



Drug susceptibility of influenza A/H3N2 strains co-circulating during 2009 influenza pandemic: First report from Mumbai



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ARTICLE INFO

Article history:

Received 2 June 2014

Received in revised form 12 September 2014

Accepted 5 November 2014

Available online 11 November 2014

Keywords:

Influenza
Amantadine
Oseltamivir
Surveillance
Phylogenetic analysis
Mumbai

ABSTRACT

Objective: From its first instance in 1977, resistance to amantadine, a matrix (M2) inhibitor has been increasing among influenza A/H3N2, thus propelling the use of oseltamivir, a neuraminidase (NA) inhibitor as a next line drug. Information on drug susceptibility to amantadine and neuraminidase inhibitors for influenza A/H3N2 viruses in India is limited with no published data from Mumbai. This study aimed at examining the sensitivity to M2 and NA inhibitors of influenza A/H3N2 strains isolated from 2009 to 2011 in Mumbai.

Methods: Nasopharyngeal swabs positive for influenza A/H3N2 virus were inoculated on Madin–Darby canine kidney (MDCK) cell line for virus isolation. Molecular analysis of NA and M2 genes was used to detect known mutations contributing to resistance. Resistance to neuraminidase was assayed using a commercially available chemiluminescence based NA-Star assay kit.

Results: Genotypically, all isolates were observed to harbor mutations known to confer resistance to amantadine. However, no known mutations conferring resistance to NA inhibitors were detected. The mean IC₅₀ value for oseltamivir was 0.25 nM. One strain with reduced susceptibility to the neuraminidase inhibitor (IC₅₀ = 4.08 nM) was isolated from a patient who had received oseltamivir treatment. Phylogenetic analysis postulate the emergence of amantadine resistance in Mumbai may be due to genetic reassortment with the strains circulating in Asia and North America.

Conclusions: Surveillance of drug susceptibility helped us to identify an isolate with reduced sensitivity to oseltamivir. Therefore, we infer that such surveillance would help in understanding possible trends underlying the emergence of resistant variants in humans.

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1. Introduction

Influenza virus belongs to the family *Orthomyxoviridae* with a genome makeup of seven or eight single-stranded, negative-sense RNA segments (Bouvier and Palese, 2008). Influenza A viruses are a major cause of acute respiratory infections, responsible for annual epidemics and irregular pandemics in humans worldwide. These incidences can be attributed to the high frequency of antigenic changes occurring in the major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (Cox and Kawaoka, 1998). Vaccination is the primary approach to control influenza infections

in humans. However, for individuals who have not been vaccinated or when vaccines are not available, it becomes impossible to respond to sudden antigenic variants. At such times, antiviral agents provide an alternate strategy for controlling influenza infections (Deyde et al., 2007; Jonges et al., 2009).

Adamantane derived drugs such as amantadine and rimantadine have been globally used for treatment and prevention of influenza A virus infection. These drugs block the M2 protein, which is the proton channel of the virion. They inhibit the pH change necessary for virus replication and thus prevent release of viral RNA (Lan et al., 2010). The molecular mechanism of viral resistance to these drugs has been well characterized and is associated with various amino acid substitutions at positions L26F, V27A, A30T, S31N or G34E in the transmembrane region of the M2 protein (Belshe et al., 1988; Hay et al., 1986). A significant increase in amantadine resistance among influenza A/H3N2 circulating in Asia, Europe,

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North America, and Australia was noticed in recent antiviral surveillance studies (Bauer et al., 2009). Amantadine resistant in A/H3N2 has increased significantly in India, from 30% in 2004 to 100% in 2009 (WHO, 2010a).

Haffkine Institute for Training, Research and Testing is National Influenza Center under World Health Organization (WHO) for the surveillance of influenza viruses in the Mumbai region. During the influenza season 2009–2011, in the “2009 influenza pandemic period”, co-circulation of 2009 pandemic influenza A/H1N1 and influenza A/H3N2 virus was evident from the epidemiology data generated. As per the CDC interim guidelines for pandemic and seasonal influenza, the use of neuraminidase inhibitors oseltamivir and zanamivir was recommended for patients with severe or high risk of complications and hospitalized patients with suspected or confirmed pandemic H1N1 infection (CDC, 2009). Zanamivir is administered by inhalation while oseltamivir is available as an oral formulation. Neuraminidase (NA) plays a major role in influenza virus propagation (Ferraris et al., 2005). These drugs bind to the highly conserved NA active site, inhibiting enzyme neuraminic acid receptor activity, preventing release of progeny virions budding from infected host cell membranes (Bauer et al., 2009; Ferraris et al., 2005; Okomo-Adhiambo et al., 2010). In the subtype N2 viruses, mutations at catalytic site (R292K) and framework sites (E119V and N294S) in NA residues have been reported in earlier study (WHO, 2012).

