Virological perspectives and characterization of seasonal influenza B viruses in Mumbai

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Acute respiratory infections caused by influenza viruses are major life-threatening infections affecting the global population. Information on influenza viruses in Mumbai region in India is limited. With an estimated population above one crore, it becomes imperative to identify the predominant circulating strains, seasonal evolution, epidemiology and impact of influenza virus infection on the public health of Mumbai. The present study is aimed to isolate and identify the prevalent influenza B virus strains circulating in the Mumbai region. Virological analysis of 482 throat and nasal samples from patients presenting with suspected influenza-like illness was conducted from January to December 2012 at a research institute in Mumbai. Isolation of positive influenza B samples was performed on Madin Darby Canine Kidney (MDCK) cell line. The isolates were further characterized by hemagglutination inhibition assay and one-step reverse transcriptase polymerase chain reaction. Among all the specimens tested, 71% were positive for influenza virus. The positivity for influenza B virus, influenza A (H1N1) pdm 09 and seasonal influenza A virus was 38%, 22% and 11% respectively. Of the 80 influenza B specimens cultured on MDCK, virus was isolated from 38 (48%) samples. Influenza B/Wisconsin/1/2010-like serotype belonging to the B/Yamagata/18/88 lineage was the predominant serotype identified. Influenza was a significant public health concern in the Mumbai region during the study period. Young children were the most affected age group. The study data generated will help understand the epidemiology of the disease in the Mumbai region as well as generate information for global influenza surveillance.

Keywords: Acute respiratory infections, influenza virus, serotype, virological perspectives.

INFLUENZA virus belongs to the family Orthomyxoviridae with a genome make-up of seven or eight single-stranded, negative-sense RNA segments[1]. Influenza viruses are a major cause of acute respiratory infections in humans, causing significant morbidity and mortality worldwide[2,3]. Influenza A viruses (IAVs) and influenza B viruses (IBVs) are responsible for annual epidemics across several regions of the world[4]. The incidences of epidemics of influenza can be attributed to the high frequency of antigenic changes occurring in the major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA)[5]. Both IAVs and IBVs undergo antigenic drift. In addition, IAVs undergo antigenic shift, which causes genetic reassortment between different subtypes. IAVs are divided into several antigenic subtypes and they infect a variety of host species[6,7]. Unlike IAVs, IBVs infect mainly humans and do not have distinct subtypes, except for a strain isolated from a seal in the Netherlands[6,8]. However, multiple lineages of IBVs are known to circulate globally[5].

The evolutionary analysis of the hemagglutinin gene since 1983 represents two major phylogenetic lineages of IBVs – Victoria and Yamagata – represented by the reference strains B/Victoria/2/1987 (Vic87) and B/Yamagata/16/1988 (Yam88) respectively. Each of these two viral lineages achieved pre-dominance at different time periods and in different geographic regions. IBVs of the Victoria lineage were primarily found in the 1980s, while the viral strains of Yamagata lineage have been circulating globally since 1990s[6,9].

The evolution and antigenic drift of IBVs occur due to various mechanisms of insertion, deletion and re-assortment within diverse lineages. This phenomenon leads to increase in the genetic diversity of IBVs. The IBVs undergo frequent segment re-assortment between different lineages, resulting in high genome diversity, which in turn leads to co-circulation of multiple genotypes at a particular time period in a particular area[4]. Furthermore, the IBVs isolated between 1979 and 2003 belong to at least 14 genotypes resulting from genomic re-assortments between the descendants of Vic87 and Yam88 viruses[10]. Thus, monitoring antigenic and genetic variations of circulating influenza viruses is essential for the selection of annual vaccine strains[7].

Since 1991 until 2007, sporadic, seasonal as well as epidemic occurrence of IBVs have been detected around the world; these strains belonged to different lineages and had diverse genotypes[4,5,8,9]. It is therefore imperative to comprehend the seasonal patterns of influenza infections for the development of effective prevention and control strategies[11].

Limited surveillance on IBV has been conducted in western India. In the Pune region, B/Victoria/2/87 lineage

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was found circulating during 1987–89, while B/Yamagata/16/88 lineage was found circulating during 1990 and 2001. During 2002, reappearance of B/Victoria/2/87 lineage in the community has highlighted the need for regular surveillance of the influenza strain[5]. This study characterizes the prevalent strain of IBVs circulating in Mumbai in 2012.

Methodology

Ethics committee approval

This project was approved by the Institutional Ethics Committee of the Research Institute (vide letter No. HITRT/IEC/012/2012; dated 12 March 2012).

