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RESEARCH ARTICLE

Genetic characterization of oseltamivir-resistant seasonal influenza A (H1N1) virus circulating during 2009 pandemic influenza in Mumbai

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Abstract

Background: Oseltamivir is an important antiviral agent for controlling the transmission and dissemination of influenza viruses. The emergence and widespread occurrence of oseltamivir-resistant A (H1N1) viruses are of major concern. Information on drug susceptibility of seasonal influenza A viruses in India is limited. To understand the genetic background of antiviral drug-resistance, we performed sequence analysis of seasonal influenza A viruses circulating during 2009 pandemic influenza in Mumbai.

Methods: Nasopharyngeal swabs positive for seasonal influenza A virus by real time reverse transcriptase polymerase chain reaction were inoculated on Madin-Darby canine kidney (MDCK) cell line for virus isolation. Molecular analysis of hemagglutinin (HA), neuraminidase (NA) and matrix (M2) genes was used to detect known mutations contributing to resistance. Resistance to neuraminidase was assayed using a commercially available chemiluminescence based NA-Star Influenza Neuraminidase Inhibitor Resistance Detection kit.

Results: Investigations showed that oseltamivir-resistant virus was obtained from an untreated person with H275Y mutation in the NA gene. The dominant variant also acquired additional substitutions, including G189S, A193T and H196N in receptor binding site of HA1 domain and D354G in NA gene. Phylogenetic analysis revealed that HA and NA gene of oseltamivir-resistant isolate from Mumbai exhibited close homology to human A (H1N1) A/Brisbane/59/2007 vaccine strain.

Conclusion: Resistance to oseltamivir was observed in seasonal influenza A (H1N1) isolate co-circulating in Mumbai during the 2009 pandemic influenza. The study highlights the importance of continual surveillance on influenza in India for early detection and identification of mutations conferring antiviral resistance

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INTRODUCTION

Antiviral agents confer significant prophylactic and therapeutic benefits not only during seasonal influenza outbreaks but also in unexpected pandemic (Bazet *et al.*, 2010). In spring 2009, a novel strain of influenza virus from swine to humans emerged in Mexico in March and spread globally (Chen *et al.*, 2009). As per the CDC interim guidelines for pandemic and seasonal influenza, use of neuraminidase inhibitors (oseltamivir and zanamivir) had been recommended for patients with severe or high risk of complications and hospitalized patients with suspected or confirmed pandemic H1N1 infection (CDC, 2009a).

Before the influenza season of 2007, sporadic cases of resistance to oseltamivir were very low (Renaud *et al.*, 2011; Diaet *et al.*, 2013). The emergence of oseltamivir-resistant seasonal influenza A (H1N1) [influenza A (sH1N1)] was first reported during the 2007-08 influenza season in Norway. An unexpectedly high proportion of natural resistance to oseltamivir has evolved among the prevailing influenza A (H1N1) virus circulating in humans (Chen *et al.*, 2009; Haugeet *et al.*, 2009). The most common mutation conferring resistance to oseltamivir is at position 275; that of substitution of Histidine (H) to Tyrosine (Y) (H275Y) in the N1 subtype of the neuraminidase protein (Zaraketet *et al.*, 2010). In Europe, this resistance of an H275Y mutation has been detected in the human A/Brisbane/59/2007 (H1N1)-like viruses (A[H1N1] Brisbane-like viruses) (Meijer *et al.*, 2009). These resistant viruses have been subsequently reported in many other regions of the world (Yang *et al.*, 2011; Njouomet *et al.*, 2010). Initial analysis indicated that 2009 pandemic influenza A (H1N1) viruses were sensitive to neuraminidase inhibitors but resistant to adamantanes having the S31N (serine to asparagine) mutation in the M2 ion channel protein (CDC, 2009b; Calatayudet *et al.*, 2011). During 2009-2010 influenza seasons, resistance to oseltamivir has been reported in 285 cases of pandemic (H1N1) influenza (WHO, 2010a).

