Journal of
Antivirals & Antiretrovirals
ISSN: 1948-5964

Gohil et al., J Antivir Antiretrovir 2015, 7:4
DOI: 10.4172/jaa.1000126

Research Article Open Access

Oseltamivir Resistant Influenza A (H1N1) Virus Infection in Mumbai, India

Devanshi Gohil[1*], **Sweta Kothari**[1], **Pramod Shinde**[2], **Anand Chintakrindi**[3], **Rhuta Meharunkar**[4], **Rajas Warke**[1,4], **Meena Kanyalkar**[3], **Abhay Chowdhary**[1] and **Ranjana Deshmukh**[1]

[1]*Department of Virology and Immunology, Haffkine Institute for Training, Research and Testing, Parel, Mumbai*

[2]*Department of Bioinformatics, Guru Nanak Khalsa College, Matunga, Mumbai*

[3]*Department of Pharmaceutical Chemistry, Principal K. M. Kundnani College of Pharmacy, Colaba, Mumbai*

[4]*Molecular Biology and Virology, HiMedia Laboratories, Ghatkopar, Mumbai*

[*]**Corresponding author**: Devanshi Gohil, Department of Virology and Immunology, Haffkine Institute for Training, Research and Testing, Acharya Donde Marg, Parel, Mumbai-400012, Maharshtra, India, Tel: +91-22-2416-0947; Fax: +91-22-2416-1787; E-mail: devanshi.gohil@gmail.com

Received date: October 16, 2015; **Accepted date**: November 13, 2015; **Published date**: November 20, 2015

Abstract

Objectives: Oseltamivir is an important antiviral agent approved for treatment of infections caused by Influenza virus. The emergence of an Oseltamivir resistant seasonal Influenza A (H1N1) virus in 2007 in Norway and pandemic Influenza A (H1N1) virus in 2009 [Influenza A (H1N1) pdm 09] is of major concern. This study aimed for examining antiviral drug resistance of Influenza A viruses circulating during 2009 Influenza season in Mumbai.

Methods: Nasopharyngeal swabs positive for Influenza A virus were inoculated on Madin-Darby canine kidney cell line for virus isolation. Molecular analysis of neuraminidase gene and matrix gene was carried out to detect known mutations contributing to resistance. Resistance to neuraminidase was assayed using a commercially available chemiluminescence based NA-Star assay kit.

Results: Genotypically, a total of 47 isolates of Influenza A (H1N1) pdm 09 isolates were observed to harbor mutations known to confer resistance to Adamantane. However, no known mutations conferring resistance to neuraminidase (NA) inhibitors were detected. Resistance to Oseltamivir was observed in a single isolate of seasonal Influenza A (H1N1) isolate with an extremely high IC_{50} value of 1261 nM. Conversely, the isolate was sensitive to Adamantane. Phylogenetic analysis revealed that NA gene of this Oseltamivir-resistant isolate from Mumbai was antigenically related to human A (H1N1) A/Brisbane/59/2007 vaccine strain.

Conclusions: Surveillance on drug susceptibility of circulating strains in Mumbai helped us to identify Oseltamivir resistance in seasonal Influenza virus. The study highlights the importance of continual surveillance on Influenza in India to ensure that antiviral prescribed by clinicians is effective when treating patients for Influenza infections.

Key words:

Influenza; Antiviral; Surveillance; Oseltamivir; Resistant; Mumbai

Introduction

Influenza virus belongs to the family Orthomyxoviridae with a genome makeup of seven or eight single-stranded, negative-sense RNA segments. Influenza A viruses are a major cause of acute respiratory infections, responsible for annual epidemics and irregular pandemics in humans worldwide [1]. In the spring of 2009, novel swine-origin Influenza A virus was detected in Mexico. On June 11, 2009, the world health organization (WHO) declared a phase-6 alert, indicating first pandemic of the 21st century caused by Influenza A (H1N1) virus [2].

Antiviral agents confer significant prophylactic and therapeutic benefits during seasonal Influenza outbreaks and unexpected Influenza pandemics [3]. The development and occurrence of antiviral drug resistance in human Influenza viruses has been extensively studied over the last decade [4]. Initial analysis of Influenza A (H1N1) pdm 09 revealed that the virus was resistant to the Adamantanes class of drugs that inhibit the M2 ion channel [5]. Therapeutic options were limited to two neuraminidase inhibitors (NAI), viz. Oseltamivir (TamifluTM) and Zanamivir (RelenzaTM) [6]. As per the CDC interim guidelines for pandemic and seasonal Influenza, the use of NAIs was recommended for patients with severe or high risk of complications and hospitalized patients with suspected or confirmed pandemic H1N1 infection [7]. Preliminary analysis indicated that Influenza A (H1N1) pdm 09 viruses were sensitive to neuraminidase inhibitors [8,9]. As of April 2010, resistance to Oseltamivir had been reported in 285 cases of pandemic (H1N1) Influenza [10].

