Characterization of Seasonal Influenza Virus Type and Subtypes Isolated from Influenza Like Illness Cases of 2012

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ABSTRACT

Background

Seasonal influenza is one of the increasing public health burdens in Nepal.

Objective

The objective of this study was to isolate and characterize the influenza virus type and subtypes of Nepal.

Method

A total of 1536 throat swab specimens were collected from January to December 2012. Total ribonucleic acid was extracted using Qiagen viral nucleic acid extraction kit and polymerase chain reaction assay was performed following the US; CDC Real-time PCR protocol. Ten percent of positive specimens were inoculated onto Madin-Darby Canine Kidney cells. Isolates were characterized by using reference ferret antisera.

Result

Of the total specimens (n=1536), influenza virus type A was detected in 196 (22%) cases; of which 194 (99%) were influenza A (H1N1) pdm09 and 2 (1 %) were influenza A/H3 subtype. Influenza B was detected in 684 (76.9%) cases. Influenza A (H1N1) pdm09, A/H3 and influenza B virus were antigenically similar to the recommended influenza virus vaccine candidate of the year 2012. Although sporadic cases of influenza were observed throughout the year, peak was observed during July to November.

Conclusion

Similar to other tropical countries, A (H1N1) pdm09, A/H3 and influenza B viruses were co-circulated in Nepal.

KEY WORDS

A (H1N1) Pdm09, influenza, real time PCR

INTRODUCTION

Respiratory tract infections, including influenza, are a leading cause of death worldwide. Influenza, commonly referred to as the flu, is are spiratory illness caused by influenza viruses.[1] The rapid, global spread of pandemic influenza may be a relatively modern development related to increases in population and the growth of transportation systems necessary for the global transmission of the novel virus.[2] Influenza epidemics also occur annually in many parts of the world and cause high mortality and morbidity.[3] Each year, seasonal epidemics of influenza cause serious illness and deaths throughout the world. It is estimated that about 10-20% of the world's population is affected by seasonal influenza each season, with an average of 2,50,000-5,00,000 deaths annually.[4] The influenza A virus undergoes a minor antigenic change, namely antigenic drift from year to year and may also undergo a major changes, termed an antigenic shift, which can cause a pandemic.[5]

In temperate regions, influenza activity peaks during the winter months. In Northern Hemisphere, influenza outbreaks and epidemics typically occur between November and March, whereas in the Southern Hemisphere and in tropical regions; influenza activity occurs between April and September and throughout the year respectively.[6] Influenza virus types A and B are two of the most important causes of human respiratory infection.[7] Although the epidemiology of influenza has been studied for many years, certain features such as its seasonality, the precise mechanism for the emergence of new variants and the factors that influence the spread of disease are not well understood.[6] Rapid diagnosis of influenza virus infection is important for prevention and control of influenza epidemics and timely initiation of antiviral treatment.[8] In this study we had attempt to identify and characterize the influenza virus types and subtypes circulating during the year 2012 in Nepal.

METHODS

This is a laboratory based descriptive study conducted from January to December 2012. This study was approved by Nepal Health Research Council. The study population consisted of all suspected cases of influenza like illness (ILI) visiting at health care center, hospitals and medical institutes. Specimens were collected from patient meeting ILI case definition. The data included patient's demographic characteristics (age and sex); geographic location, type and subtypes of influenza virus confirmed by Real Time polymerase chain reaction (rRT-PCR) assay. Statistical analysis was performed using SPSS version 11.5. Descriptive statistics were calculated for categorical variables.

Throat swab specimens were collected from patient meeting ILI case definition. Specimens were transported in triple package cold box to National Influenza Center (NIC) and aliquoted in micro-centrifuge tubes (1.8 ml) following standard biosafety protocol. One aliquot was processed for RNA extraction and remaining specimen were kept in -80^0 C freezers. Ten percent of PCR positive specimens were randomly selected on the basis of different geographical area and inoculated onto Madin-Darby Canine Kidney (MDCK) cell line for isolation. Identification and antigenic characterization of influenza virus was performed along with reference ferret antisera.

RNA extraction and PCR amplification

Total RNA was extracted using QIAamp Viral RNA extraction Kit (QIAGEN, Germany), according to the manufacturer's instructions. Influenza A, B and their sub-types were confirmed by using US, CDC real time PCR assay protocol.[9] The final concentration of master mix components includes forward and reverse primer (0.8 µM), probe ((0.2 µM), reverse transcriptase (RT) enzyme (AgPath-ID™ one step RT-PCR, US), 2x RT-PCR buffer (Ambion, Applied Biosystem, USA) and nuclease free water. Finally, 5 µl of viral RNA template of different samples was added onto master mix preparation for real time RT-PCR assay. Briefly, reverse transcription at 50°C for 30 minutes, Taq inhibitor inactivation 95°C for 10 minutes followed by 45 cycles at 95°C for 15 seconds, and 55°C for 30 seconds. Furthermore, positive and negative controls were included along with mock RNA extraction control in PCR assays. Real Time PCR amplification, detection and analysis were performed on Rotor-Gene 6000, Corbett Life Science, Australia.

