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Original Article

Amantadine-Resistant among Seasonal H1N1 and 2009 Pandemic Isolated of Influenza A Viruses in Iran

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Abstract

Background and Aims: Influenza A viruses are important pathogens for humans especially in pandemic episodes. Two adamantane derivates, amantadine and rimantadine, are used for prophylaxis and treatment of influenza A virus infections. However, single amino acid substitutions in the M2 transmembrane domain which lead to amantadine resistance of these viruses occur at residues 26, 27, 30, 31 or 34. Rates of resistant viruses have been increasing globally.

Methods: In this report, 21 specimens of seasonal H1N1 and pandemic influenza A viruses which grew on MDCK cell line were studied for detection of amantadine resistant viruses. After RT-PCR M2 gene of samples were sequenced. In addition, as confirmatory assay, amplification of pandemic influenza A viruses on amantadine treated MDCK cell line and evaluation of TCID50 assay, were accomplished.

Results: All seasonal influenza A viruses were amantadine sensitive but none of the 2009 pandemic influenza A viruses where us none of the 2009 pandemic influenza A virus were sensitive.

Conclusion: Considering emergence of new influenza A virus variant, and resistance to amantadine, it is noteworthy that application of amantadine in new variant A/H1N1 influenza viruses might not be effective.

Keywords: Amantadine; influenza A virus; Iran

Introduction

Influenza A viruses cause respiratory infection during winter epidemics and pandemics (1, 2, 3). These viruses are associated with significant rates of devastating impact on human health and universal economy (2).There are two kinds of medicine that are being used for treatment of influenza A virus infection, which one of them is amantadine compound. Amantadine inhibits the replication of influenza A viruses by blocking the ion channel activity of the M2 protein which prevents the viral uncoating (2, 4-6). Although it has some advantages like decreasing the course of illness and severity of symptoms, amantadine resistant viruses rapidly emerge during treatment as a result of single amino-acid substitutions at position 26, 27, 30, 31 and 34 in the transmembrane domain of the M2 protein (5, 7, 8). During recent decade, it has been shown that the rate of mutation in influenza A/H3N2 is more than A/H1N1 viruses (16). According to the WHO report, 2009 pandemic influenza A virus carry S-31-N substitution. Resistance to this drug has compromised their effectiveness against many

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influenza strains, including 2009 pandemic H1N1 viruses (9). Amantadine resistant influenza A/H3N2 viruses have been detected in Iran (10). In this report, mutation in seasonal influenza A/H1N1 and 2009 pandemic influenza A H3N2 viruses were assessed and compared using TCID50/0.2ml titration method for detection of amantadine resistant influenza A viruses.

Methods

Specimen collection

We studied 14 seasonal influenza A/H1N1 and 7 pandemic influenza A virus isolated from the specimens of patients which were sent to the Iranian National Influenza Center by the Ministry of Health.

RT-PCR and sequencing

Viral RNA was extracted from 150µl of virus culture supernatant of the inoculated MDCK cell culture supernatant, using a Nucleospin Viral RNA extraction kit (MN, Germany). Forward primer was (5'- GGC CAT GGA GGT TGC TAG TC-3') and the reverse one was (5'- CTC TGG TAC TCC TTC CGT AG-3') primers were designed using OLIGO6 software and designated M2F and M2R, respectively. After reverse transcription (RT) by M2F primer for cDNA synthesis, PCR was performed by M2F and M2R primers to amplify a 295 length region in M2 gene from nucleotide positioned at 628 to 923, which contains the coding region for transmembrane domain of the M2 gene. The PCR products were visualized under UV light after electrophoresis with 1.5% agarose. All positive PCR products were subjected to sequencing. The sequences were studied to find probable mutations at positions 26, 27, 30, 31 and 34 in transmembrane region of M2 gene.

Biological assay

The confluent monolayers of the cell cultures were prepared in 96-well tissue culture microplates. The confluent monolayers of cell cultures in one of the series of microplates was pretreared with 2µg/ml of amantadine for 30 min. Tenfold dilutions of viruses in triplicate, containing 2µg/ml of TPCK-trypsin, were added to each of two series of cell monolayers. Following one hour adsorption period, virus medium was replaced by culture medium containing the respective concentration of amantadine. After 48 hour incubation period at 37˚C, virus cytopathic effects (CPE) were recorded and the viral titer was calculated by Reed and Muench method.