Emergence of Oseltamivir resistance to seasonal Influenza A (H1N1) viruses was detected in Norway in 2007. These resistant viruses have evolved into the dominant Influenza A (H1N1) in humans [11]. The most common mutation conferring resistance to Oseltamivir is at position 275 by substitution of histidine (H) to tyrosine (Y) (H275Y) in the N1 subtype of the neuraminidase protein [12]. In Europe, H275Y mutation in the human A/Brisbane/59/2007 (H1N1)-like viruses (A [H1N1] Brisbane-like viruses) had been detected to cause resistance to Oseltamivir [13]. These resistant viruses were subsequently reported in many other regions of the world [14,15].

By 2009, all seasonal Influenza A (H1N1) viruses were resistant to Oseltamivir [5,9]. Genomic analysis revealed that Oseltamivir-resistant Influenza A (H1N1) pdm 09 viruses also contained this specific H275Y mutation in the N1 numbering of the NA protein [16]. Recent findings of a community transmission of the Oseltamivir-resistant Influenza A (H1N1) pdm 09 viruses raise the concern that such viruses might spread globally and become predominant [9]. However, no evidence of genetic reassortment between Influenza A (H1N1) pdm 09 and seasonal Influenza A (H1N1) viruses strain has been detected [17].

After the first case report of Influenza A (H1N1) pdm 09 virus in India in May 2009, and subsequent reports from many other places in the country, Ministry of Health and Family Welfare, Government of India initiated guidelines for control of Influenza [18,19]. Antiviral therapy with Oseltamivir was recommended to all high risk and seriously ill patients [20]. In India, majority of seasonal Influenza A (H1N1) isolates have been Amantadine sensitive, however in 2009 Amantadine resistant A (H1N1) viruses were found to be in circulation. By 2008-2009, resistance to neuraminidase inhibitors in seasonal Influenza A (H1N1) viruses was detected [3].

Haffkine Institute for training, research and testing is the National Influenza Center under world health organization (WHO) for the surveillance of Influenza viruses in the Mumbai region. In Mumbai, during 2009 Influenza season, co-circulation of Influenza A (H1N1) pdm 09 and seasonal Influenza A virus was evident from the epidemiology data generated at Institute. The present study was envisaged to monitor the incidence of Oseltamivir resistance in Influenza A (H1N1) pdm 09 viruses and seasonal Influenza A viruses circulating in Mumbai between 2009 and 2011 using sequence based genotypic assessment, and the phylogenetic relationship was determined between circulating strains. Adamantane and neuraminidase susceptibility was determined genotypically by sequencing while neuraminidase susceptibility was also determined phenotypically using the chemiluminescence based enzyme inhibition assay.

Materials and Methods

Viral culture

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, USA) supplemented with 10% fetal bovine serum (Gibco, by Life Technologies, USA), 100 U/ml Penicillin and 0.5 mg/ml Streptomycin (Hi-Media Laboratories, India). During 2009-2011, a total of 150 samples positive for Influenza A (H1N1) pdm 09 and 75 samples positive for seasonal Influenza A 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 [21]. These clinical samples were inoculated onto confluent MDCK cells in serum free medium containing 2 µg/ml of tosyl phenylalanyl chloromethyl ketone (TPCK) trypsin for virus isolation. The samples were passaged twice to reach sufficient titers. Tissue culture fluid was harvested after observing MDCK cells for cytopathic effect. Virus stocks were aliquoted and stored at -80°C until use [22].

Hemagglutination inhibition (HIT) assay

The presence of Influenza virus in the cell culture supernatant was determined by hemagglutination assay using Guinea pig RBCs [23]. The identification of Influenza A virus subtype was analyzed by HAI assay. Each isolate was confirmed for its subtype using specific antiserum panel using Guinea pig RBCs as per the WHO Collaborating centre for reference and research on Influenza VIDRL, Australia protocol for typing of human Influenza isolates for 2010.

Reverse transcription polymerase chain reaction (RT-PCR)

Viral RNA was extracted from 140 µl of viral cell culture supernatant using QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. NA and M genes were amplified using the oligonucleotide primers as described in Table 1.

