

Original Article

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

Ghaderi M¹, Mokhtari-Azad T^{1*}, Shahmehmoodi Sh¹, Samimi-Rad K¹, Yavarian J¹, Naseri M¹, Abbasi S¹, Norozbabaei M¹, Fazeli M²

1. National Influenza Center, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.
2. Department of Virology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

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

*Corresponding author: Talat Mokhtari Azad, Ph.D, Porsina Ave, Keshavarz Blv, Tehran, Iran.
PO: 14155-6446
Tel: (+98) 21 88 96 23 43
Email: mokhtari@sina.tums.ac.ir

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 150 μ l 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 pretreated with 2 μ g/ml of amantadine for 30 min. Tenfold dilutions of viruses in triplicate, containing 2 μ g/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).

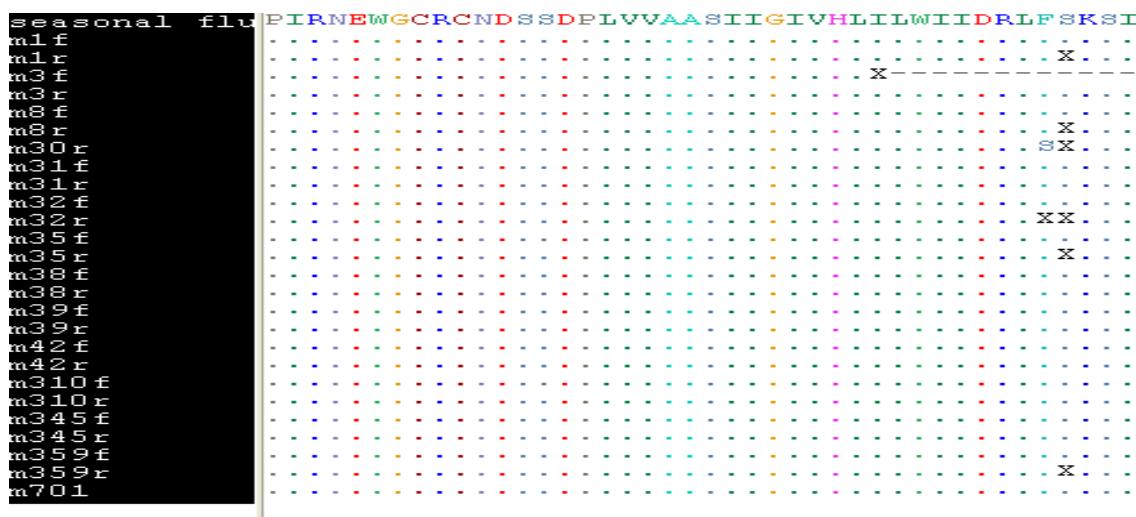
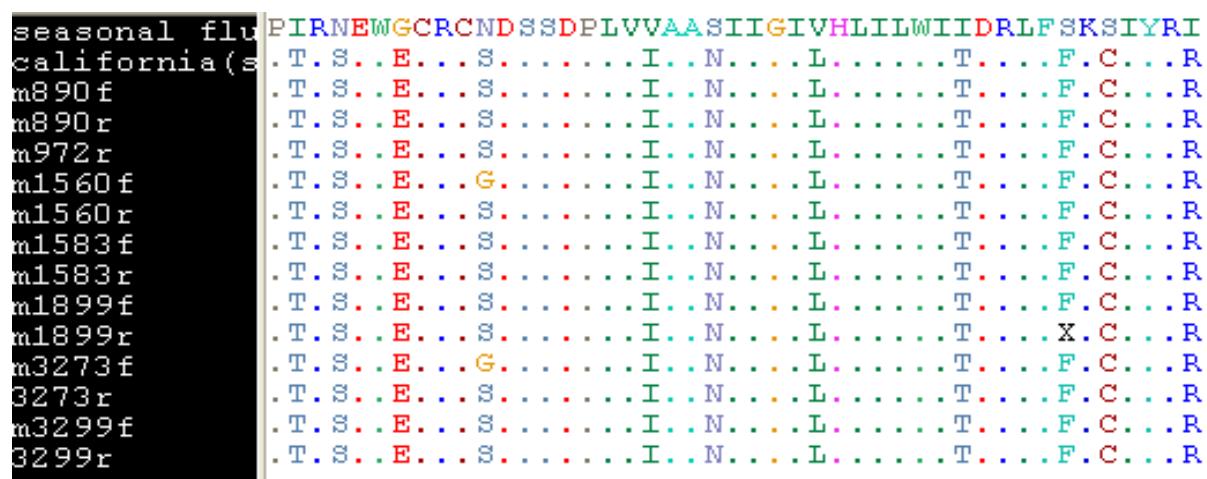


Fig. 1. 2009 pandemic influenza A RT-PCR bands. Lane M: Ladder and Other Lanes: Samples



Fig. 2. Seasonal influenza A/H1N1 RT-PCR bands. 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

**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

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).

