
Email: alamtanwirghy@gmail.com

Abstract
The virus that has posed a serious public health challenge world-wide was pandemic 2009 H1N1 influenza A. This prospective study was undertaken to analyze the clinical profile of 2009 pandemic H1N1 influenza A virus [A (H1N1) pdm 09] infection in patients presenting with influenza like illness from January 2015 to December 2015. A total of 116 respiratory samples were collected from the patients attending the hospitals, which included 71 males and 45 females, ranging in age from one to 79 years. Respiratory specimens were tested for A (H1N1) pdm 09 virus by one step real time reverse transcription polymerase chain reaction. In real-time PCR, the A (H1N1) pdm 09 virus was detected in 14 patients (12.07%) and highest percent of positivity (50.0%) was detected in the age group 25-40 years with the mean age being 40.07 ±4.43 years (SD). Most common presenting symptoms were fever (92.86%) and cough (92.86%) followed by shortness of breath (57.14%), nasal catarrh (50.00%) and sore throat (35.71%). From the study it has been seen that there is prevalence of A (H1N1) pdm 09 virus infection in Assam and thus it needs urgent attention to prevent further spread of the virus in the community.

Keywords: Pandemic, Influenza virus, H1N1, Real-time RT-PCR.

Introduction
Among the respiratory viruses which can cause epidemic outbreaks, influenza A viruses are particularly dangerous. Influenza A virus belongs to the family *Orthomyxoviridae* is a single stranded negative sense RNA virus. The virus causes a highly contagious acute respiratory illness and is a major health threat throughout the world (Scott et al., 1996). Due to the presence of a segmented genome of influenza A virus, with eight RNA segments and the virus undergoes to high rate of mutation in the surface hemagglutinin (HA) and neuraminidase (N) proteins. These unique molecular features coupled with the ability of the virus to cause infection in a wide host range of humans, domestic animals and birds renders it a potential pandemic agent. Domestic pigs and birds because of their proximity to humans provide a great opportunity for the occurrence of mixed influenza infections.

In India, the first laboratory-confirmed case of A (H1N1) pdm 09 virus infection was reported on 16 May 2009; a 23-year-old man who had travelled from United States of America to Hyderabad in South India and by end of the year 2010, 20,604 cases with 1763 deaths were reported (Gupta et al., 2011). Since December 2014, A (H1N1) pdm 09 virus reappeared in several states of the country including northeastern region of India, leading to over 30,000 cases and 2000 deaths countrywide (as of 28th March, 2015). The A (H1N1) pdm 09 virus infection demonstrates the need for reliable tools that enable rapid laboratory confirmation and follow-up of infections. Moreover, to limit community or hospital transmission and to initiate antiviral therapy in time as recommended by the World Health Organization (WHO), the rapid detection of the virus in suspected cases remains crucial.

Real Time RT-PCR for detection of sequence specific templates can be achieved by using fluorogenic probes, specificity is ensured by an inherent hybridization reaction, and cross-contaminations are largely avoided (Heid et al., 1996). Moreover, use of one step real-time PCR reduces the chance of carryover contamination and disables post-PCR processing as a potential source of error. Detection of influenza A virus by real time RT-PCR is based on the conserved matrix protein 2 gene (M2) and the respective subtype identification is based on detection of the hemagglutinin HA1 gene. One step real-time RT-PCR targeting the M2 and HA1 gene of influenza A virus genome was used in the present study to confirm the A (H1N1) pdm 09 virus infection in clinical samples.

Materials and Methods
Specimen Collection and Processing
This is a prospective study, conducted from January 2015 to December 2015 for a period of one year. The study is based on the respiratory samples collected from the patient sent to the Virology Laboratory, Department of Microbiology, Gauhati Medical College & Hospital in Assam, located in the city of Guwahati. The respiratory specimens consist of 20 nasal swabs and 97 throat swabs were collected from patients of all age groups with acute respiratory illness who fit into the WHO's influenza like illness (ILI) case definition. As per WHO 2008, cases were defined as ILI in patients presenting with sudden onset of fever > 38°C or history of sudden onset of fever in the recent past (less than three days), cough or sore throat and/or rhinorhea in the absence of other diagnosis. A questionnaire containing questions inquiring about any sign or symptom related to respiratory infection during the samples collection was completed for each patient. Collected swab specimens were then placed in sterile screw-capped containers with viral transport media (VTM) and transported in ice to the laboratory. Swab specimens were gently agitated and extracted for further processing. The extracts were kept at -70°C until RNA extraction.