One-Step RT-PCR was performed using access quick RT-PCR System (Promega Corporation, USA) in accordance with the manufacturer's instructions [24]. Each segment was amplified using 5 µl of template RNA to 20.5 µl of the RT-PCR mixture consisting of nuclease free water 3.5 µl, forward primer 2 µl, reverse primer 2 µl, AMV Reverse Transcriptase (5U) 0.5 µl and Master Mix (2X) 12.5 µl. The segments were amplified in fragments in order to obtain appropriate sequence coverage. PCR cycling conditions were divided into holding stage and cycling stage. In the holding stage reverse transcription was carried out at 48°C for 45 minutes, followed by RT inactivation at 94°C for 2 minutes. PCR cycling conditions were as follows: 29 cycles of 94°C for 20s, 56°C for 30s and 72°C for 1 min and a final cycle of 72°C for 7 min followed by holding at 4°C.

PCR product purification and sequencing

Amplified products were purified using HiPurATM PCR product purification kit (Hi Media Laboratories Pvt. Ltd, India) as per the manufacturer's instructions and stored at -20°C until sequencing. Sequencing was performed using an automated sequencer (ABI 3730Xl Applied Biosystems, USA) with corresponding forward and reverse primers [24].

NA inhibitor

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

NA inhibition assay

The 50% inhibitory concentration (IC_{50}) of Oseltamivir for the isolates was determined using the NA-star Influenza neuraminidase inhibitor resistance detection Kit (Applied Biosystems, USA). Briefly, 25 µl of half-log dilutions (0.03 nM 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 370C for 20 mins. For negative controls, two-wells contained only assay buffer (instead of NI) and culture medium (instead of virus) were included. 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 IC_{50} 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. The negative control well contained assay buffer and culture medium.

Primer	Gene	Sequence (5'- 3')	Size of amplified product
M forward	M	TGT AAA ACG ACG GCC AGT AGC AAA AGC AGG TAG	1027 bp
M reverse	M	CAG GAA ACA GCT ATG ACC AGT AGM AAC AAG GTA GT	
NA 1 forward	NA	TGT AAA ACG ACG GCC AGT AGC AAA AGC AGG AGT	600 bp
NA 1 reverse	NA	CAG GAA ACA GCT ATG ACC CTG GAC CRG AAA TTC C	
NA 2 forward	NA	TGT AAA ACG ACG GCC AGT GGT CAG CAA GCG CAT GYC ATG A	810 bp
NA 2 reverse	NA	CAG GAA ACA GCT ATG ACC GCT GCT YCC RCT AGT CCA GAT	
NA 3 forward	NA	TGT AAA ACG ACG GCC AGT AAT GGR CAR GCC TCR TAC AA	726 bp
NA 3 reverse	NA	CAG GAA ACA GCT ATG ACC AGT AGA AAC AAG GAG	

Table 1: Primers sequences used for RT-PCR amplification of regions of M and NA genes

Nucleotide sequence deposition

All the sequences identified in this study have been submitted to national center for biotechnology information (NCBI) GenBank. The accession numbers of 47 complete sequences of the M gene (982 bp) obtained in this study are KJ942136-KJ942182, and 47 complete sequences for NA gene (1421 bp) are KJ958934-KJ958980, 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 [25]. Sequences were assembled and aligned with the reference sequences of the same season, and for the same gene to generate consensus sequence. 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 resistant/sensitive variants found in Mumbai with those found in other regions, sequences of drug resistant/sensitive strains were obtained from the Influenza virus resource, national center for biotechnology information (NCBI). These sequences were included as references in the sequence driven analysis considering it as training data.

Results

A total of 47 isolates of Influenza A (H1N1) pdm 09 virus and 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 that 16 isolates obtained during 2009-2011 were antigenically close to A/Perth/16/2009(H3N2)-like virus. During 2009-2010, one isolate was however antigenically close to A/Brisbane/54/2007 (H1N1)-like virus. Drug susceptibility was investigated for all the isolates. All the H3N2 isolates were resistant to Amantadine and sensitive to Oseltamivir which is in agreement with other reports [3,19]. As the seasonal Influenza A (H1N1) isolate was contrary to our expected finding, a detailed characterization was carried out. Neuraminidase inhibition assay was performed in combination with NA and M gene sequence analysis.