As soon as the influenza cases were reported from multiple regions in the country, Ministry of Health and Family Welfare, Government of India initiated guidelines for control of influenza in India (Suri and Sen, 2011). These guidelines recommended antiviral therapy with oseltamivir to all high risk and seriously ill patients (Government of India, 2009). Information on drug susceptibility to amantadine and neuraminidase inhibitors for seasonal influenza A/H3N2 viruses in India is limited, while no published data from Mumbai. Understanding the susceptibility of circulating influenza A viruses to the existing antiviral agents is a crucial objective of influenza surveillance (Jonges et al., 2009). Monitoring resistance to the NA inhibitors is based mainly on testing viruses by using an NA activity inhibition assay in combination with an NA sequence analysis (Sheu et al., 2008).

In the present study, we examined M2 and NA inhibitor sensitivity of influenza A/H3N2 strains isolated from Mumbai during the season of 2009–2011, and determined the phylogenetic relationship between those strains. Neuraminidase susceptibility of isolates was determined using the chemiluminescence based enzyme inhibition assay.

2. Materials and methods

2.1. 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), 100 U/ml Penicillin and 0.5 mg/ml Streptomycin (Hi-Media Laboratories, India). Clinical samples positive for influenza A/H3N2 were inoculated onto confluent MDCK cells with serum free medium containing 2 µg/ml of Tosyl phenylalanyl chloromethyl ketone (TPCK) trypsin and passaged twice to reach sufficient titers. During 2009–2011 influenza season, 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, 2010b). Tissue culture fluid was harvested after observing MDCK cell line for cytopathic effect. Virus stocks were aliquoted and stored at -80°C until use (Balish et al., 2006). The presence of influenza virus in the cell culture supernatant was determined by hemagglutination assay using Guinea pig RBCs (Hirst, 1942; Hsiung and Fong, 1982).

2.2. RNA extraction and 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, Hilden, Germany) according to the manufacturer's instructions. The NA and M genes were amplified using the oligonucleotide primers as described elsewhere (Galiano et al., 2012; WHO, 2011) listed in Table 1. One-Step 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. The 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 min, followed by RT inactivation at 94°C for 2 min. PCR cycling conditions were as follows: 29 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 1 min and 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.

2.3. 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 3730XI Applied Biosystems, USA).

2.4. NA inhibitor

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

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

| Primer | Gene | Binding site (nucleotide position) | Sequence (5'–3') |
|--------------|------|------------------------------------|----------------------------|
| M forward | M | 1–26 | AGCAAAAGCAGGTAGATATTGAAAGA |
| M reverse | M | 1002–1027 | AGTAGAAACAAGGTAGTTTTTACTC |
| NA 1 forward | NA | 1–24 | AGCAAAAGCAGGAGTGAAAATGAA |
| NA 1 reverse | NA | 754–777 | TTAGTATCAGCTTTTTCTGAAGCA |
| NA 2 forward | NA | 387–410 | AGCAAAAGCAGGAGTGAAAATGAA |
| NA 2 reverse | NA | 1081–1104 | ATCCACACGTCATTTCCATCATCA |
| NA 3 forward | NA | 754–777 | TGCTTCAGGAAAAGCTGATACTAA |
| NA 3 reverse | NA | 1424–1447 | TTCTAAAATTGCGAAAAGCTTATAT |

2.5. 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 μ l of half-log dilutions (0.03 to 1000 nM) of NA inhibitor were mixed with 25 μ l of a virus dilution with a HA titer equal to 16 and incubated at 37 °C for 20 min. For negative controls, two-wells contained only assay buffer (instead of NI) and culture medium (instead of virus) were included. Diluted substrate (10 μ l) was added to each well and was incubated at room temperature for 15 min, followed by addition of 60 μ l 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 μ l of diluted virus was mixed with 25 μ l of assay buffer instead of neuraminidase inhibitor. Viruses were diluted fivefold where their signal-to-noise ratio was found to be 40:1. For a negative control, one well contained assay buffer and culture medium.

