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SHORT COMMUNICATION

Characterization of influenza virus among influenza like illness cases in Mumbai, India

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Abstract The present study was carried out to monitor influenza viruses by identifying the virus and studying the seasonal variation during 2007–2009 in Mumbai. A total of 193 clinical respiratory samples (nasal and throat swab) were collected from patients having influenza like illness in Mumbai region. One-step real-time reverse-transcriptase PCR (rRTPCR) was used to detect Influenza type A (H1 and H3) and Influenza type B virus. Isolation of the virus was carried out using in vitro system which was further confirmed and typed by hemagglutination assay and hemagglutination inhibition assay. Out of 193 samples 24 (12.4 3%) samples tested positive for influenza virus, of which 13 (6.73 %) were influenza type A virus and 10 (5.18 %) were influenza type B virus, while 1 sample (0.51 %) was positive for both. By culture methods, 3 (1.55 %) viral isolates were obtained. All the three isolates were found to be Influenza type B/Malaysia (Victoria lineage) by Hemagglutination Inhibition Assay. The data generated from the present study reveals that both Influenza type A and B are prevalent in Mumbai with considerable activity. The peak activity was observed during monsoon season.

Keywords Influenza virus · ILI cases · rRT PCR · Phylogeny

Introduction

Influenza virus belongs to *Orthomyxoviridae* family and has an enveloped, segmented single-stranded negative sense RNA genome consisting of three distinct types, influenza type A, B and C [1, 2]. Considering the public health importance of influenza, WHO in 1948 initiated a global network of surveillance centers. Currently, there are 112 National Influenza Laboratories in 83 countries that participate in the WHO influenza surveillance system Influenza surveillance is carried out throughout the year, to detect new antigenic variants emerging in different regions. Monitoring the antigenic drift of viruses in global populations is essential for optimal selection of component strains for the annually updated trivalent influenza vaccine [3, 6]. With the aim of keeping a track on the prevalence of influenza virus, the present study was carried out in Mumbai which will be helpful in identifying the predominant circulating types and strains of influenza.

Nasal swab/throat swab specimens from 193 individuals showing characteristic (ILI) determined by their clinical history were collected from B. J. Wadia Hospital, Nair Hospital and Western Railway Unit hospital, Mumbai, India during July 2007 to July 2009 according to the WHO guidelines [10]. The clinical samples were collected in the vial containing virus transport medium (VTM). Thorough cold chain was maintained while transporting the samples to the laboratory. The samples were processed in biosafety cabinet type 2/A2, and further stored as conveniently sized aliquots in a −80 °C deep freezer until further use. The study was approved by the Institutional Ethics Committee of Haffkine Institute, Mumbai after thoroughly reviewing the research plan. A written informed consent was obtained before collecting clinical samples from suspected patients. The Samples were processed in Madin Darby Canine Kidney (MDCK) cell lines. MDCK cell lines was maintained in Modified Eagles Medium, 100 IU/mL penicillin 100 mg/mL streptomycin and 2 mM L-glutamine and 1.5 g/L sodium bicarbonate, supplemented with 10 % fetal bovine serum

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(FBS). The clinical sample (0.5 mL) along with the uninfected cell control were then inoculated onto the cell line and allowed to adsorb for 45 min. After incubation virus growth medium (TPCK trypsin 2 μg/mL and Nystatin 50 units/mL in MEM) was added and flasks were kept in CO_2 incubator up to 3–4 days till cytopathic effect (CPE) is observed [5]. Hemagglutination assay (HA) and hemagglutination inhibition assay (HIA) was performed by using 0.75 % guinea pig and 0.5 % fowl RBCs using WHO reference HIA kits. This included ferret antisera influenza A (H3N2), influenza A (H1N1), influenza B/Florida/4/2006 like (Yamagata lineage), influenza B/Malaysia/2506/2004 like (B Victoria lineage). The viral RNA was extracted from clinical samples using the spin column based QAIamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) as per the manufacturer's instructions. Reverse transcriptase real time PCR (rRT-PCR) was carried out using a StepOne Real Time-PCR instrument (M/s ABI) using 2× PCR mix and SuperScript™IIIRT/Platinum Taq Enzyme mix (M/s Invitrogen) and influenza A (H1), (H3) and influenza B primers and probes as previously published [8] (Table 1).