Results

We studied 14 isolated of seasonal H1N1 and 7 isolated of 2009 pandemic influenza A viruses; All 21 samples included in this study were positive in RT-PCR assay of M2 gene amplification (figure 1, 2).



Fig. 1. 2009 pandemic influenza A RT-PCR bonds. Lane M: Ladder and Other Lanes: Samples

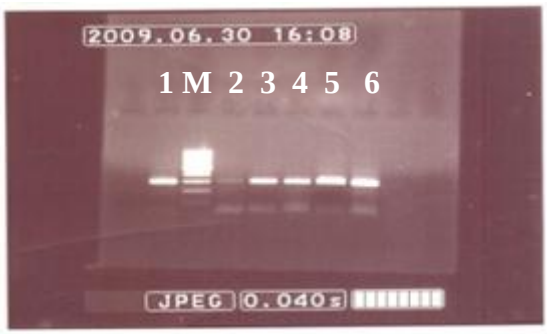


Fig. 2. Seasonal influenza A/H1N1 RT-PCR bonds. Lane 1: Positive Control, Lane M: Ladder and other lanes: Samples

In sequencing survey, none of the the 14 seasonal H1N1 viruses had mutation in the transmembrane domain of M2 gene (fig. 3). All of seven samples of 2009 pandemic

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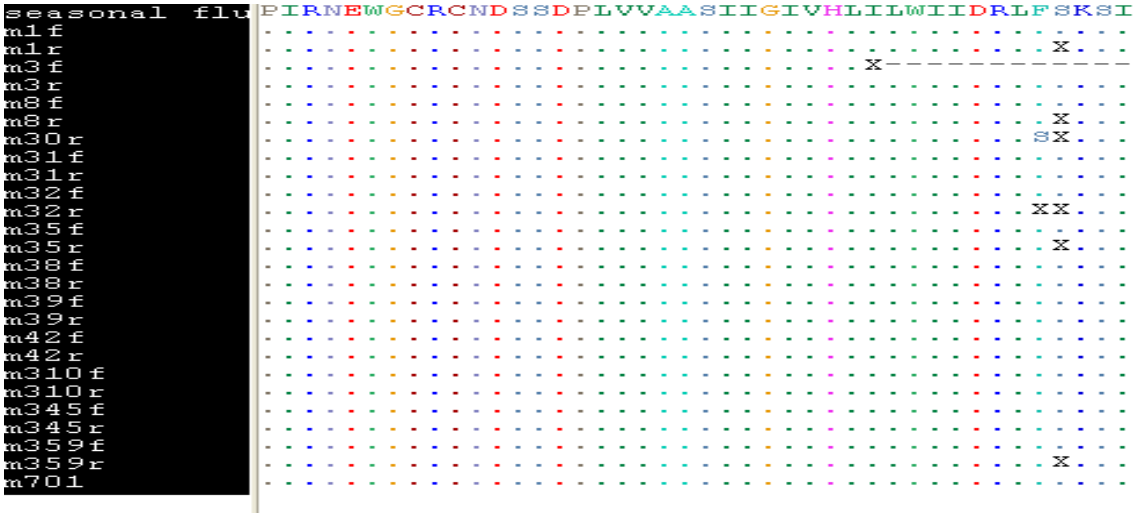


Fig. 3. seasonal influenza A/H1N1 amino-acid sequences.

