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

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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 know mutations conferring resistance to NA inhibitors were detected. The mean IC_{50} value for oseltamivir was 0.25 nM. One strain with reduced susceptibility to the neuraminidase inhibitor (IC_{50} = 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.

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 3730Xl 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	AGTAGAAACAAGGTAGTTTTTTACTC
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	TTCTAAAATTGCGAAAGCTTATAT