Isolation of RNA from Clinical Samples

All samples in the laboratory were processed in bio-safety level 3 facilities. Viral RNA was extracted using the viral nucleic acid extraction kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 200 µl samples were added to 250 µl binding buffer with poly (A) and proteinase K, vortex and incubated for 10 min at 72ºC. After 10 min, the solution was mixed with 100 µl binding buffer and the mixture was centrifuged through a spin column. Then inhibitor removal buffer (500 µl) was added and centrifuged at 8000 g for 1 min. After washing the column twice with the appropriate buffer, finally RNA was eluted using 50 µl elution buffer and stored at -20º C.

Primers and Probe Sequences

For the one step real-time RT-PCR assay, a set of primers and probes described earlier (Panning *et al.,* 2009) targeting the M2 and HA1 gene sequence of influenza A (H1N1) virus was used. The genome position and sequences of the primers and probes used in this study are presented in table 1. The genome position according to influenza A (H1N1) virus (Genbank accession number FJ966082) and all the primers and probes were synthesized by TIB Molbiol, Berlin, Germany. The probe was labeled with 6-carboxyfluorescein (FAM) at the 5' end as reporter dye and barbeque quencher as non-fluorescent quencher at the 3' end.

Table 1: Primer and probes used real-time RT-PCR

Name	Sequence (5'→3')	Genome position
Primer M_InfA F	AAG ACC AAT CCT GTC ACC TCT GA	175-197
Primer M_InfA R	CAA AGC GTC TAC GCT GCA GTC C	269-248
Probe M_InfA TM	FAM- TTT GTG TTC ACG CTC ACC GC- BBQ	215-234
Primer H1 SWS	CAT TTG AAA GGT TTG AGA TAT TCC C	380-404
Primer H1 SWAs	ATG CTG CCG TTA CAC CTT TGT	457-437
Probe H1 SWP	FAM-ACA AGT TCA TGG CCC AAT CAT GAC TCG-BBQ	409-435

One step TaqMan Real Time RT-PCR

One step real-time RT-PCR assay was carried out using the commercially available real time ready Influenza A/ H1N1 detection set (Roche Diagnostics GmbH, Germany). The real time RT-PCR assay was optimized using a total volume of 20 µl. Briefly, for a single sample 7.6 µl nuclease free water, 4 µl 5x reaction buffer, 0.4 µl 50x enzyme blend and 3 µl preheated primer/probe mixture (Inf A/M2 or Human NA or Inf A/HA1) were pooled as a master mix. Finally, viral RNA 5.0 µl was added and real time RT-PCR was carried out in a LightCycler® 480 II (Roche Diagnostic GmbH, Mannheim, Germany). The temperature profile was reverse transcription at 58ºC for 8 min, initial denaturation at 95ºC for 30 s and followed by 45 cycles of amplification (denaturation at 95ºC for 1 s, annealing at 60ºC for 20 s and extension at 72ºC for 1 s) and finally cooling at 40ºC for 30 s. During amplification, PCR amplification by quantitative analysis of the fluorescence emissions was monitored by the LightCycler® 480 II real time PCR system. The cycle threshold (C_T) represented the refraction cycle number at which a positive amplification was measured and was set at 10 time the standard deviation of the mean baseline emission calculated for PCR cycle 3 to 15. The complete run takes approximately 1h.

Statistical Analysis

EPI INFO 7 software program was used to test the correlations of the age, sex and each presenting symptom of the patients with their viral loads. A P-value of <0.05 between two variables was considered as statistically significant.

Results and Discussions

During this study period, a total of 116 respiratory samples were received in the Virology Laboratory, Department of Microbiology, Gauhati Medical College & Hospital, which included 71 (61.21%) male and 45 (38.79%) female, ranging in the age from 1 to 79 years [mean ± standard deviation (SD): 35.2 ± 6.8 years]. Based on the clinical examination and collected data from the questionnaires, initial presentations included 85 (73.27%), 74 (63.79%), 58 (52.25%), 52 (46.84%) and 49 (42.24%) were with cough, fever, sore throat, nasal catarrh and shortness of breath, respectively of the study population. However, 31.53% of the cases had simultaneous fever, cough, sore throat and nasal catarrh. Not a single patient had evidence of severe lower respiratory tract illness or unusual symptoms of influenza such as diarrhea. On the other hand, 27 (23.27%) cases resides in infected community, 21 (18.10%) cases travel to infected community, 19 (16.37%) cases and 18 (15.51%) cases had the history of contact with infected person and visited the other country respectively and 81.86% had no history of obvious contact with infected people.