The first case of 2009 pandemic H1N1 influenza in India was reported in May 2009 (Sarkaret *et al.*, 2011). Immediately after the influenza cases were reported in Maharashtra, Pune and other places in the country, Ministry of Health and Family Welfare, Government of India put into effect, the guidelines for control of influenza in India (Suri *et al.*, 2011). It recommended antiviral therapy with oseltamivir to all high risk and seriously ill patients (GOI-MOHFW, 2009). In India, majority of seasonal (H1N1) isolates were amantadine sensitive but in 2009 amantadine resistant seasonal (H1N1) viruses were found to be in circulation. For seasonal (H1N1) influenza virus, resistance to neuraminidase inhibitors was detected by the end of December 2008 (WHO, 2010b). Reports from USA, Europe and other parts of the world state that the 2009 pandemic H1N1 influenza had completely overtaken seasonal influenza A viruses. While, in India both pandemic and seasonal influenza A virus strains were found to be in co-circulation (Sarkaret *et al.*, 2011).

Haffkine Institute for Training, Research and Testing, Mumbai is the National Influenza Center under World Health Organization (WHO) for the surveillance of influenza viruses. During 2009 influenza season in Mumbai, co-circulation of pandemic H1N1 and seasonal influenza A virus was evident from the epidemiology data generated by the institute. Information on drug susceptibility of seasonal influenza A viruses is limited in India while no published data from Mumbai. The present study examines drug susceptibility of seasonal influenza A viruses circulating in Mumbai.

METHODS:

Ethics Clearance

The project was approved by the Institutional Ethics Committee and Institutional Animal Ethics Committee of Haffkine Institute for Training, Research and Testing, vide certificate number HITRT/IEC/04/2011.

Cells and viruses

Madin-Darby canine kidney (MDCK) cells, obtained from National Center for Disease Control (NCDC) were maintained in Minimal Essential Medium (MEM, Gibco, by Life Technologies) supplemented with 10% fetal bovine serum (Gibco, by Life Technologies), 100U/ml Penicillin and 0.5 mg/ml Streptomycin (Hi-Media Laboratories, India). Clinical samples positive for seasonal influenza A were inoculated onto confluent MDCK cells in serum free medium containing 2µg/ml of Tosyl phenylalanyl chloromethyl ketone (TPCK) trypsin, and were passaged twice to reach sufficient titers. A total of 75 samples were selected based on the cycle threshold value ($C_t < 35$), different age groups and geographical settings, maximum volume of the samples available and complete clinical history of the patient (WHO, 2010c). Tissue culture fluid was harvested after observing MDCK cell line for cytopathic effect. Virus stocks were aliquoted and stored at -80°C until use (Balishet *et al.*, 2006).

Hemagglutination and Hemagglutination Inhibition assay

The presence of influenza virus in the cell culture supernatant was determined by hemagglutination assay using Guinea pig RBCs (Hirst *et al.*, 1942; Hsiung *et al.*, 1982). Each isolate was confirmed for its subtype using specific antisera panel by the hemagglutination inhibition (HAI) assay as per the WHO kit protocol for typing of human influenza isolates for 2010 (WHO Collaborating Centre for Reference and Research on Influenza VIDRL, Australia).

RNA Extraction and Reverse Transcription Polymerase Chain Reaction

Viral RNA was extracted from 140µl of viral cell culture supernatant using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The HA, NA and M gene were amplified using the oligonucleotide primers as described elsewhere (WHO, 2009). One-Step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed using Access Quick RT-PCR System (Promega Corporation, Madison, WI, USA) in accordance with the manufacturer's instructions. The segments were amplified in three fragments in order to obtain appropriate sequence coverage. PCR conditions included reverse transcription at 48°C for 45 minutes, followed by RT inactivation at 94°C for 2 minutes. The cycling conditions comprised of 29 cycles of 94°C for 20s, 56°C for 30s and 72°C for 1 min with a final cycle of 72°C for 7 min, followed by holding at 4°C. The resulting amplicons were analyzed by 1.5 % agarose gel electrophoresis.

PCR product purification and sequencing

Amplified products were purified using HiPurA™ PCR product purification kit (Hi Media Laboratories Pvt. Ltd) as per the manufacturer's instructions and stored at -20°C until sequencing. Sequencing was performed using an automated sequencer (ABI 3730X1 Applied Biosystems, USA).

NA inhibitor

Oseltamivir carboxylate, the active form of the active metabolite of the prodrug oseltamivir phosphate, was procured from Clearysyn Labs Pvt. Ltd, Mumbai.