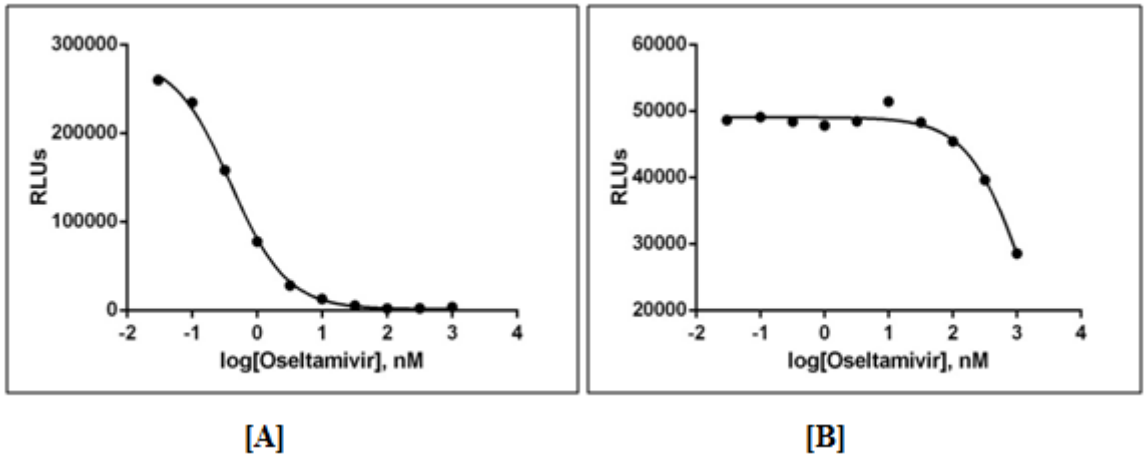
Neuraminidase activity of Influenza A (H1N1) pdm 09 and seasonal Influenza A (H1N1) isolates

Neuraminidase susceptibility of the isolates to Oseltamivir was tested using NA inhibition chemiluminescence based assay. The IC_{50} values of the neuraminidase inhibitor were determined by regression analysis using GraphPad Prism Software (Figure 1).

A/Pune isolate/2009(H1N1) (seasonal virus) sensitive to Oseltamivir was used as a control. The IC_{50} value of the control virus was determined to be 0.51 nM. The mean IC_{50} value of Oseltamivir for Influenza A (H1N1) pdm 09 isolates was determined to be 0.39 nM ± 0.124 nM. However, the seasonal Influenza A (H1N1) isolate circulating during 2009-2010 season was resistant to Oseltamivir with extremely high IC_{50} value of 1261 nM. This IC_{50} value was 2473-fold high when compared with the IC_{50} value of the control virus. On retrospective investigation, it was observed that 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.

Detection of molecular markers in the neuraminidase of seasonal and pandemic H1N1 viruses

Oseltamivir susceptibility was determined by N1 sequence analysis for the substitutions at residue 275. Neuraminidase protein of all Influenza A (H1N1) pdm 09 isolates possessed Histidine at amino acid position 275, conferring sensitivity to Oseltamivir. NA of the Oseltamivir-resistant seasonal Influenza A (H1N1) isolate possessed the substitution H275Y, well known marker which confers a resistance to Oseltamivir [15]. Additionally, the isolate was also characterized by the substitution of aspartic acid (D) by glycine (G) at residue 354 (D354G), typically of the majority of Oseltamivir-resistant H1N1 viruses that emerged in Europe [13].



[A] [B]

Figure 1: Assessment of the IC_{50} for Oseltamivir, using chemiluminescent neuraminidase inhibitor assay. (A) Oseltamivir- sensitive A/Pune isolate/2009 (H1N1) virus; (B) Oseltamivir-resistant A/Mumbai/3212/2009 (H1N1) virus; x axis, Oseltamivir concentrations (nM) on a logarithmic scale, y axis, NA activity in relative light units (RLUs). Data points indicate actual activity measured at a single point using a plate reader Synergy HT Multi-Mode Microplate Reader. The data line represents the best-fit curve generated using GraphPad Prism software.

Detection of molecular markers in M2 protein of seasonal and pandemic H1N1 viruses

Adamantane susceptibility of seasonal and pandemic H1N1 viruses was screened for the amino acid substitution in the M2 protein, conferring resistance to the Adamantane class of anti-Influenza drugs. Seasonal Influenza A (H1N1) isolate did not exhibit any variation at amino acid residues, viz. L26F, V27A, A30V, A30T, S31N and G34E [26]. Genomic analysis of all Influenza A (H1N1) pdm 09 isolates indicated the typical S31N mutation conferring resistance to Adamantanes. Of these viruses, three of the isolates also exhibited substitution of leucine (L) by phenylalanine (F) at residue 26 (L26F). Additionally, one of the isolate contained substitution of glycine (G) to glutamic acid (E) at residue 34 (G34E).