2.6. 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 the sequences of the M gene obtained in this study are KJ511817–KJ511832, and those for NA gene are KJ511833–KJ511848, and are considered as testing data in further analysis.

2.7. 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. 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, NCBI (<http://www.ncbi.nlm.nih.gov/nucleotide/>). These sequences were included as references in the sequence driven analysis considering it as training data.

Table 2

IC₅₀ value of oseltamivir determined from various virus isolates.

| Influenza virus isolate | Influenza season isolated | Oseltamivir IC ₅₀ (nM) |
|-------------------------|---------------------------|-----------------------------------|
| A/Mumbai/5460/2010 | 2010–2011 | 4.08 |
| A/Mumbai/5257/2010 | 2010–2011 | 0.17 |
| A/Mumbai/5256/2010 | 2010–2011 | 0.17 |
| A/Mumbai/5420/2010 | 2010–2011 | 0.17 |
| A/Mumbai/3983/2009 | 2009–2010 | 0.32 |
| A/Mumbai/261/2009 | 2009–2010 | 0.47 |
| A/Mumbai/3258/2009 | 2009–2010 | 0.21 |
| A/Mumbai/3196/2009 | 2009–2010 | 0.17 |
| A/Mumbai/3150/2009 | 2009–2010 | 0.47 |
| A/Mumbai/3103/2009 | 2009–2010 | 0.17 |
| A/Mumbai/3067/2009 | 2009–2010 | 0.21 |
| A/Mumbai/3021/2009 | 2009–2010 | 0.51 |
| A/Mumbai/2832/2009 | 2009–2010 | 0.24 |
| A/Mumbai/2516/2009 | 2009–2010 | 0.14 |
| A/Mumbai/2559/2009 | 2009–2010 | 0.22 |
| A/Mumbai/2349/2009 | 2009–2010 | 0.14 |

3. Results

A total of 16 isolates (out of 75 samples cultured) of influenza A/H3N2 were obtained and were further characterized using RT-PCR. All the isolates were amplified by their NA and M genes; and their nucleotide sequences were determined.

3.1. Molecular screening of drug susceptibility

Genomic analysis showed that all isolates contained the S31N (serine to asparagine) substitution known to confer resistance to amantadine. None of the other amino acid residues, viz. L26, V27, A30 and G34, exhibited any variation (Bright et al., 2005). Neither of the mutations (R292K, E119V and N294S) that confer resistance to NA inhibitors was detected in any of the isolates.

3.2. Emergence of influenza A/H3N2 with reduced sensitivity to neuraminidase inhibitors after oseltamivir treatment

Susceptibility to oseltamivir for all the isolates was tested using NA Inhibitor chemiluminescence based assay. The IC₅₀ values for oseltamivir were determined by regression analysis using GraphPad Prism Software (Fig. 1). The standard sensitive strain referring as Pune isolate was obtained from National Institute of Virology (NIV) as a control strain. The IC₅₀ value of the control virus was determined to be 0.51 nM. The mean IC₅₀ value (barring the outlier A/Mumbai/5460/2010) for oseltamivir was determined to be 0.25 nM, as summarized in Table 2. In isolate A/Mumbai/

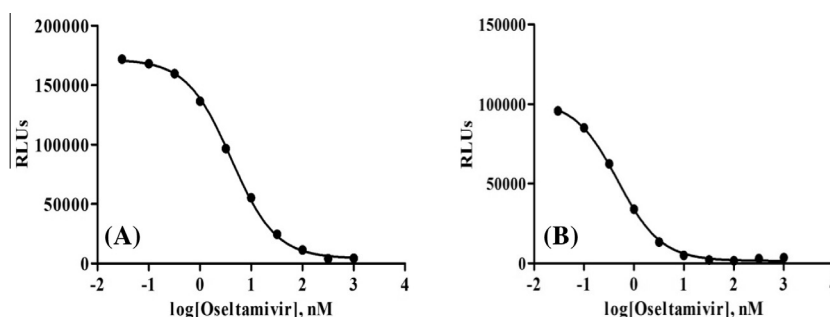


Fig. 1. Assessment of the IC₅₀ for oseltamivir using the chemiluminescent neuraminidase inhibitor assay. (A) Oseltamivir-sensitive A/Mumbai/5460/2010 (H3N2) virus with reduced susceptibility to oseltamivir; (B) Oseltamivir-sensitive A/Mumbai/3150/2009 (H3N2) virus. x axis, oseltamivir concentrations (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.