NA inhibition assay

The 50% inhibitory concentration of oseltamivir for the isolates was determined using the NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Briefly, 25 µl of half-log dilutions (0.03 to 1,000 nM) of NA inhibitor were mixed with 25 µl of a virus dilution with a HA titer equal to 16 and incubated at 37°C for 20 mins. Twowells containing only assay buffer (instead of Neuraminidase Inhibitor) and culture medium (instead of virus) were used as negative controls. Diluted substrate (10 µl) was added to each well and was incubated at room temperature for 15 min, followed by addition of 60 µl of accelerator, and the emitted chemiluminescent signal was measured immediately. The 50% inhibitory concentration (IC₅₀) was determined by regression analysis (Prism; version 6.00; GraphPad Software). For the NA activity determination, 25 µl of diluted virus was mixed with 25 µl of assay buffer instead of neuraminidase inhibitor. For a negative control, one well contained assay buffer and culture medium.

Nucleotide sequence deposition

All the sequences identified in this study have been submitted to National Center for Biotechnology Information (NCBI) GenBank. The accession number of the sequences for HA gene obtained in this study is KJ909523, NA gene is KJ909524, and for M gene KJ909525, and are considered as testing data in further analysis.

Sequence driven phylogenetic analysis

Multiple sequence alignment was performed using Molecular Evolutionary Genetics Analysis (MEGA) 6.0.5 (Tamura *et al.*, 2013). Sequences were assembled and aligned with the reference sequences of the same season, and for the same gene to generate consensus sequence. The consensus sequences of drug resistant and drug sensitive isolates for each gene type were further studied. Phylogenetic tree was constructed by Maximum-Parsimony method with Subtree-Pruning-Regrafting (SPR) method where a tree topology is searched heuristically reducing the number of topologies searched. To compare the drug susceptibility pattern of circulating strain in Mumbai, reference sequences of drug resistant/sensitive strains were obtained from the Influenza Virus Resource of NCBI. The reference sequences were obtained from India and other regions worldwide and were included as references in the sequence driven analysis considering it as training data.

RESULTS:

A total of 17 isolates of seasonal influenza A virus were obtained and were further characterized. In HAI tests using specific antisera panel and two prototype vaccine viruses we observed 16 isolates to be antigenically close to A/Perth/16/2009(H3N2)-like virus. One isolate was however antigenically close to A/Brisbane/54/2007 seasonal (H1N1)-like virus. Drug susceptibility was investigated for all the isolates. All the H3N2 isolates were resistant to amantadine and sensitive to oseltamivir (Gohil *et al.*, 2015). As the seasonal H1N1 isolate was contrary to our expected finding, a detailed characterization was carried out. Neuraminidase inhibition assay was performed in combination with HA, NA and M gene sequence analysis.

Neuraminidase activity of Influenza A (sH1N1) virus

NAI susceptibility of the isolates to oseltamivir was tested using NA inhibition chemiluminescence based assay. The IC₅₀ values of the neuraminidase inhibitor were determined by regression analysis using GraphPad Prism Software. A/Pune isolate/2009(H1N1) (seasonal virus) sensitive to oseltamivir was used as a control. The IC₅₀ value of the control virus was determined to be 0.51 nM. However, influenza A (sH1N1) isolate was resistant to oseltamivir with extremely high IC₅₀ value of 1261 nM, which was 2473-fold increase when compared with that of the control virus. This virus was obtained from a 20-year-old female who did not receive oseltamivir treatment. Gene sequencing was further carried out to understand the molecular basis of amino acid substitution.

Genetic characterization

Oseltamivir susceptibility was determined based on N1 sequence analysis for the substitutions at residue 275. NA of the oseltamivir resistant influenza A (sH1N1) isolate possessed the substitution H275Y, a marker documented to confer a high level of resistance to oseltamivir (Njouomet *et al.*, 2010). Additionally, the isolate also showed substitution of D354G, typical of the many of oseltamivir resistant H1N1 viruses that emerged in Europe (Meijer *et al.*, 2009). This dominant variant furthermore presented substitutions in the HA gene. HA1 protein segment revealed amino acid substitutions of G189S, A193T and H196N. Amantadine susceptibility of influenza A (sH1N1) isolate was also assessed by conventional sequencing. M2 sequence analysis of the isolate was also analyzed for the amino acid substitution conferring resistance to the adamantane class of anti-influenza drugs. None of the amino acid residues, viz. L26, V27, A30, S31 and G34, exhibited any variation (Deyde *et al.*, 2010).