The cycling condition for the r-RT-PCR were 50 °C for 30 min of reverse transcription followed by Taq inhibitor inactivation for 95 °C for 10 min and PCR amplification (45 cycles) of 95 °C for 15 s, 55 °C for 30 s. The amplified products were purified using Qiagen PCR purification kit. The cleaned amplicons were sequenced with a 3730×1 DNA analyser (M/s ABI). Phylogenetic analysis was done using the MEGA 5 software.

The present study was carried out to determine the prevalence of influenza virus in Mumbai. In this study, total 193 Throat and nasal swab samples were collected from patients attending the Out Patient's Departments of Nair Hospital, Western Railway Health Unit Hospital and B. J. Wadia Hospital with characteristic influenza like illness (ILI) symptoms. These were processed by rRT-PCR method, 7.25 % (14) were positive for influenza type A virus, of which 2.59 % (5) and 4.66 % (9) were positive for influenza type A (H1) and influenza type A (H3) virus respectively. Whereas 5.70 % (11) were positive for influenza type B virus, (0.52 %) one sample was positive for dual infection of influenza type A (H1) and influenza type B virus. Whereas 1.55 % (3) was found to be influenza virus type B/Malaysia/2506/04 (Victoria lineage) by culture methods. Statistically, positivity by rRT-PCR method is significantly higher than culture method, using two tailed test of proportion (Fig. 1a). Similar findings were obtained during the year 2007–2008 in a study was carried out on National hospital based surveillance in Bangladesh and National Institute of Cholera and Enteric Disease (NICED) in Kolkata [1, 12].

The most common clinical features observed in patients with ILI were fever (87.50 %), nasal discharge (83.33 %) and sore throat (50 %). We encountered 75.0 % (18/24) of influenza positive samples were male and 25.0 % (6/24) were female; however out of 193 clinical samples, statistically there was no significant difference observed with respect to influenza positivity in gender-wise distribution as seen previously [4, 11, 13].

Table 1 Influenza type A, (AH1) and (AH3), influenza type B and RNaseP primer and probe sequence

Influenza type A virus	Primers and probes	Sequence (5′ > 3′)
Influenza type A	Forward	GAC CRA TCC TGT CAC CTC TGA C
	Reverse	AGG GCA TTY TGG ACA AAK CGT CTA
	Probe	TGC AGT CCT CGC TCA CTG GGC ACG
Influenza type A (H1)	Forward	AAC TAC TAC TGG ACT CTR CTK GAA
	Reverse	CCA TTG GTG CAT TTG AGK TGA TG
	Probe	TGA YCC AAA GCC"T"CT ACT CAG TGC GAA AGC
Influenza type A (H3)	Forward	AAG CAT TCC YAA TGA CAA ACC
	Reverse	ATT GCR CCR AAT ATG CCT CTA GT
	Probe	CAG GAT CAC ATA TGG GSC CTG TCC CAG
Influenza type B	Forward	TCC TCA AYT CAC TCT TCG AGC G
	Reverse	CGG TGC TCT TGA CCA AAT TGG
	Probe	CCA ATT CGA GCA GCT GAA ACT GCG GTG
RNase P	Forward	AGA TTT GGA CCT GCG AGC G
	Reverse	GAG CGG CTG TCT CCA CAA GT
	Probe	TTC TG ACCT GAA GGC TCT GCG CG

Within the age group of 0–12 years, 6 % (6/100) were positive for influenza type A virus and 5 % (5/100) were positive for influenza type B virus by rRT-PCR method, out of which 3 % (3/100) were isolated by culture methods. From age group of 13–24 years, 16.12 % (5/31) were positive for influenza type A virus and 3.22 % (1/31) were positive for influenza type B virus. From age group of 25–36 years, 3.33 % (1/30) were positive for influenza type A virus and 10 % (3/30) were positive for influenza type B virus. From age group 37–48 years, 6.25 % (1/16) were positive for both influenza type A (H1) and influenza type B virus (dual infection). From age group of 49–60 years, none were found to be positive for influenza virus. From age group 61–72 years, 33.33 % (1/3) were positive for influenza type A and type B virus (Fig. 1b).