In the real-time RT-PCR, fourteen patients (12.07%) were positive for A (H1N1) pdm 09 virus genome. Out of 14 positive patients, male and female potive patient were 9 (64.28%) and 5 (35.71%) respectively. The A (H1N1) pdm 09 virus infection affected all age groups (11 to 79 years), with the mean age being 40.07 ± 4.43 (SD) years. Out of 14 positive cases, highest number of positive cases was detected in the 25-40 years age group (50.0%) followed by 50-55 years (21.42%), 14.28% in both 10-15 years and more than 60 years age groups. The youngest positive patient was 11 years old and the oldest one 79 years old. All the patients positive for A (H1N1) pdm 09 virus were sensitive to

oseltamivir. However, 92.86% positive patients had fever and cough at the time of sample collection. Other respiratory symptoms like shortness of breath (57.14%), nasal catarrh (50.0%) and sore throat (35.71%) were seen in the patients. On the other hand, no fever and other respiratory symptoms were observed in one A (H1N1) pdm 09 positive patient. On radiography, one patient had bilateral pneumonia and the other patients displayed a normal chest X ray. No correlation was observed between presence of the virus and age or sex. However, a significant correlation was found between the presence of virus and fever (P = 0.001). None of our cases had history of H1N1 vaccination.

The set of primers and probes used in real-time RT-PCR detected all the positive samples in below 35 cycles and no positives were detected after 35th cycle. Further there was no nonspecific amplification of negative samples and no template control (NTC) even when the real time PCR assay was extended up to 45th cycle, hence the cut off cycle for interpretation of results of positive or negative samples was kept at a cycle threshold (Ct) of 35 (Fig. 1). Analysis of the Ct values of the H1N1 real-time PCR positive samples indicated that out of 14 positive samples, 8 samples detected with Ct values between 25 to 30 for both HA1 and M2 gene. On the other hand 6 samples were detected with Ct values between 31 to 35 for both HA1 and M2 gene in real-time PCR. However, M2 gene was absent in all the A (H1N1) pdm 09 negative samples.

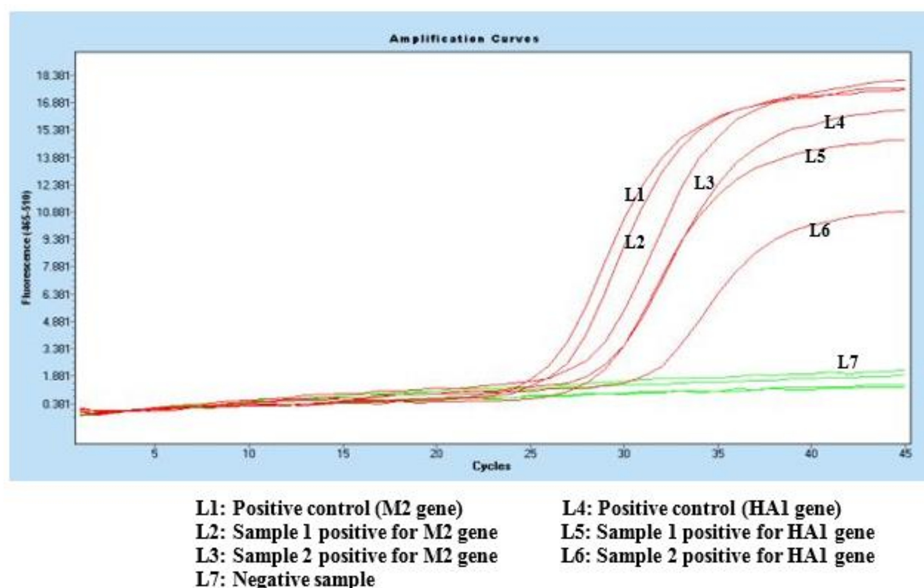

Fig. 1: The amplification plot showing detection of pandemic H1N1 influenza A virus from suspected clinical samples by Real-Time RT-PCR

The influenza A virus is highly prone to mutations and re- assortment. It is responsible for recurrent seasonal epidemics and pandemics. During the 20th century, four major pandemics, Spanish flu in 1918, Asian flu in 1957, Hong Kong flu in 1968-69 (Schafer et al., 1993) and the recent pandemic 2009 H1N1 influenza A virus has occurred (Panning et al., 2009).

After the global spread of the new swine-origin influenza virus A (H1N1), many countries, including India, organized a network for the report, diagnosis and treatment of influenza A (H1N1) infection. The province's health care system provides primary medical care in small rural medical centers where general practitioners or physicians without a specialty offer medical services, while secondary care services are provided by hospitals located in Guwahati, the capital of Assam.