Phylogenetic analysis

Sequence analysis of all Influenza A (H1N1) pdm 09 Indian isolates from Mumbai revealed nucleotide identity ranging from 98.1%-99.4% with A/California/07/2009 (H1N1) prototype strain. Interestingly, only 78.3% amino acid identity was noted in seasonal Influenza A (H1N1) isolate with A/California/07/2009 (H1N1) strain. Phylogenetic analysis was performed for NA gene of Influenza A (H1N1) pdm 09 and seasonal Influenza A (H1N1) viruses in Mumbai, to study the genetic mechanism for the emergence of antiviral resistance. 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 105 nucleotide sequences with composite training and testing dataset. All positions containing gaps and missing data were eliminated in the data preprocessing steps. There were a total of 1403 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [25].

The tree was constructed using reference vaccine strain and reference sequences for the NA gene which were obtained from the Influenza virus database. These sequences were treated as the training data whereas sequenced data was considered as testing data. Figure 2 represents phylogenetic relationship of the NA gene segments of Influenza A (H1N1) viruses in Mumbai. The phylogenetic analysis of NA gene revealed two distinct clades, Influenza A (H1N1) pdm 09 viruses grouped in Clade I while seasonal Influenza A (H1N1) viruses were grouped in Clade II. All the Influenza A (H1N1) pdm 09 isolates in Clade I were sensitive to Oseltamivir and were homologous to WHO-recommended vaccine strain A/California/07/2009(H1N1)-like virus, used in the Northern hemisphere during the 2010-2011 season. The isolates also exhibited close homology with the strains circulating in United States and Indian isolates circulating during the same Influenza season of 2009.

The Oseltamivir-sensitive and Oseltamivir-resistant isolates in Clade II were homologous to WHO-recommended vaccine strain A/Brisbane/59/2007(H1N1)-like virus used in the Northern hemisphere during the 2008-2009 season. The first introduction of Oseltamivir resistance was observed in Norway and Europe during the Influenza season of 2007-2008[11,13]. Similar genetic reassortment events were then noted in isolates obtained from Asia, United States and Europe in the subsequent Influenza seasons of 2008-2009. The emergence of a new variant of resistant Influenza viruses may be due to antigenic drift (point mutation) or genetic reassortment. Oseltamivir-resistant viruses in Clade II constituted of strains circulating during 2007-2009 seasons which 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 viruses that circulated in Europe during 2007-2008 and other countries during the 2008-2009 seasons [12]. 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. Sequence analysis of seasonal Influenza A (H1N1) isolate from Mumbai revealed >98% nucleotide identity with A/Brisbane/59/2009 (H1N1) prototype strain. 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 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.

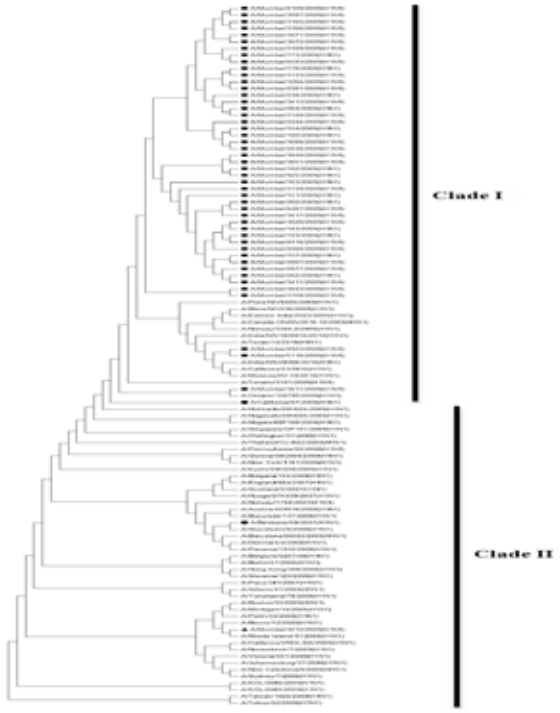


Figure 2: Phylogenetic tree constructed on the basis on NA gene nucleotides. The circle indicates the reference vaccine strains. Oseltamivir-resistant seasonal Influenza A (H1N1) isolate from Mumbai (closed triangle). Oseltamivir-sensitive Influenza A (H1N1) pdm 09 isolates from Mumbai (closed square).