Positivity of influenza virus was observed from June to October, while highest positivity was observed in the month of August during 2007–2009. Influenza type B virus was dominant during 2007, later influenza type A and influenza type B was well distributed in the population. Highest

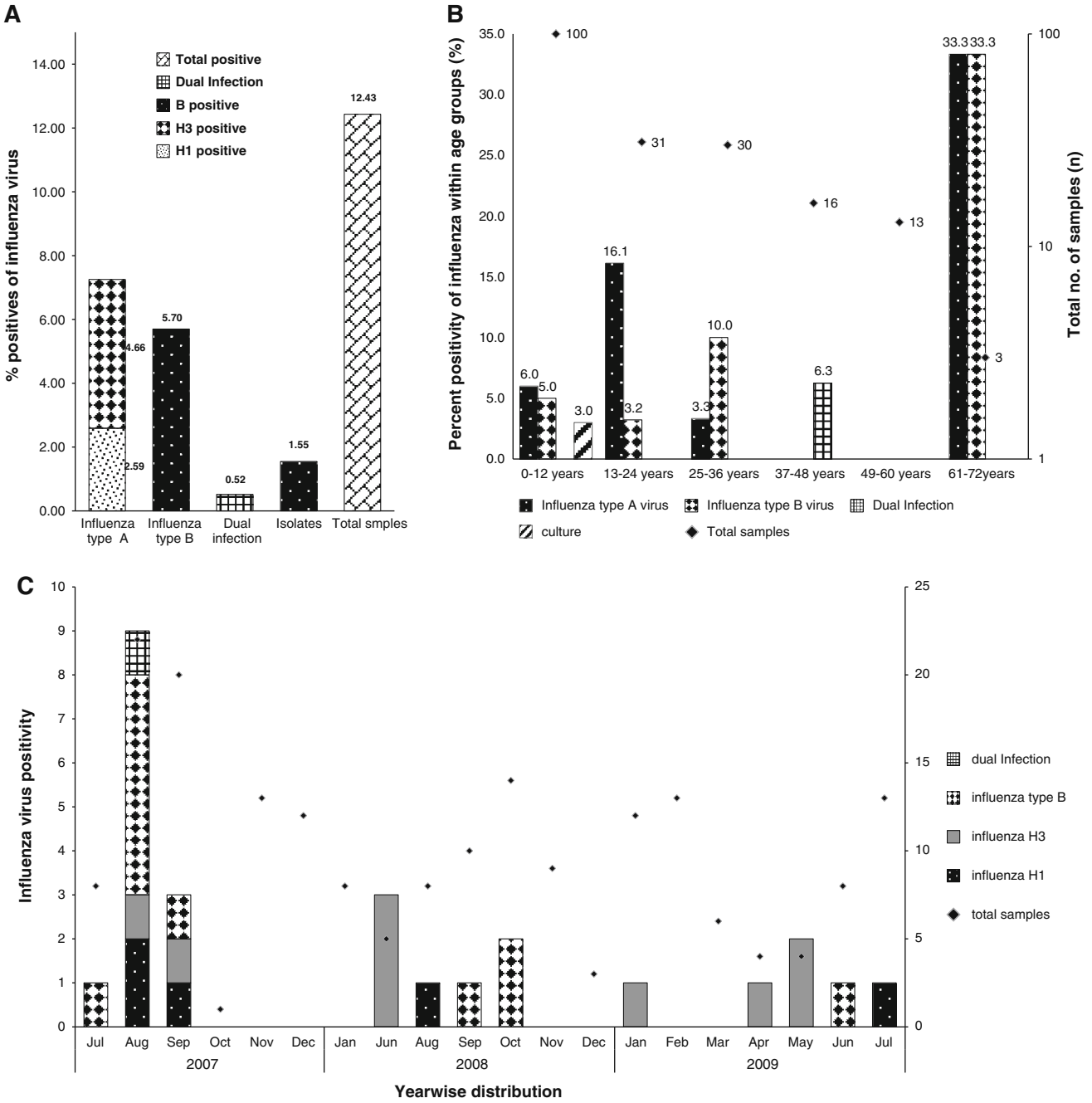

Fig. 1 **a** Percent positivity of influenza A/H1 (2.59 %), A/H3 (4.66 %) and influenza B (5.70 %) virus by rRT-PCR and culture methods (1.55 %); **b** Percent positivity of influenza samples within each age group; **c** Year-wise distribution of samples with respect to viral subtype positivity for the years 2007, 2008 and 2009

positivity of influenza virus was found during the monsoon season (Fig. 1c). Similar findings were observed in Pune, which has a tropical climate, and showed that influenza outbreaks occurred predominately during the rainy months [1, 7]. In Taiwan and Harbin city in China [13], highest positivity was observed in early spring and winter season, whereas in Nigeria and Singapore during the hot and rainy seasons which speaks of the distribution of influenza virus positivity in different season [2, 3]. Increased cases of influenza activity were also reported during the winter in northern India (December–February), and the hot and wet season in eastern or western India, as in Kolkata. Sequencing and phylogenetic analysis of the hemagglutination gene of the Mumbai isolates of influenza type B virus revealed that all the three strains were related to the Reference Influenza B/Victoria/87 strain. Influenza B/Yamagata/88 Reference strain was the out-group. Interestingly, the isolates were more closely related to the B/Brisbane/60/2008 strains which