Though several authors have reported the presence of the virus in India during 2009-2010 (Gupta et al., 2011), but till 2014 no report was found from the northeastern region of India for the presence of A (H1N1) pdm 09 influenza virus. It is important to study the present scenario to determine the current trend of the circulating virus and to take appropriate action in order to prevent morbidity and mortality. The incidence of A (H1N1) pdm 09 influenza virus among patients presenting with ILI, diagnosed during the study period was 12.07%.

According to sex distribution, males (64.28%) were affected more by A (H1N1) pdm 09 virus infection. However, other studies have reported different or equal infection rate in both males and females. Several authors have noted that the mean age of people affected by A (H1N1) pdm 09 virus to be 39.7 years (Mukherjee et al., 2010) and of those affected which is approximately similar to our reports. In our study, the percentage of people affected in the age group of 25-40 years was 50.00%. Fever was the commonest feature (92.86%) in A (H1N1) pdm 09 cases similar to other reports (Siddharth et al., 2012). Cough was a common complaint in 92.86%, with accompanying sore throat in 35.71% of cases.

Influenza is a complex virus and the basic epidemiology is quite intricate. With testing services

becoming available, it has provided an opportunity to study the epidemiological pattern of the virus in detail especially community transmission, secondary attack rate and vaccine effectiveness.

In summary, fever, cough and sore throat are early clinical clues to suspect influenza infection. For confirmation, the one-step real-time RT-PCR assay provides a simple, fast, highly sensitive and specific method for detection of pandemic H1N1 influenza A virus infection in clinical samples. The protocol described above for this assay is easy to perform and also this diagnostic tool can be used for rapid identification thus saving lot of time when control measures have to be applied to prevent virus transmission, after the first detection of the virus. This tool should prove useful during outbreaks when diagnosis needs to be made rapidly to curtain disease transmission.

Acknowledgements

The authors gratefully acknowledge the Department of Microbiology, Gauhati Medical College, for providing all necessary help to carry out the research. The authors also thank Department of Health Research, Govt. of India for all the help and support rendered for carrying out the research.

Conflict of Interest: None.

References

1. Centers for Disease Control and Prevention (CDC). Update: swine influenza A (H1N1) infections--California and Texas, April 2009. *Morb Mortal Wkly Rep 1* 2009;58(16):435-437.
2. Gupta, S. D., Lal, V., Jain, R. and Gupta, O. P. Modeling of H1N1 outbreak in Rajasthan: Methods and approaches. *Ind J Community Med* 2011;36:36-38.
3. Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. Real time quantitative PCR. *Genome Res* 1996;6:986–994.
4. Mukherjee, A., Roy, T., Agrawal, A. S., Sarkar, M., Lal, R. and Chakrabarti, S. Prevalence and epidemiology of pandemic H1N1 strains in hospitals of Eastern India. *J Public Health Epidemiol* 2010;2:171-174.
5. Panning M., Eickmann M., Landt O., Monazahian M., Olschlager S., Baumgarte S., et al. Detection of influenza A (H1N1) virus by real-time RT-PCR. *Eur Surveill* 2009;14: pii_19329. http://www.eurosurveillance.org.
6. Schafer J.R., Kawaoka Y., Bean W.J., Suss J., Senne D, Webester R.G. Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. *Virology*, 1993;94:781-788.
7. Scott C. B., Ratcliffe D. R. and Cramer, E. B. Human monocytes are unable to bind to or phagocytize IgA and IgG immune complexes formed with Influenza Virus in vitro. *J Immunol* 1996;157:351-359.
8. Siddharth, V., Goyal, V. and Koushal, V. K. Clinical Epidemiological Profile of Influenza A H1N1 Cases at a Tertiary Care Institute of India. *Ind J Com Med* 2012;37:232-235.
9. Smith G. J, Vijaykrishna D., Bahl J S., Lycett S. J., Worobey W., Pybus O., et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nat* 2009;459:1122–1125.
10. World Health Organization (WHO). 2008. A practical guide to harmonizing virological and epidemiological influenza surveillance 2008. Available from: http://www.wpro.who.int/emerging_diseases/documents/Guide to Harmonizing Influenza Surveillance revised2302/en/index.html.
11. World Health Organization (WHO). 2009. Pandemic (H1N1) 2009 - update 64. 04 September, 2009. Available from: http://www.who.int/csr/don/2009_09_04.

How to cite this article: Alam ST, Rajbongshi G, Rabha D, Sharma A. Recent trends of pandemic 2009 H1N1 virus infection in Guwahati, Assam: A prospective study. *Indian J Microbiol Res* 2019;6(1):18-21.