5460/2010 (H3N2), the IC_{50} value was observed to be 16-fold higher of the mean IC_{50} value. On retrospective investigation, it was observed that this virus was obtained from a 29-year-old resident pathologist who had received oseltamivir immediately after diagnosis. The virus with reduced sensitivity to the neuraminidase inhibitor was isolated from a throat swab on day 5 after initiation of oseltamivir therapy. However, no difference was observed in the amino acid sequence of the neuraminidase protein.

3.3. Phylogenetic analysis of amantadine-resistance

Matrix gene sequences of all influenza A/H3N2 isolates in this study were used to construct the phylogenetic tree. Multiple sequence alignment of these strains revealed that all these sequences were homologous with sequence identity more than 95%, thus Maximum Parsimony (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 60 nucleotide sequences 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 MEGA 6.0.5 (Tamura et al., 2013).

The tree was constructed using reference vaccine strains and reference sequences for the M gene which were obtained from the Influenza virus database treating it as the training data whereas sequenced data was treated as testing data. The phylogenetic analysis reveals two distinct clades of the S31N mutation from 2005 to 2010 (Fig. 2). The first introduction of amantadine-resistance (Sub-clade 1a) observed in these data occurred in Singapore in 2005 [isolate A/Singapore/70/2005(H3N2)]. The emergence of new variant of the influenza virus in the same season may be due to antigenic drift (point mutation) or genetic reassortment. Similar genetic reassortment events were noted in isolates obtained from Thailand and United States in the subsequent influenza seasons of 2006–2008.

The amantadine-sensitive and amantadine-resistant isolates in the first clade were homologous to reference vaccine strains. Indian isolates obtained from Mumbai region also showed close homology with reference vaccine strains. These isolates were further divided into different sub-clades. Sub-clade (1b) comprised of isolates that were antigenically homologous to strains circulating in United States [i.e., A/California/12/2008(H3N2), A/Boston/99/2009(H3N2) and A/Vermont/22/2009(H3N2)] during 2008–2009. Isolates in the sub-clade (1c) were homologous to the strains circulating in United States during influenza season of 2010–2011. The second introduction of S31N appeared from amantadine-resistant isolates circulating in United States in 2008. In clade 2, isolates from Mumbai showed homology with sequences of isolates obtained from multiple regions, circulating globally during 2008–2010.

3.4. Phylogenetic analysis of oseltamivir-sensitivity

Neuraminidase gene sequences of all influenza A/H3N2 isolates in this study were used to construct the phylogenetic tree. Multiple sequence alignment of these strains also had a lower evolutionary divergence. Therefore, MP method was selected for construction of phylogenetic tree, as mentioned earlier for M gene. The tree was constructed using reference vaccine strains and other reference sequences for the NA gene which were obtained from the Influenza virus database. The phylogenetic analysis revealed two distinct clades with isolates from Mumbai deviating completely (Fig. 3). Nine of sixteen Mumbai isolates were categorized in the first clade showing homology to oseltamivir-sensitive reference vaccine

strains. The oseltamivir-sensitive isolates in Mumbai region were antigenically homologous to the isolates recognized in Kolkata during the influenza season of 2009, thus subsiding into the same sub-clade. Indian isolates from Mumbai also exhibited close homology with the strains circulating in United States, Australia and Asia in 2009. In the second clade, isolates from Mumbai were homologous to the reference isolates circulating globally during the influenza season of 2009–2010.

4. Discussion

Antiviral therapy plays an important role in the management of influenza virus infection. Rapid increase in the prevalence of amantadine and oseltamivir resistant influenza A/H3N2 strains has been reported worldwide (Deyde et al., 2007; Okomo-Adhiambo et al., 2010; Bright et al., 2005; Dapat et al., 2010; Nelson et al., 2009; Melidou et al., 2009; Salter et al., 2011).