Virus Isolation

Monolayer of Madin-Darby Canine Kidney (MDCK) cell-line was grown (80-100%) in T25 flask. Approximately 10% of specimens tested positive for influenza A (H1, H3, or H1N1pdm09) and or B were inoculated into MDCK cell-line, incubated at 37^{o}C in presence of 5% CO_2 for 3-7 days. The cell-line showing four plus cytopathic effect (CPE) was harvested and Hemagglutination (HA) test was performed using human 'O' group RBC following WHO standard protocol.[10] Briefly, 50 µl of culture supernatant was added to U shaped micro-well plate containing 50 µl of phosphate buffer solution (pH 7.2) and serial two fold dilution was made. Equal volume (50 µl) of RBC suspension (0.75%) was added to all micro-wells and incubated for one hour at room temperature. A positive reaction was observed by mat formation in U-bottom plate (Greiner, Germany) and settled RBCs in the form of circular button shape were considered as negative reaction. The specimen showing HA titer ≥ 1:32 were processed for antigenic characterization by Hemagglutination inhibition (HI) assay.

Antigenic Characterization

The cell line showing four plus CPE were harvested and screened for influenza virus using respiratory virus screening Immunofluorescence Assay (IFA) and confirmed by HI assay with reference ferret antiseraas per test protocol.[10] Briefly, 25 µl of phosphate buffer solution (PBS) was added in a U-shape 96 well plate followed by

addition of 25 µl RDE-treated reference ferret antisera in the first column of the plate respectively. Two-fold serial dilution was made by transferring 25 µl from well 1 to 10, the last two wells 11 and 12 were considered as control well. Equal volume (25 µl) of standardized test antigen was added in all corresponding wells from 1 to 10. Similarly, as an alternative of test antigen, same volume of PBS was added in control wells and incubated at room temperature for 30 minutes. Standardized RBCs suspension (0.75%) was added to all micro-wells, incubated for one hour at room temperature, result of HI titer was recorded in a standard test format.

RESULTS

Of the total throat swab (n=1536) specimens, 901 (58.7%) were from males and 635 (41.3%) from females. Influenza virus was detected positive in 890 (58%) specimens. Influenza A virus was detected in 196 (22%) specimen; of which 194 (99%) were influenza A/H1N1 pdm09 and 2 (1 %) were influenza A/H3 subtype. Influenza B was identified in 684 (76.9%) specimens. Co-infection of influenza A (H1N1) pdm09 and influenza B was observed in 10 (1.1%) cases.

A total of 82 PCR positive specimens were inoculated onto MDCK cell-line for virus isolation, of them influenza virus were isolated in 40 (48.8%) specimens comprising 12 (30%) influenza A (H1N1) pdm09, 2 (5%) influenza A/H3, 13 (32.5%) influenza B/F/4/2006-Y like and 13 (32.5%) were influenza B/B/60/2008-V like. All isolates of influenza A (H1N1) pdm09, influenza A/H3 and influenza B virus were antigenically similar to the influenza A/California/07/2009 (H1N1); A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010 like viruses respectively. Phylogenetic tree of HA1 gene of influenza A (H1N1) pdm09 viruses could be divided into eight genetic clades. The phylogenic analysis of HA1 gene of influenza virus circulated in Nepal were clustered with clades 6 and 7 as reported globally and did not showed divergence further from neighboring viruses circulating in India, China and Pakistan (fig. 1). Similarly, phylogenetic trees of HA1 gene of influenza B Yamagata lineage were constructed. Phylogenetic analysis revealed that the influenza B (Yamagata lineage) viruses circulated in Nepal belonged to clade-2 which was transmitted globally during the influenza season (fig. 2). Nucleotide sequences of HA1 genes were used to construct the phylogenetic tree for better understanding on genetic diversity of influenza A (H1N1) pdm09 and influenza B (Yamagata lineage) isolates of Nepal (fig. 1 and 2). Results were generated based on pair-wise analysis using the Maximum Composite Likelihood method in Molecular Evolutionary Genetic Analysis (MEGA) version 6 as described previously.[11] The sequencing of HA-1 gene of influenza A (H1N1) pdm09, influenza B (Yamagata lineage) and susceptibility of viruses to four nuraminidase (NA) inhibitors (oseltamivir, peramivir, zanamivir and laninamivir) were performed at Influenza Virus Research Center, National Institute of Infectious