Sample collection

During January–December 2012, a total of 438 nasal and/or throat swab samples were collected in viral transport medium (VTM) by trained clinicians of King Edward Memorial (KEM) Hospital and L. H. Hiranandani Hospital, Mumbai, as a part of Laboratory-based Influenza (SARI/ILI) Surveillance Plan India under Integrated Disease Surveillance Project. The specimens were transported in a triple-layer packing in a styrofoam box with ice pack and the clinical history of the patient with respect to the demographic characteristics and clinical symptoms were obtained.

Real-time RT-PCR (qRT-PCR)

Viral RNA was extracted from throat and nasal secretions using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One-step real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using AgPath^{TM} One-Step RT-PCR Kit (Applied Biosystems, Life technologies) on ABI Step One platform, as previously described in WHO-CDC protocol, Geneva[12].

Virus isolation

A total of 80 clinical samples positive for influenza B by qRT-PCR were inoculated onto confluent Madin Darby Canine Kidney (MDCK) cells with the serum-free minimal essential medium containing 2 µg/ml of trypsin. The samples were selected based on the cycle threshold value (Ct < 35), different age groups and geographical settings, maximum volume of the samples available and complete clinical history of the patient[13]. Tissue culture fluids were harvested after observing the MDCK cell line for cytopathic effect, usually on the seventh day of infection. Virus stocks were aliquoted and stored at –80°C until further use[14].

Hemagglutination assay

The presence of influenza virus in the cell culture supernatant was determined by hemagglutination assay (HA). A serial two-fold dilution of all specimens (50 µl) was prepared in *U*-bottom 96-microtitre plates using phosphate buffer saline (PBS). Guinea pig RBCs (0.75%) were added (50 µl) to each well and the plate was incubated at 22–24°C for 45–60 min. The HA titre of the samples was indicated as the reciprocal of the dilution of virus in the last well with complete hemagglutination[14–16].

Hemagglutination inhibition assay

The identification of IBV lineage was analysed by hemagglutination inhibition (HAI) assay. Each isolate was confirmed for its subtype using specific antiserum panel according to the WHO kit protocol for typing of human influenza isolates for 2012. All the isolates were standardized to 4 HA units/25 µl. The back titration was also performed and was only accepted when both replicates yielded matching results. A serial twofold dilution of specific sera (25 µl) was prepared in *U*-bottom 96-microtitre plates using PBS, followed by the addition of 25 µl/well of each isolate of the standardized antigen. This mixture was incubated for 45 min at the room temperature. Guinea pig RBCs (0.75%) were added (25 µl) to each well and the plate was incubated at room temperature for 45–60 min. The HAI titre results were indicated as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination.

One-step RT-PCR

Viral RNA was extracted from 140 µl of cell culture supernatant using the QIAamp viral RNA Mini Kit, according to the manufacturer's instructions. RT-PCR assay was performed using the AgPath^{TM} One-Step RT-PCR Kit in a final volume of 25 µl using the Takara Gradient Thermal Cycler. Primers used in the study were targeted to influenza B subtype specific to HA gene segment[17]. Five microlitres of the extracted RNA sample was added to 20 µl of the reaction mixture. The amplification conditions were divided into holding stage and cycling stage. In the holding stage, reverse transcription was carried out at 48°C for 45 min, followed by *Taq* inhibitor activation at 94°C for 2 min. In the cycling stage, PCR amplification (29 cycles) was at 94°C for 20 sec, 56°C for 30 sec and 72°C for 1 min. The final extension was carried out at 72°C for 7 min.

Results

Viral surveillance

Of the total 482 throat and nasal swab samples received with acute respiratory infections during January 2012 to

December 2012, 71% were positive for influenza virus. Among these, 184 (38%) samples were positive for IBV, 106 (22%) samples were positive for influenza A (H1N1) pdm 09 and 51 (11%) samples were positive for seasonal influenza A.

Among the 184 samples positive for influenza B virus, 91 (49.45%) were from males and 93 (50.54%) were from females; however, no gender specificity to influenza infectivity was observed. Age-wise analysis of positive cases revealed that maximum positivity belonged to the age group of 1–10 years (Figure 1).

The clinical presentation in influenza-positive population included fever 145 (79%), cough 145 (79%), sore throat 131 (71%), nasal catarrh 107 (58%), headache 88 (48%), shortness of breath 62 (34%) and vomiting 30 (16%).