Discussion

Influenza viruses cause annual epidemics in various countries and irregular worldwide pandemics. Antiviral drugs play an important role in preventing Influenza infections and reducing the impact of epidemics and pandemics. However, emergence and spread of resistant viruses has created challenges for treating and preventing Influenza virus infections. Antiviral resistant human Influenza A subtypes, Adamantane-resistant Influenza A (H3N2) viruses in 2005-2006 and Oseltamivir-resistant seasonal H1N1 viruses during 2007-2008 have occurred and circulated globally [9]. Mumbai being a major migration center of people nationally and internationally, the infections transferred herein and circulating strains become critically important to be identified and reported. Information on drug susceptibility to Amantadine and neuraminidase inhibitors for Influenza viruses in India is limited. Thus, monitoring of the susceptibilities of Influenza A (H1N1) pdm 09 and seasonal Influenza A (H1N1) viruses to antiviral drugs is essential in Mumbai region. In the present study, an overview of the antiviral resistance patterns was identified in pandemic and seasonal Influenza viruses co-circulating during the 2009 pandemic H1N1 Influenza season. The data demonstrate that all the viruses tested were resistant to one of the two types of antiviral drugs.

Before the Influenza season of 2007-2008, only few clinical data describing Oseltamivir-resistant seasonal H1N1 were available. During 2007-2008, 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 [12]. In Europe, resistance due to H275Y mutation has been detected in the human A [H1N1] Brisbane-like viruses [13]. 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 [15]. 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 [27]. On the other hand, majority of circulating Influenza A (H1N1) pdm 09 viruses remained sensitive to Oseltamivir. Despite the widespread of 2009 pandemic virus, the number of reported cases of Oseltamivir-resistant Influenza A (H1N1) pdm 09 viruses remained very low [8]. However, the emergence of Oseltamivir-resistant seasonal Influenza A (H1N1) virus in 2009 season projects that there could be the possibility that Oseltamivir-resistant Influenza A (H1N1) pdm 09 virus may also become dominant during the next Influenza season [3].

In the present study a chemiluminescence-based assay was utilized in conjunction with NA sequence analysis. Seasonal Influenza A (H1N1) strain showed 2473-fold reduction in Oseltamivir susceptibility. A recent study conducted in New Zealand revealed all 2009 seasonal Influenza A (H1N1) viruses to be resistant to Oseltamivir with the IC_{50} values between 305 nM to 7912 nM. Similar study conducted in Thailand reported IC_{50} values between 165.76 nM to 840.77 nM [4,28]. The Oseltamivir-resistant isolate obtained from Mumbai also displayed extremely high IC_{50} value of 1261 nM which was similar to 2009 seasonal Influenza A (H1N1) viruses circulating in New Zealand and Thailand. The IC50 value for Oseltamivir for Influenza A (H1N1) pdm 09 isolates obtained in the present study ranged from 0.23 nM to 1.01 nM. This was consistent with earlier study observations [29].

In spite of the highly conserved nature of the M gene segment among Influenza A viruses, the region comprising five amino acid markers of Adamantane resistance in M2 is variable among the different lineage and subtypes [26]. A single point mutation in the sequence coding for amino acids at positions L26F, V27A, A30V/T, S31N, and G34E of the M2 protein confers resistance to Adamantanes [26]. M2 protein coding region of the seasonal Influenza A (H1N1) and Influenza A (H1N1) pdm 09 viruses in the present study revealed that all pandemic viruses contained substitution at amino acid 43 (I43T) which is not present in seasonal Influenza viruses. This was consistent with the previous study [26]. Thus; a mutation at residue 43 differentiates pandemic H1N1 viruses from seasonal Influenza viruses. M2 protein coding region of all Influenza A (H1N1) pdm 09 viruses were resistant to Adamantane. Conversely, seasonal Influenza A (H1N1) virus showing high level of resistance to Oseltamivir was sensitive to Adamantane. In the present study, all Influenza A (H1N1) pdm 09 isolates exhibited S31N mutation as reported in earlier studies [26,29]. Additionally, the isolates also exhibited L26F and G43E mutations associated with Adamantane resistance.