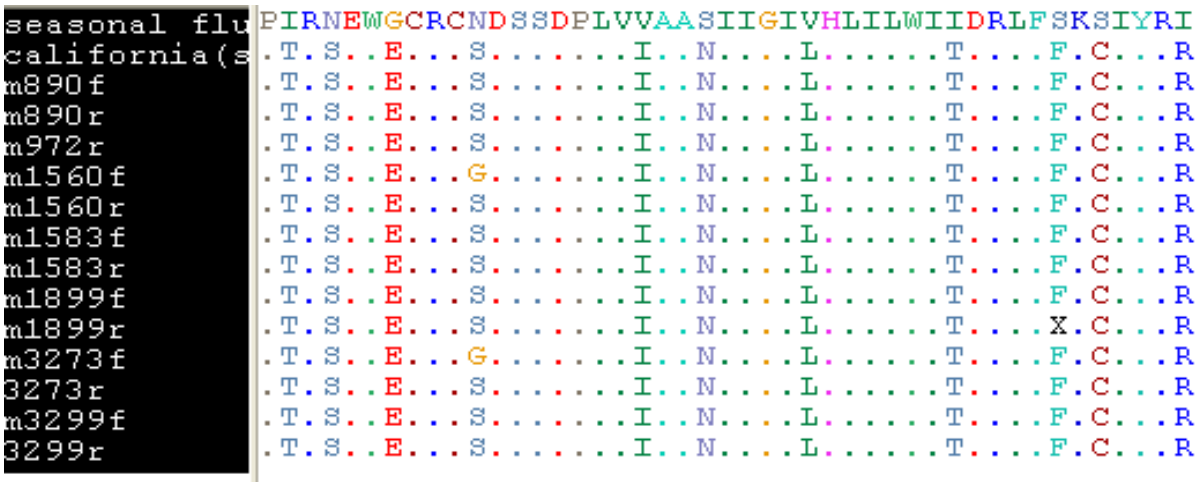


Fig. 4. 2009 pandemic influenza A amino-acid sequences.

Table 1. Amantadine-resistant phenotype of 2009 pandemic influenza A viruses.

Sample's Code	TCID50/0.2ml(Am-)	TCID50/0.2ml(Am+)	phenotype
m890	5.75	5.5	R
m972	5.75	5.5	R
m1560	5.75	5.5	R
m1583	6.5	6.25	R
m1899	6.5	6.25	R
m3273	5.5	5.75	R
m3299	6.5	6.25	R

influenza A viruses the same as WHO report had mutation at position Ser-31-Asn (fig. 4), and in biologic assay their TCID50/0.2ml titer differences in presence or absence of the drug

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were less than 2 fold, which showed these viruses were resistant to amantadine (table1).

Discussion

The homotetrameric M2 channel protein is composed of 97 residues in per subunit. Each unit follows an extracellular N-terminal domain (24 residues), a transmembrane (TM) domain (19 residues), and an intracellular C-terminal domain (54 residues) (11, 6). The viral M2 protein functions as a proton optional channel which is activated by low pH environments as found in endosomes. The main machinery selective proton channel function of M2 is believed to lie within the TM helical bundle that exhibits proton conductive activity (6, 9). In the TM region, the ionizable His37 acts as a proton selectivity with the indole side chain of Trp41 acts as a proton gate in blocking the pore (6, 12, 13). After the virus enters the infected cell by endocytosis, the proton channel acidifies the viral core, which triggers the dissociation of the virus matrix and subsequent viral gene expression (12, 13). Amantadine is used for prophylaxis and treatment of influenza virus infection worldwide, but the emergence of resistant strains of influenza virus, restricted the use of this drug in some cases. It has been shown that amantadine resistant rates among influenza A/H1N1 viruses varies in different regions (14). In this study, no mutation was observed in transmembrane domain of M2 gene in seasonal circulating influenza A/H1N1 viruses in Iran. This may be due to the lack of amantadine treatment of patients in Iran. However, it was shown that there are resistant influenza A/H3N2 viruses with Ser-31-Asn mutation in M2 gene in Iran. In 2009, we found amantadine resistant in all of seven samples and because of circulating of these viruses in the world, we extrapolated that our detection of the mutation at position of Ser-31-Asn is not far from mind. Regarding to emergence of new influenza A viruses, and resistant to amantadine, prevention and treatment of influenza A viruses with amantadine must be limited. Meanwhile monitoring the circulating viruses for drug resistant is necessary because of the potential cause of pandemic with avian influenza A viruses and emergence of amantadine resistant strains (15).

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