Phylogenetic analysis

To further study the genetic mechanism of the emergence of antiviral resistance among seasonal A (H1N1) viruses in Mumbai, phylogenetic analysis was performed on the HA and NA genes of the isolate. Multiple sequence alignment of these strains revealed that all these sequences were homologous having sequence identity of more than 95%. Maximum-Parsimony (MP) method is considered as an important and accurate optimal criterion for the evolution of phylogenetic trees when the datasets are at lower evolutionary divergence and have more inclination with the rule of association (Gregoret *et al.*, 2013). Thus, MP method was tailored for the construction of phylogenetic tree. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 0 in which the initial trees were obtained by the random addition of sequences (5 replicates). The analysis involved 62 nucleotide sequences for NA gene and 55 nucleotide sequences for HA gene with composite training and testing dataset. All positions containing gaps and missing data were eliminated in the data preprocessing steps. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013)

The tree was constructed using reference vaccine strain sequence and reference sequences for the HA and NA gene, which were obtained from the Influenza virus database treating it as the training data whereas sequenced data was treated as testing data. (Figure 1) represents phylogenetic relationship of the NA gene segments of influenza A (H1N1) virus in Mumbai. The phylogenetic analysis of NA gene revealed two distinct clades characterized by a difference in amino acid residue 275 (H275Y) as well as 354 (D354G) mutation from 2007 to 2009. The oseltamivir-sensitive and oseltamivir-resistant isolates were homologous to WHO-recommended vaccine strain used in the Northern hemisphere during the 2008-09 season. The first introduction of oseltamivir-resistance (Sub-Clade IA) observed in these data occurred in Norway and Europe during the influenza season of 2007-08. Similar genetic reassortment events (Sub-Clade IB and Sub-Clade ID) were noted in isolates obtained from Asia, United States and Europe in the subsequent influenza seasons of 2008-09. The emergence of a new variant of the influenza virus may be due to antigenic drift (point mutation) or genetic reassortment. Clade II entirely constituted of oseltamivir-resistant viruses from the 2008-09 seasons that possessed the H275Y and D354G mutation in their NA gene. These viruses are represented by A/Brisbane/59/2007-like lineage, the major oseltamivir-resistant lineage that circulated in Europe during 2007-08 and other countries during the 2008-09 seasons (Zararet *et al.*, 2010).

Indian isolate from Mumbai was categorized in the Clade II showing homology to oseltamivir-resistant and antigenically related to A/Brisbane/59/2007-like vaccine virus. The isolate also exhibited close homology with the strains circulating in United States (i.e., A/Illinois/12/2009(H1N1) and A/Rhode Island/17/2009(H1N1)). However, Indian isolates obtained from Kolkata (Sub-Clade IC) which emerged in the same influenza season of 2009 were also characterized by H275Y and D354G mutation, yet did not exhibit close homology to the isolate circulating in Mumbai region.

The phylogenetic topology of HA gene was similar. (Figure 2) represents phylogenetic relationship of the HA gene segments of influenza A (H1N1) virus in Mumbai. Clade I was constituted majorly by the viruses from the 2007-08 season which possessed oseltamivir-sensitive and oseltamivir-resistant isolates. The viruses in Clade I were homologous to A/Brisbane/59/2007-like vaccine virus. Clade II harbored drug resistant viruses from the 2007-08 seasons. HA gene of Indian isolate from Mumbai was categorized in the Clade II showing close homology to oseltamivir-resistant viruses circulating in other different countries during the 2008-09 seasons. The isolate was antigenically related to A/Brisbane/59/2007-like vaccine virus which exhibited close homology with the strains circulating in United States (i.e., A/California/VRDL366/2009(H1N1) and A/Rhode Island/17/2009(H1N1)).

Figure 1. Phylogenetic relationship of the NA gene segments of influenza A (H1N1) virus in Mumbai. The closed triangle indicates the WHO recommended reference vaccine strain. Oseltamivir-resistant strain from Mumbai (closed square).