Information on Oseltamivir-resistant seasonal Influenza A (H1N1) 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. Phylogenetic analysis of NA gene of Oseltamivir-resistant isolate from Mumbai was 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 revealed that the isolate in Mumbai exhibited close relationship with the strains circulating in United States, Australia, Europe and Asia. This study additionally attempts to group Oseltamivir-sensitive Influenza A (H1N1) pdm 09 viruses isolated in Mumbai region and compare them with various strains circulating worldwide. By sequence analysis, majority of the isolates in this study were antigenically close to Oseltamivir sensitive WHO recommended reference vaccine strains. The isolates also exhibited close relationship with the strains circulating in different parts of India (Pune, Bangalore and Eastern India) and United States.

Recent in vitro genetic reassortment studies have demonstrated that by coinfecting Influenza-permissive cells with the seasonal A (H1N1) Brisbane-like virus and novel Influenza A (H1N1) 2009, 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 [30]. Hence, continued Influenza surveillance on anti-viral drug resistance is essential to monitor especially in city like Mumbai where co-circulation of these strains has been identified.

Lack of immunity to Influenza A (H1N1) pdm 09 viruses in human population, combined with the Adamantane resistance of the virus, indicates that neuraminidase inhibitors constitute the main treatment regimen for susceptible groups of individuals. Thus, there is a great concern that Oseltamivir-resistant strains of Influenza A (H1N1) pdm 09 viruses may emerge and circulate in the community similar to Oseltamivir-resistant seasonal Influenza A (H1N1) virus [8]. Thus, intensive surveillance of resistant viruses is essential for monitoring genetic changes that can optimize viral fitness. Such emergence of resistant viruses in the community usually becomes predominant and spreads globally through evolution. Therefore, active surveillance for antiviral resistance needs to be maintained to ensure that prescribed neuraminidase inhibitors by clinicians are effective when treating patients for Influenza especially during Influenza pandemics.

Acknowledgements

We acknowledge National Center for Disease Control (NCDC), New Delhi, for providing us with Madin-Darby canine kidney (MDCK) cell line. We would also like to Dr. G Arunkumar form Manipal Virus Research Center for providing laboratory facility and WHO HAI kit for typing of human Influenza isolates 2010, WHO Collaborating Centre for Reference and Research on Influenza VIDRL, Australia. We also thank National Institute of Virology for providing us with seasonal Influenza A (H1N1) Pune Isolate for neuraminidase inhibition assay.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