Antigenic characterization

Out of 80 clinical samples, 38 showed cytopathic effect in first viral passage on MDCK cell line (Figure 2). Of the 38 isolates obtained, 33 (86.84%) belonged to the B/Yamagata lineage and were characterized as B/Wisconsin/1/2010-like, while five isolates (33.7%) were characterized as influenza B/Brisbane/60/2008-like belonging to the B/Victoria lineage.

Conventional RT-PCR confirmed that five isolates belonged to the B/Victoria/2/87 lineage, while the remaining belonged to the B/Yamagata/18/88 lineage (Figure 3).

Discussion

In India, respiratory infections caused by influenza virus have been generally ignored in the healthcare facilities. Information about influenza strains circulating in the Indian subcontinent is majorly unknown due to lack of systemic studies. The available information on epidemiological and clinical features of influenza virus is entirely from research studies alone. Etiology-specific diagnosis requires laboratory tests which are not widely available in India, except for a few metro cities.

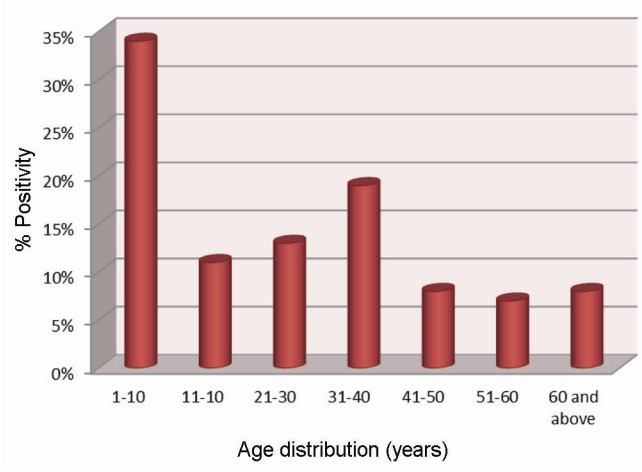

Figure 1. Age distribution in population diagnosed as influenza B (%).

The present study reports the activity of IBVs in Mumbai during January–December 2012 as a part of the National Influenza Surveillance Network, which was extended to India in 2005. During this surveillance period, a total of 482 samples were collected by the clinicians from in-patient department of KEM Hospital and L. H. Hiranandani Hospital in Mumbai, of which 184 (38%) were positive for influenza B. During the 2012 influenza season in India, the infection rate of IBVs remained more or less similar to those reported in other temperate countries. According to the Centres for Disease Control and Prevention report on influenza activity in the United States from September 2012 to May 2013, 29% of the specimens were positive for IBVs[18]. According to the National Influenza Centers and other national influenza laboratories from 82 countries, more than 19,000 specimens were tested by May 2012, of which 26% of the viruses were typed as influenza B[19]. The epidemiological data of seasonal influenza in Bangkok between 2009 and 2012 demonstrated that 30% of the specimens were positive for IBV[2]. Majority of the individuals were reported to be between 1 and 10 years of age, which is consistent with previous reports[20].

Of the 38 influenza virus isolates tested, 33 (86.84%) belonged to the B/Yamagata lineage and were characterized as B/Wisconsin/1/2010-like, and 5 (33.7%) were characterized as influenza B/Brisbane/60/2008-like, which belonged to the B/Victoria lineage. Majority of the IBVs detected worldwide have been of the Yamagata lineage; nearly all the strains were antigenically similar to B/Wisconsin/1/2010-like virus contained in the trivalent seasonal vaccine. However, a substantial number of the Victoria lineage viruses were also reported, accounting up to 30% of the B viruses in several countries[18,19,21]. These data are consistent with the findings in the present study. However, complete gene sequencing of the isolates would be ideal for an exact confirmation of the lineage.

Viral culture in combination with HAI is considered as the gold standard method for typing and subtyping of influenza virus. The long-time consumption of viral culture limits its effectiveness for public health responses to potential influenza epidemics and pandemics[22]. Molecular methods, including one-step PCR and RT-PCR have provided a convenient and sensitive approach for the identification and subtyping of influenza virus[1].

In the temperate zone, transmission of human influenza virus usually occurs in the winter months, but the exact timing and duration of the influenza season vary. The influenza season in the temperate zones usually begins early in October, and typically peaks around January[21].