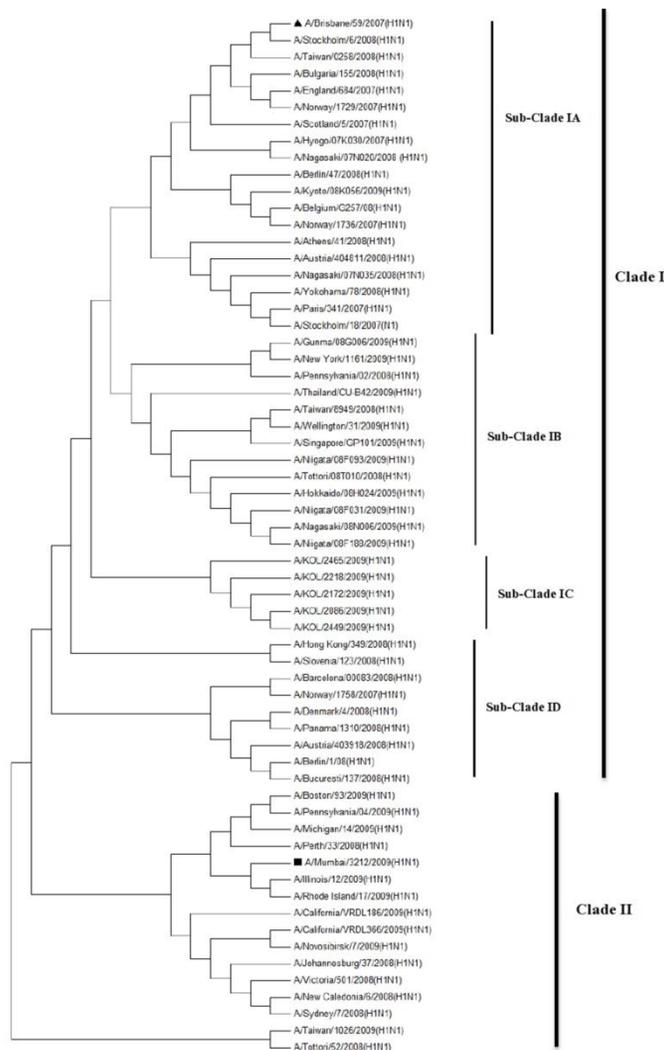
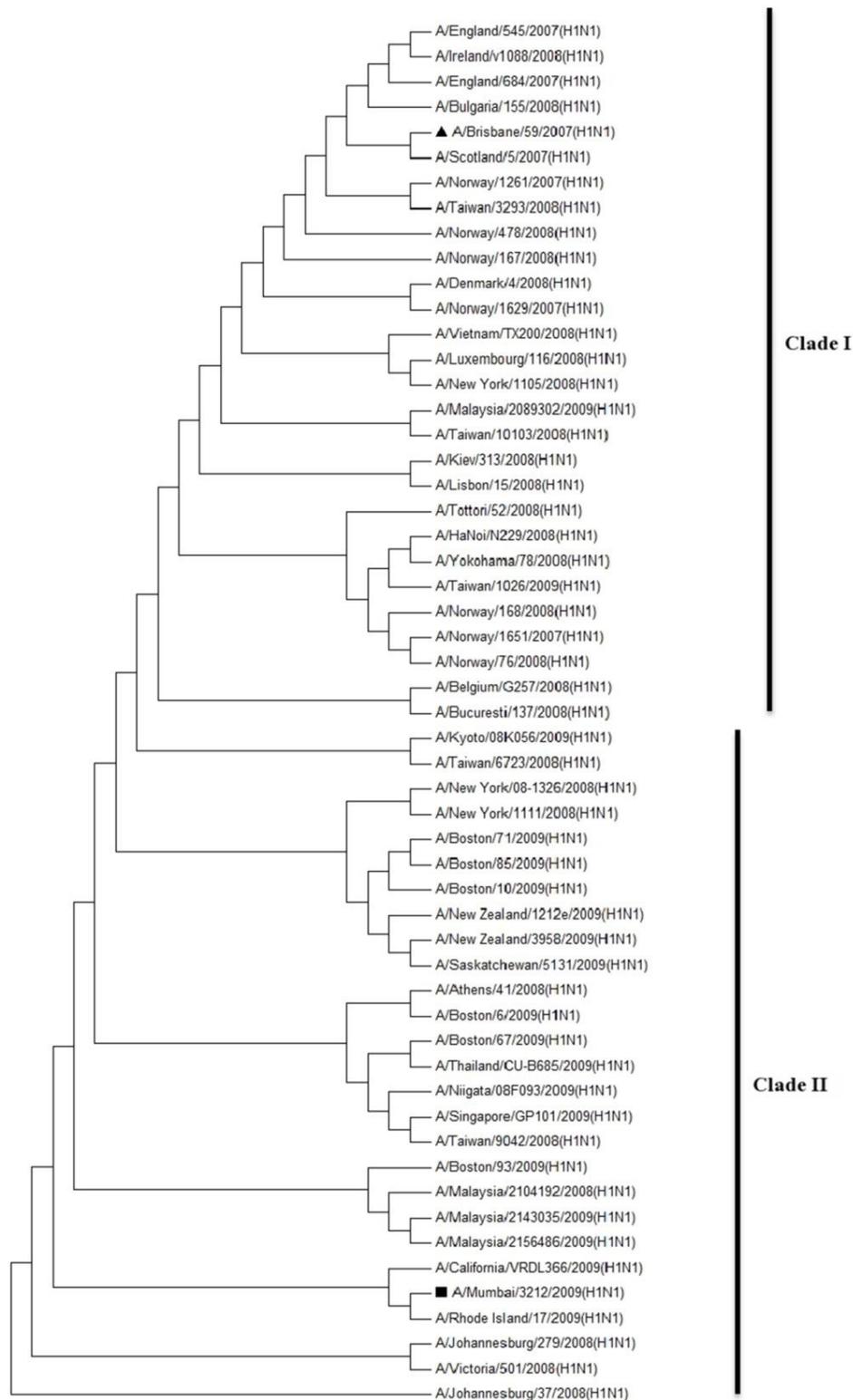