Acknowledgement

We thank from those persons in local and state health departments of Ministry of Health and Medical Education (MOHME) of Iran for its assistance in collection of data and respiratory samples.

References

1. Saito R, Li D, and Suzuki Y. High Prevalence of Amantadine-Resistance Influenza A (H3N2) in Six Prefectures, Japan, in the 2005–2006 Season. *Journal of Medical Virology*. 2007; 79:1569–1576.
2. Abed Y, and Goyette N. Generation and Characterization of Influenza A (H1N1) Viruses Harboring Amantadine Resistance Mutations. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*. 2005;8: 556-559.
3. Schmidtke M, Zell R, and Bauer K. Amantadine Resistance among Porcine H1N1, H1N2, and H3N2 Influenza A Viruses Isolated in Germany between 1981 and 2001. *Intervirology*. 2006; 49:286–293.
4. Rick A, Bright, David K, and Shay MP. Adamantane Resistance Among Influenza A Viruses Isolated Early During the 2005-2006 Influenza Season in the United States *JAMA*. 2006; 295:891-894.
5. Hata M, Tsuzuki M, and Goto Y. High Frequency of Amantadine-Resistant Influenza A (H3N2) Viruses in the 2005-2006 Season and Rapid Detection Of Amantadine-Resistant Influenza A (H3N2) Viruses by MAMA-PCR. *Jpn. J. Infect.* 2007; 60:202-204.
6. Intharathep P, and Laohpongspaisan C. How amantadine and rimantadine inhibit proton transport in the M2 protein channel.

Journal of Molecular Graphics and Modelling. 2008; 27:342–348.

- 7. Bright A, Medina M, Xu X, and Perez-Oronoz G. incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 29005: a cause for concern. *Articles*. 2005; 366.
- 8. Masuda H, and Suzuki H. Incidence of Adamantane-Resistance Influenza A Viruses in Sentinel Surveillance Sites and Nursing Homes in Niigata, Japan *Microbiol. Immunol.* 2000; 44(10):833-839.
- 9. Sarah D, Schmidt-Rohr K, and Wang J. Structure of the amantadine binding site of influenza M2 proton channels in lipid bilayers. Vol 463|4 February 2010| doi:10.1038/nature08722.
- 10. Yavarian J, and Mokhtariazad T. Amantadine-resistant influenza A (H3N2) viruses in Iran. *Acta virologica*. 2009; 53:135-138.
- 11. Rafal M. James J. and Chou P. Influenza M2 proton channels. *J. Mol. Biol.* 2010; 4-15.
- 12. Sarah D, Luo W, Hu F, and Hong M. Structure and Function of the Influenza A M2 Proton Channel. *Biochemistry*. 2009;48(31): 7356–7364.
- 13. Sarah D, Tatiana V, and Hong M. Structure of Amantadine-Bound M2Transmembrane Peptide of Influenza A in Lipid Bilayers from Magic-Angle-Spinning Solid-State NMR: The Role of Ser31 in Amantadine Binding. *J. Mol. Biol.* 2009; 385: 1127–1141.
- 14. Barr IG, Deng YM, Iannello P, Hurt AC, and Komadina N. Adamantane resistance in influenza A(H1) viruses increased in 2007 in South East Asia but decreased in Australia and some other countries. *Antiviral Research*. 2008; 200–205.
- 15. He G, Qiao J, and Dong C. Amantadine-resistance among H5N1 avian influenza viruses isolated in Northern China. *Antiviral Research*. 2008; 77:72–76.
- 16. Barr IG, Hurt AC, Deed N, and Iannello P. The emergence of adamantane resistance in influenza A (H1) viruses in Australia and regionally in 2006. *Antiviral Research*. 2007, 75:173–176.