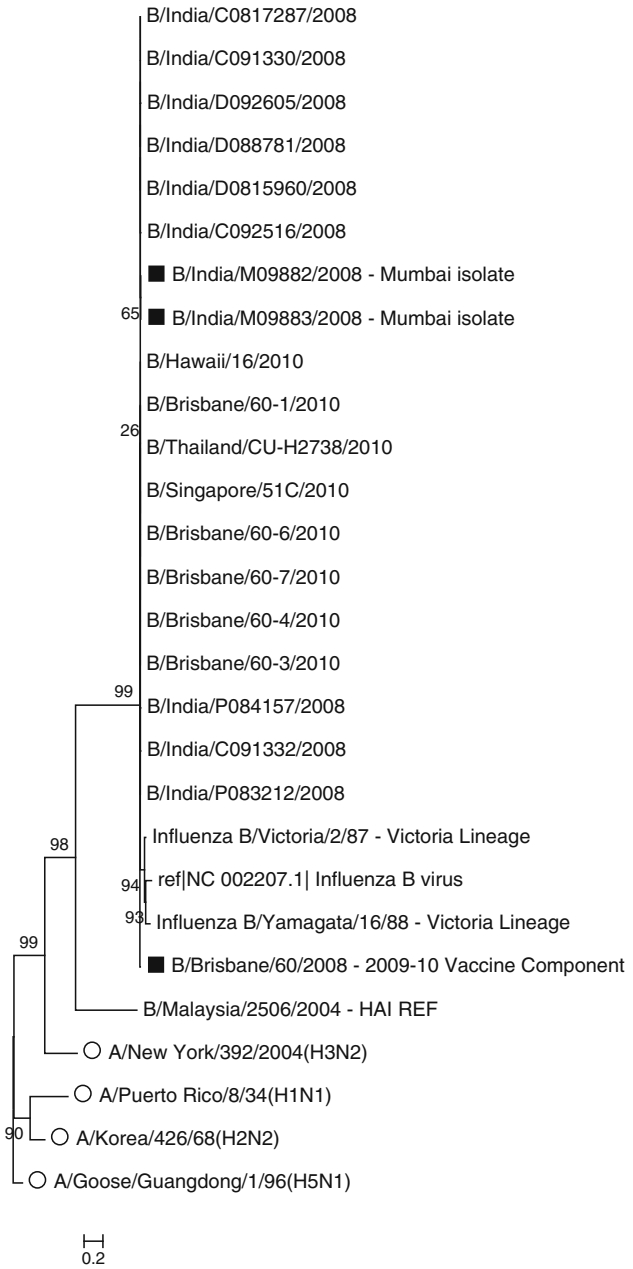

Fig. 2 Phylogenetic tree of influenza virus strains (Mumbai isolates, related strains and reference strains) using the HA gene sequences The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method with the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) shown next to the branches. The tree was rooted with a non-related influenza strain (H5N1). Evolutionary analyses were conducted in MEGA 5. *Black squares* indicate the relatedness of Mumbai isolates with the vaccine component of 2009–2010; *open circles* indicate influenza A strains

formed the part of the trivalent influenza vaccine component of 2009–2010. A phylogenetic tree comprising the Mumbai isolates and closely related strains (India, Thailand, Malaysia, Singapore) with influenza A (H1N1, H2N2, H3N2 and H5N1) as outliers was also created to elucidate the evolution of the isolates obtained in this study (Fig. 2) [9]. This demonstrates the significance of continuous influenza surveillance as one of its main objective to provide region wise data of circulating virus which can be globally compared and appropriate strains are then chosen to form the vaccine component. Keeping the track of the evolution of the virus in the society is critical in prevention and control of further pandemics. Thus the Influenza virus surveillance is necessary, as this can provide information regarding the presence of influenza viruses in the community.

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