Since their first appearance in 1997, the percentage of adamantane resistant influenza A/H3N2 viruses has notably increased. During 1997 to 2007, viral isolates sampled from different countries revealed point mutation at position 31 in the M2 protein, conferring resistance to amantadine. In the early 2005, the overall prevalence of adamantane resistance was 11% in Hong Kong, 0.6% in New Zealand/Australia and 1.7% in New York. Subsequently, the emergence of the adamantane resistant strains increased to 88% in Hong Kong, 67% in New York and 38% in New Zealand/Australia (Nelson et al., 2009). In the United States, the incidence of amantadine resistance was reported to have reached 97% to 100% by 2005–2006. By 2007, the S31N mutation was detected in 100% of influenza isolates sampled from multiple Asian countries (Deyde et al., 2007).

Five amino acid substitutions in the M2 protein are associated with adamantane resistance in influenza A viruses (Bright et al., 2005). It is indicated that the presence of S31N is a major determinant for amantadine resistance. In accordance to this, S31N mutation in the present study was consistent with earlier study observations (Bai et al., 2009). The variants containing the S31N substitution might possess an important advantage in viral replication, leading to more efficient circulation (Bai et al., 2009). No reports of prevalence of amantadine resistant influenza A/H3N2 viruses isolated from humans have been reported from Mumbai prior to our study. Mumbai being a major migration hub of people nationally and globally, the infections transferred herein and the circulating strains become critically important to be identified and reported.

The emergence of amantadine resistant influenza A/H3N2 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. 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 (Gregor et al., 2013). Previous studies illustrated that the emergence and spread of amantadine resistance appeared to be a complex process which included geographical variable selection pressures, frequent reassortment and extensive global migration (Nelson et al., 2009). It was also hypothesized that South-East Asia may serve as a reservoir and epicenter for influenza virus activity before disseminating globally to other temperate areas. It also suggested that the Hong Kong strain to be a most probable representative of the South-East Asian region (Russell et al., 2008). Phylogenetic analysis in this study suggest Singapore to be more likely a representative strain for global circulation of influenza A/H3N2 virus in India. The study postulate the emergence of amantadine resistance in Mumbai may be due to antigenic drift or