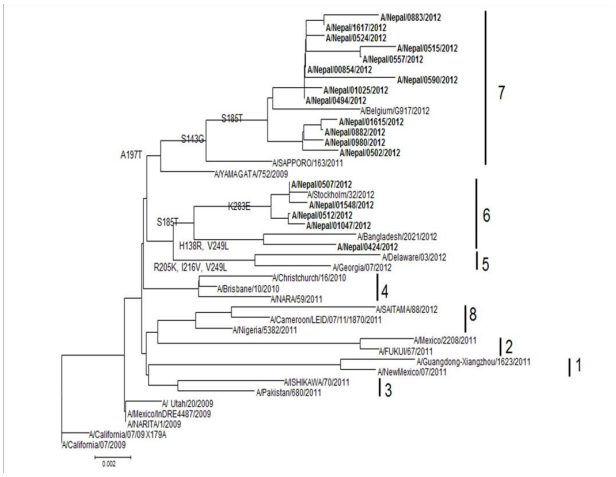

Figure 1. Phylogenetic tree of A (H1N1) pdm09 HA1 gene. Haemagglutinin (HA) genes of influenza A (H1N1) pdm09 viruses with eight clades indicated by the bars on the right. The Nepalese viruses used in this study are shown in bold font. The tree was constructed using the Neighbor-Joining method using mega software version 6.

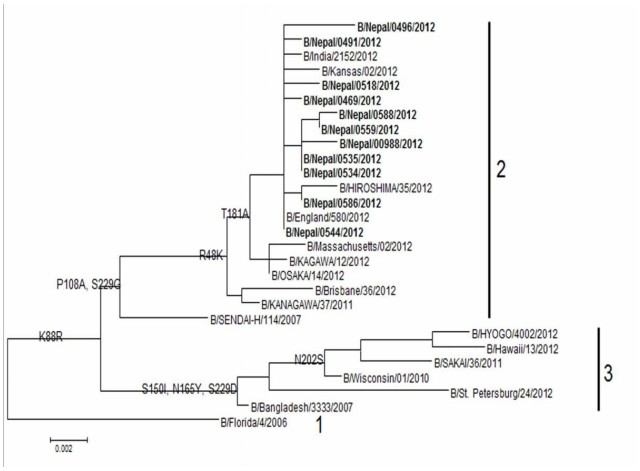

Figure 2. Phylogenetic tree of B (Yamagata lineage) HA1 gene. Haemagglutinin (HA) genes of influenza B (Yamagata-lineage) of Nepal used in this study shown in bold font. The tree was constructed using the Neighbor-Joining method using mega software version 6.

Diseases (NIID), WHO-CC, Japan. Each of sequences was registered in GISAID, a public database (http://www.gisaid.org).

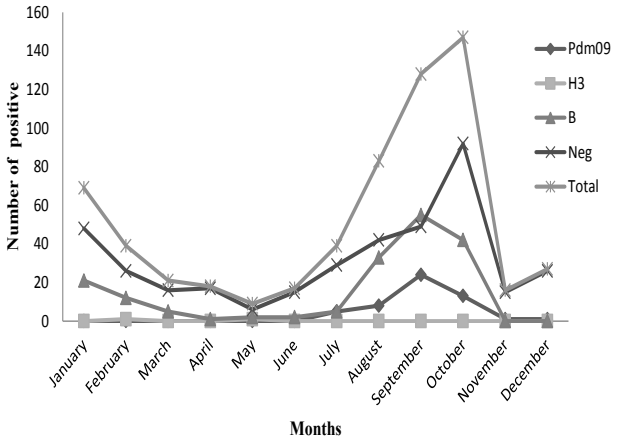

Figure 3. Month-wise distribution of influenza type & subtyphes (2012)

Although sporadic influenza was observed throughout the year, peak was observed during July to November 2012 (fig. 3). Of the 75 districts, infection of influenza virus was reported in 29 districts of Nepal (fig. 4). Outbreak of influenza was reported at Bharatpur, Pokhara and Kathmandu cities of Nepal. The highest number of influenza A (H1N1) pdm09 and influenza B positive were found in August and September 2012. Infection of influenza virus was found highest in children (5-14 years) followed by 15-45, 0-4 and more than 45years old age (fig. 5).

The map of Nepal was retrived from https://www.google.com.np and modified for symbolic repesentation of influenza virus type and subtypes.