Figure 2. Phylogenetic relationship of the HA gene segments of influenza A (H1N1) virus in Mumbai. The closed triangle indicates the WHO recommended reference vaccine strain. Oseltamivir-resistant strain from Mumbai (closed square).



DISCUSSION:

Oseltamivir is an important antiviral drug for controlling the transmission and dissemination of pandemic influenza viruses, before a vaccine is made available (Hall *et al.*, 2012). Emergence of oseltamivir resistance in seasonal and pandemic influenza A (H1N1) has created challenges for diagnosis and clinical management of influenza virus infections. Rapid increase in the prevalence of oseltamivir-resistant influenza A (sH1N1) strains has been reported worldwide (Hauge *et al.*, 2009; Meijer *et al.*, 2009; Ujike *et al.*, 2010). Unfortunately, since the past decade, knowledge of prevalent influenza strains in India has been limited, mainly due to lack of systemic study data. Mumbai being a major migration hub of people nationally and globally, the infections transferred herein and circulating strains become critically important to be identified and reported. To our knowledge, this is the first report of genetic characterization of oseltamivir-resistant strain circulating in Mumbai.

Before the influenza season of 2007-08, only few clinical data describing oseltamivir-resistant seasonal H1N1 were available. The rate of oseltamivir resistance was very low before 2007, ranging between 0.5% and 1%. Between 2005 and 2007, a study conducted in 11 children infected with seasonal influenza H1N1 found 3 cases (27.3%) in whom the H275Y mutation was detected (Renaud *et al.*, 2011). In 2007-08, oseltamivir resistance was first reported in seasonal influenza H1N1 in Japan with documented positivity of 2.6% (Hauge *et al.*, 2009; Ujike *et al.*, 2010). By early 2008, unprecedented high levels of oseltamivir resistance were reported in many countries with reported rates ranging from 56% in Europe and 67.3% in Norway. During 2008-09, 99.7% of cases reported in Japan were resistant while in many other countries the reported rates of oseltamivir resistance in seasonal influenza H1N1 were 95%-100% (Esposito *et al.*, 2010; Casalegno *et al.*, 2010; Dharanet *et al.*, 2009).