References

1. Cox NJ, Kawaoka Y (1998) Orthomyxoviruses: Influenza. In: Collier L, Balows A, Sussman M, Mahy BWJ (eds.) Topley & Wilson's Microbiology and Microbial infections (9th edn.), London.
2. Harvala H, Gunson R, Simmonds P, Hardie A, Bennett S, et al. (2010) The emergence of Oseltamivir-resistant pandemic Influenza A(H1N1) 2009 virus amongst Hospitalised immunocompromised patients in Scotland, November-December, 2009. Euro Surveill 15: 19536.
3. Potdar VA, Dakhave MR, Kulkarni PB, Tikhe SA, Broor S, et al. (2014) Antiviral drug profile of human Influenza A & B viruses circulating in India: 2004-2011. Indian J Med Res 140: 244-251.
4. Hall RJ, Peacey M, Ralston JC, de Joux DJ, Bocacao J, et al. (2012) Tracking oseltamivir-resistance in New Zealand Influenza viruses during a medicine reclassification in 2007, a resistant-virus importation in 2008 and the 2009 pandemic. Western Pac Surveill Response J 3: 71-77.
5. Hurt AC, Deng YM, Ernest J, Caldwell N, Leang L, et al. (2011) Oseltamivir-resistant Influenza viruses circulating during the first year of the Influenza A(H1N1)2009 pandemic in the Asia-Pacific region, March 2009 to March 2010. Euro Surveill 16: 19770.
6. Chen H, Cheung CL, Tai H, Zhao P, Chan JF, et al. (2009) Oseltamivir-Resistant Influenza A Pandemic (H1N1) 2009 Virus, Hong Kong, China. Emerg Infect Dis 15: 1970-1972.
7. http://www.cdc.gov/h1n1flu/recommendations.htm
8. Gioula G, Melidou A, Exindari M, Papoutsi N, Chatzidimitriou D, et al. (2011) Oseltamivir-resistant Influenza A pandemic (H1N1) 2009 virus in Northern Greece. Hippokratia 15: 272-274.
9. Yang JR, Huang YP, Chang FY, Hsu LC, Huang HY, et al. (2013) Characterization of oseltamivir-resistant Influenza A(H1N1)pdm09 viruses in Taiwan in 2009-2011. J Med Virol 85: 379-387.
10. http://www.who.int/csr/disease/swineflu/oseltamivirresistant20100416.pdf
11. Hauge SH, Dudman S, Borgen K, Lackenby A, Hungnes O (2009) Oseltamivir-Resistant Influenza Viruses A (H1N1), Norway, 2007–08. Emerg Infect Dis 15: 155-162.
12. Zaraket H, Saito R, Suzuki Y, Baranovich T, Dapat C, et al. (2010) Genetic Makeup of Amantadine-Resistant and Oseltamivir-Resistant Human Influenza A/H1N1 Viruses. J Clin Microbiol 48: 1085-1092.
13. Meijer A, Lackenby A, Hungnes O, Lina B, van der Werf S, et al. (2009) Oseltamivir-Resistant Influenza Virus A (H1N1), Europe, 2007–08 Season. Emerg Infect Dis 15: 552-560.
14. Yang JR, Lin YC, Huang YP, Su CH, Lo J, et al. (2011) Reassortment and Mutations Associated with Emergence and Spread of Oseltamivir-Resistant Seasonal Influenza A/H1N1 Viruses in 2005–2009. PLoS One 6: e18177.
15. Njouom R, Mba SA, Noah DN, Gregory V, Collins P, et al. (2010) Circulation of human Influenza viruses and emergence of Oseltamivir-resistant A(H1N1) viruses in Cameroon, Central Africa. BMC Infect Dis 10: 56.
16. Ramirez-Gonzalez JE, Gonzalez-Duran E, Alcantara-Perez P, Wong-Arambula C, Olivera-Diaz H, et al. (2011) Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus, Mexico. Emerg Infect Dis 17: 283-286.
17. Calatayud L, Lackenby A, Reynolds A, McMenamin J, Phin NF, et al. (2011) Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus Infection in England and Scotland, 2009–2010. Emerg Infect Dis 17: 1807-1815.
18. Sarkar M, Agrawal AS, Dey RS, Chattopadhyay S, Mullick R, et al. (2011) Molecular characterization and comparative analysis of pandemic H1N1/2009 strains with co-circulating seasonal H1N1/2009 strains from eastern India. Arch Virol 156: 207-217.
19. Gohil DJ, Kothari ST, Shinde PS, Chintakrindi AS, Meharunkar R, et al. (2015) Drug susceptibility of Influenza A/H3N2 strains co-circulating during 2009 Influenza pandemic: First report from Mumbai. Infect Genet Evol 29: 75-81.
20. http://www.mohfw.nic.in/WriteReadData/l892s/804456402Categorisation.pdf
21. http://apps.who.int/csr/disease/Influenza/Influenzanetwork/2010_12_06_clinical_specimens_for_virus_isolation_and_virus_for_shipment_from_nic_to_who_collaborating_center.pdf
22. Szretter KJ, Balish AL, Katz JM (2006) Influenza: Propagation, Quantification, and Storage. Curr Protoc Microbiol 15G.1.1-15G.1.24.
23. Hsiung GD, Fong KY (1982) Hemagglutination and the Hemagglutination-Inhibition Test. In: Diagnostic Virology Illustrated by

Light and Electron Microscopy, (3rdedn), Yale University Press, New Haven-London.

24. http://www.who.int/csr/resources/publications/swineflu/GenomePrimers_20090512.pdf
25. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol 30: 2725–2729.
26. Deyde VM, Sheu TG, Trujillo AA, Okomo-Adhiambo M, Garten R, et al. (2010) Detection of molecular markers of drug resistance in 2009 pandemic Influenza A (H1N1) viruses by pyrosequencing. Antimicrob Agents Chemother 54: 1102-1110.
27. Rameix-Welti MA, Enouf V, Cuvelier F, Jeannin P, van der Werf S (2008) Enzymatic Properties of the Neuraminidase of Seasonal H1N1 Influenza Viruses Provide Insights for the Emergence of Natural Resistance to Oseltamivir. PLoS Pathog 4: e1000103.
28. Chittaganpitch M, Waicharoen S, Warachit De silva RJ, Supawat K, Pattamadilok S, et al. (2011) A study of oseltamivir-resistant Influenza viruses in Thailand, 2008-2010. Regional Health Forum 15: 57-62.
29. Centers for Disease Control and Prevention (2009) Update: Drug Susceptibility of Swine-Origin Influenza A (H1N1) Viruses, April 2009.
30. Ottmann M, Duchamp MB, Casalegno JS, Frobert E, Moules V, et al. (2010) Novel Influenza A (H1N1) in vitro reassortant viruses with oseltamivir resistance. Antivir Ther 15: 721-726.