Figure 4. District-wise distribution of influenza virus (2012)

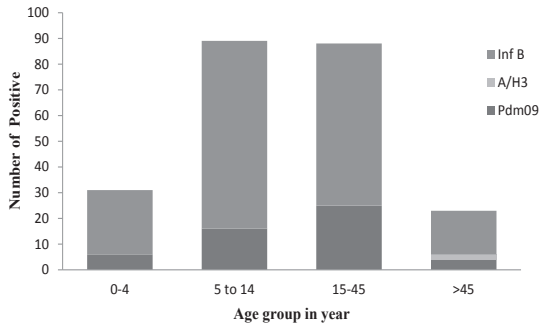

Figure 5. Age group-wise distribution of influenza type and subtypes (2012)

DISCUSSION

Nucleic acid amplification tests are the preferred methods for identification of respiratory viral infections, including influenza and PCR-based methods provide rapid, sensitive detection and most importantly, help with identifying different subtypes of influenza viruses.[7]

The highest number of influenza A (H1N1) pdm09 and Influenza B positive cases were found during August and September followed by January, February and May of 2012. However; transmission of influenza virus was recorded throughout the year. Similarly, influenza A (H1N1) pdm09 strain was predominantly circulated during the year 2009 to 2010 which we had previously published.[18] Countries belonging to tropical zones like Brazil, Cuba, Ecuador, El Salvador and Panama in the Americas (influenza A/H1N1 pdm09 and influenza B); Ghana and Madagascar in sub-Saharan Africa (influenza A/H3N2); southern China, Singapore and Viet Nam in Asia (A/H3N2, A/H1N1) had reported notable influenza activities.[12] In Nepal, the number of influenza cases reached at peak (22%) in 2012 which is similar to other countries belonging to South-East Asia. Our findings revealed that influenza A (H1N1) pdm09 and Influenza B virus were predominantly circulated in Nepal during the year 2012. Widespread and or regional outbreaks of influenza A/H3 were reported in Europe, part of Asia, North Africa, Canada and USA in February to April, however it was found very low during 2012 in Nepal.[8] At a global scale, viral migration from regions characterized by more persistent influenza transmission, notably East and South-East Asia, appears to be important in determining large-scale epidemiological pattern.[13-15]

To the best of our knowledge, the circulation of influenza virus was reported in 29 districts of Nepal. Seasonal influenza outbreak was reported in Bharatpur, Pokhara and Kathmandu which are densely populated cities of Nepal. Similar findings were reported from Srinagar (January-March), Delhi (July-September), Lucknow (June-July), Kolkata (June-July) and Pune (July-September) of India where subtype A/H3 was predominant in 2011, 2012 and 2013.[19] Annual seasonal influenza epidemics alone causes significant morbidity and mortality, affecting 5-15% of the global population, hence are of major public health concern.[20] Growth of human population, increasing density at urban areas and other ecological factors such as changing land use, agriculture and livestock intensification are important independent predictors of emerging infectious disease.[16] Even though there is inadequate infrastructure, health care and diagnostic facilities; it is important to understand the epidemiology and seasonal variation of influenza viruses within the country in comparison with viruses circulating globally.

Co-infection with A (H1N1) pdm09 and influenza B was found highest in children less than 15 years of age group. Similar findings were reported in patients with influenza like illness during the Winter/Spring season in Shanghai, China.[16] Simply few publications have reported simultaneous infection by two different types of influenza viruses in humans. Thus, the factors that may be responsible for such events are not clear yet, even though the host immune system and the virus properties have been suggested.[17]

Regular influenza surveillance is necessary to understand the epidemiology and seasonality of influenza and optimizing influenza control strategies.[19] Based on history, influenza is and will continue to be a serious threat to the health of many species including humans. Therefore, interdisciplinary research and communication between veterinary and public health professionals is essential in order to know the precise mechanism that could lead the next influenza pandemic.[21] However; there were several constrains such as short period of study time, limited number of sample, budget and diagnostic reagents and kits were main limitation of our study. Hence, this study does not reflect the entire population of the affected districts.

CONCLUSION

Similar to other tropical countries of South-East Asia; circulation of A (H1N1) pdm09, A/H3 and influenza B were found throughout year with the peak during July-November in Nepal. Antigenic characteristicsof A (H1N1) pdm09, A/H3 and influenza B virus were similar to the vaccine strain A/California/07/2009 (H1N1); A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010 like viruses respectively. Comparison of influenza types and subtypes in consecutive years is necessary to link the seasonality and viral genetic changes. The findings of our study could be useful for influenza preparedness and vaccination strategies.

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