During 2007-08, oseltamivir-resistant viruses were found to have evolved through genetic reassortment from oseltamivir-sensitive strain by acquisition of the H275Y mutation in NA gene with additional modifications in the genome (Zaraket *et al.*, 2010). In Europe, resistance due to H275Y mutation has been detected in the human A [H1N1] Brisbane-like viruses (Meijer *et al.*, 2009). In addition to the H275Y mutation, the D354G substitution was also prominent in the NA gene sequences of oseltamivir-resistant viruses isolated in other parts of the world (Njouomet *et al.*, 2010). The oseltamivir-resistant strain isolated in this study also exhibited H275Y and D354G mutation. The location of residue 354 is on the top external side of the neuraminidase tetramer and away from the enzyme binding site, thus making it unlikely to be compensating for the H275Y substitution (Rameix-Weltet *et al.*, 2008). Interestingly, the oseltamivir-resistant strain additionally acquired mutation at the residue 189, 193 and 196 in the receptor binding domain of the HA1 protein, in parallel with the H275Y resistance in the NA protein. Substitutions at residues 187-198 belong to overlapping receptor binding site 190-helix of the Sb antigenic site near the base of the globular head of HA gene (Suwannakarn *et al.*, 2010; Yang *et al.*, 2013). Mutation at residue A193T in HA1 domain of influenza A (sH1N1) has been previously described (Zaraket *et al.*, 2010). Oseltamivir-resistant virus isolated from Mumbai revealed additionally substitutions at G189S and H196N at the Sb antigenic site. It has been documented that any mutations interfering receptor binding are likely to impair viral adsorption and infectivity (Yang *et al.*, 2013). However, the role of this substitution on influenza epidemiology needs to be studied (Yang *et al.*, 2011). Although, influenza A (sH1N1) was oseltamivir-resistant, this strain remained susceptible to amantadine. This was consistent with earlier study observation (Ujike *et al.*, 2010).

In the present study a chemiluminescence-based assay was utilized in conjunction with NA sequence analysis. Influenza A (sH1N1) strain showed 2473-fold reduction in oseltamivir susceptibility. A recent study conducted in New Zealand revealed all 2009 influenza A (sH1N1) viruses were resistant to oseltamivir with the IC₅₀ values between 305 nM to 7912 nM (Ujike *et al.*, 2010). The oseltamivir-resistant isolate obtained from Mumbai also displayed extremely high IC₅₀ value of 1261 nM which is similar to 2009 influenza A (sH1N1) viruses circulating in New Zealand.

The emergence of oseltamivir-resistant influenza A (sH1N1) viruses in India has been limited. This study incorporates multi-segment sequence data sampled locally and globally and determining the evolutionary process of the virus worldwide. Genetic characterization of seasonal influenza strains circulating in Kolkata revealed higher average percentage similarity to vaccine strain A/Brisbane/59/2007-like lineage (Agrawal *et al.*, 2010). Phylogenetically, the HA and NA genes of oseltamivir-resistant isolate from Mumbai were also antigenically related to A/Brisbane/59/2007-like vaccine strain. However, the isolate did not exhibit close homology to the isolates circulating in Kolkata. The study also reveals the isolate in Mumbai exhibited close relationship with the strains circulating in United States, Australia, Europe and Asia.

Genetic characterization studies from India have reported that in spite of oseltamivir treatment during the pandemic H1N1 2009, the co-circulating strains pandemic (H1N1) 2009 as well as seasonal (H3N2) strains did not show any mutation conferring resistance to oseltamivir (Agrawal *et al.*, 2010). It has been suggested that a gene shuffle between viruses of the same subtype can occur frequently. Recent in vitro genetic reassortment studies have demonstrated that by coinfecting influenza-permissive cells, the HA segment from the novel influenza A (H1N1) 2009 virus can acquire mutated NA from the A (H1N1) Brisbane-like virus. The co-circulation of the novel influenza A (H1N1) and human A (H1N1) Brisbane-like virus might eventually produce an oseltamivir-resistant virus by gene reassortment (Ottmann *et al.*, 2010).

In conclusion, the findings suggest that continued influenza surveillance on anti-viral drug resistance is essential to monitor the emergence and spread of drug resistance. Such studies will help to ensure that prescribed neuraminidase inhibitors by clinicians are effective when treating patients for influenza especially during pandemic.

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DECLARATION OF CONFLICT OF INTEREST:

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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