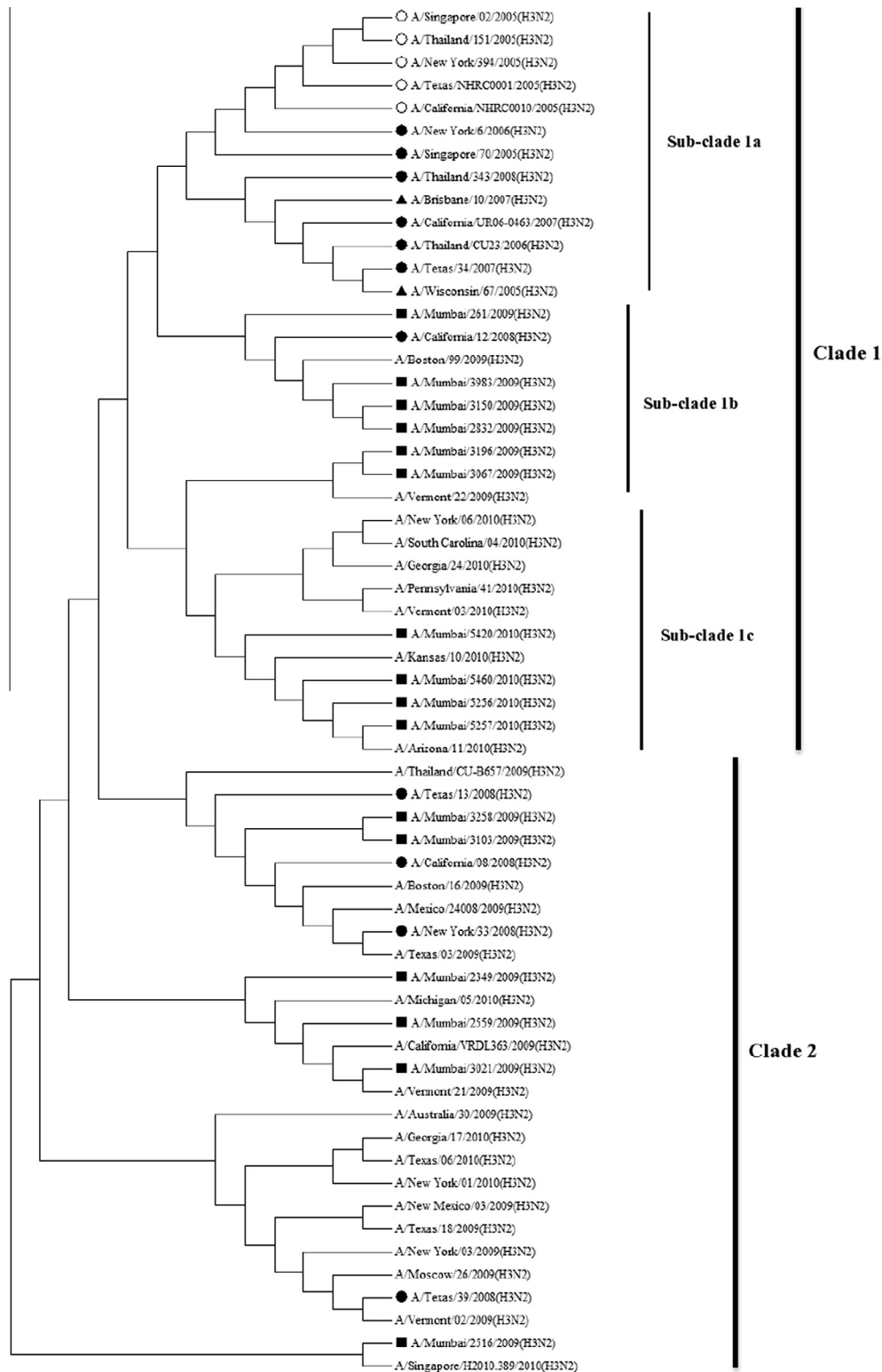


Fig. 2. Phylogenetic tree constructed on the basis on M genes (containing that overlap with the reading frames of M1–M2). The triangle indicates the reference vaccine strains. Amantadine-sensitive reference strains (open circle), Amantadine-resistant reference strains (closed circle) from 2005 to 2008, Amantadine-resistant strains from Mumbai (closed square).

genetic reassortment with the strains circulating in Asia and North America. Yet, intensive sampling from other localities is greatly needed to understand the evolutionary dynamics in Mumbai region.

Due to increase in the incidence of amantadine resistance, neuraminidase inhibitors (NAI) represent the drugs of choice for

antiviral therapy. NAIs inhibit both influenza A and B viruses and are well characterized by low resistance rates. However, specific amino acid substitution at catalytic sites and framework sites may confer drug resistance. NAI susceptibility by fluorescence as well as chemiluminescence enzyme inhibition based assays was applied in previous surveillance studies (Sheu et al., 2008;