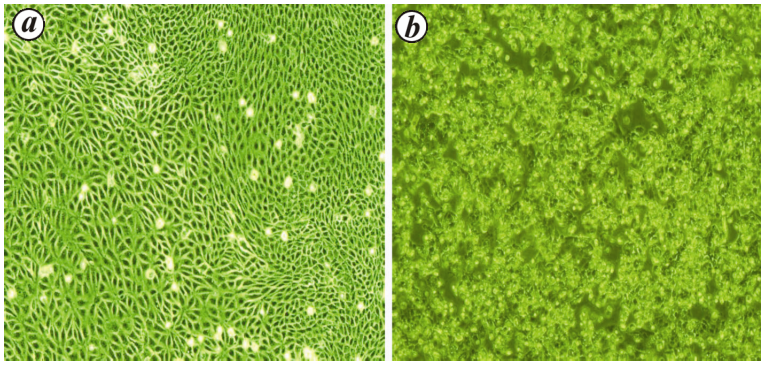

Figure 2. *In vitro* method: cytopathic effects on the MDCK cell lines. ***a***, Normal MDCK cell line. ***b***, Cytopathic effect on MDCK cell line.

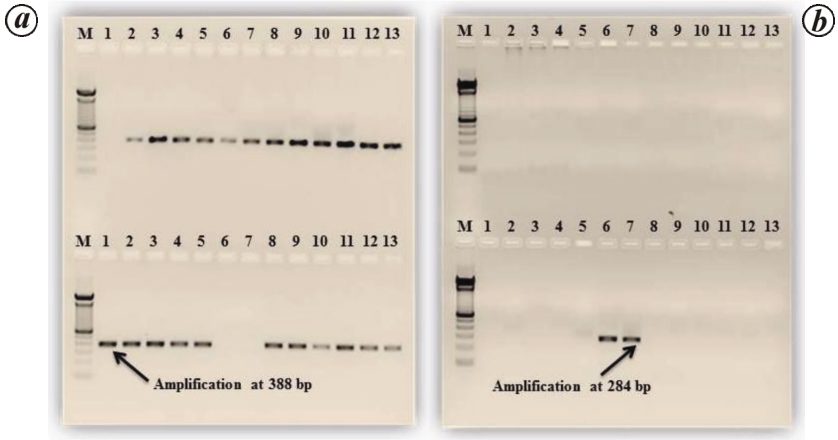

Figure 3. Serotyping of influenza B positive virus isolates by RT-PCR. ***a***, Influenza B Yamagata lineage (388 bp). ***b***, Influenza B Victoria lineage (284 bp). Amplification of influenza B virus isolates (representative gel pictures) using sequence-specific primers. Figure 3 *a* and *b* indicate that the amplification is specific for the lineage. While influenza B (Yamagata) gives a 388 bp product, the influenza B (Brisbane) yields a 284 bp product after amplification. Each isolate is respectively run using both primer sets and electrophoresed identically.

Unlike temperate climate countries, in tropical countries like India the incidence of influenza virus infection has been reported during the rainy season[23,24]. Mumbai, being located in the western region of India, has a significant tropical wet and dry climate, which experiences monsoon season from June to September and a post-monsoon season from October to December. Enhanced influenza activity during the monsoon season was observed during the study period. Pune also has tropical monsoon climate where similar outbreaks of influenza have been reported during the rainy season[5].

However, the northern part of the country is considerably cooler in the winter months. The peak influenza activity in winter with higher influenza activity during the rainy season has been noted in different cities like Delhi, Kolkata and Chennai with secondary peak of influenza infection in the winter months[25]. Thus, regional differences in the circulation of influenza virus in India may be due to the climatic diversity in the northern and southern parts of the country.

The exact mechanism of influenza activity is poorly understood. However, seasonal changes in the virus survival and its transmissibility play a key role. In addition, host susceptibility to infection, social behaviour and then interaction can also affect influenza activity[26–28]. It has also been suggested that metrological factors can directly affect influenza activity. In the temperate regions, a higher activity of influenza might be due to the cold temperatures, low indoor humidity and minimal solar radiation[11]. On the other hand, high humidity as well as rainy season in several tropical regions have revealed a link between increased influenza activities[29–31].

Conclusion

This study provides virological perspective of influenza B infection and strain diversity in Mumbai region. The study finds influenza/B/Yamagata lineage as the predominant circulating virus sub-type. The data relevant to Mumbai region may not be representative of the whole country considering the climatic diversity across India. However, considering the distinct presence of two strains of IBV, it is probable that such co-circulation may be

present in other parts of the country. This study shows that there is a scope of evaluating whether a quadrivalent vaccine should replace the existing trivalent vaccine. Since this was a fixed timeline study (January 2012–December 2012), it is limited in strength of forecasting whether such prevalence data would be obtained over the years. Therefore, continued surveillance of the circulating influenza viruses and determination of regional differences in influenza seasonality in India will help determine influenza control and possible vaccination programmes among priority populations.

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