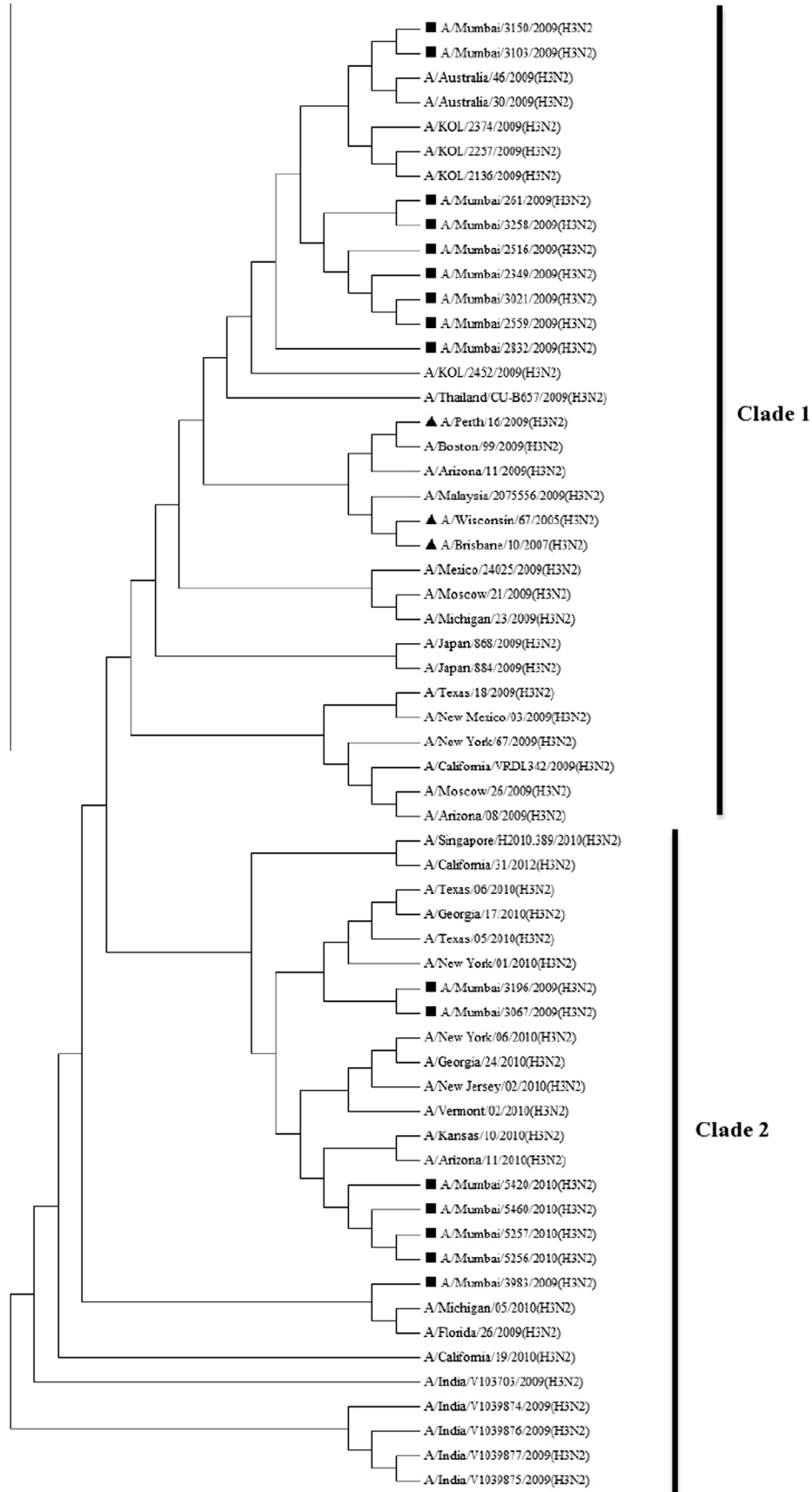


Fig. 3. Phylogenetic tree constructed on the basis on NA genes nucleotides. The triangle indicates the reference vaccine strains. Oseltamivir-sensitive strains from Mumbai (closed square).

McKimm-Breschkin et al., 2003; Monto et al., 2006; Mungall et al., 2004; Wetherall et al., 2003). In the present study a chemiluminescence-based assay was utilized in conjunction with NA sequence analysis. IC₅₀ values of the present study fully correspond with the results of the previous surveillance (Bauer et al., 2009; Sheu et al., 2008). In the present study influenza A/H3N2 strain showed 16-fold reduction in oseltamivir susceptibility although no amino acid substitution was seen in the NA protein. A recent study conducted in Japan revealed emergence of drug resistant influenza virus after treatment with oseltamivir especially in children. It was observed that by day 5–6, influenza A/H3N2 virus isolates displayed more than 100 fold reduction in oseltamivir susceptibility with R292K mutation in the NA gene sequence. The study also describes that relative underdosing of oseltamivir may contribute to post treatment resistance to N2 viruses. Further molecular studies are needed in order to characterize this strain. Thus, antiviral therapy compliance especially during pandemic should be strongly encouraged (Stephenson et al., 2009).

This study additionally attempts to group the oseltamivir-sensitive influenza 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.

5. Conclusion

The present study underlines the importance of continuous monitoring of evolution and drug susceptibility to influenza A virus strains. This study finds that influenza A/H3N2 strains circulating in Mumbai were resistant to amantadine and the single isolate with reduced susceptibility to neuraminidase inhibitor was isolated for the patient undergoing oseltamivir treatment. Continuous surveillance of the circulating influenza virus is essential to monitor the emergence and spread of drug resistance. Thus, monitoring of drug susceptibility to adamantanes and neuraminidase inhibitor is needed along with routine prevalence studies. Such surveillance would help in timely detection and identification of emerging resistant variants in human populations.

Acknowledgements

We acknowledge to National Center for Disease Control (NCDC), New Delhi, for providing us with Madin–Darby canine kidney (MDCK) cell line.

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