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Amantadine-resistant influenza A (H3N2) viruses in Iran

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Summary. – Adamantanes have been used for the prophylaxis and treatment of Influenza A virus (IAV) infections worldwide. However, they have limited use because of increasing number of resistant viruses during recent years. In investigating the frequency of amantadine-resistant IAVs (H3N2) circulating in Iran in 2005–2008, we found that M2 sequences of recently circulating viruses that were amantadine-resistant contained a Ser31Asn mutation. Thus, adamantanes should not be used for treatment or prophylaxis of recent IAVs (H3N2) infections. In future, their potential use will depend on the resistance of circulating viruses.

Keywords: Influenza A (H3N2) virus; amantadine; resistance; Iranian isolates

Introduction

IAV is a major cause of morbidity and mortality worldwide and its prevention and treatment have become an increasing priority in the world. In addition to annual influenza vaccination, influenza antiviral drug therapy has been shown to be an effective means of preventing and treating IAV infections. Adamantane derivates such as amantadine and rimantadine have been used as antiviral agents against IAV. Amantadine, the first antiviral agent available against influenza was approved by the Food & Drug Administration in 1966 and rimantadine was approved in 1993.

These drugs block ion channel of the influenza A virion and inhibit the pH change necessary for the uncoating process and maturation (Hata *et al.*, 2007; Pinto *et al.*, 2007; Satio *et al*., 2007, 2006; Fehlmann *et al.*, 2005; Wang *et al*., 1993). Antiviral therapy may also play a major role in both treatment and prophylaxis during a pandemic (Hayden *et al.*, 2004, 2001). An influenza pandemic is expected to occur within the next decade, but the emergence of resistance is a matter of concern (Mahbubur *et al.*, 2008).

Amantadine is also licensed for use in the treatment of Parkinson's disease. Resistant influenza viruses have been found everywhere amantadine is used for treating not only influenza, but also Parkinson's disease (Suzuki *et al.*, 2003). The genetic basis for resistance to adamantanes is associated with the amino acid substitutions at positions 26, 27, 30, 31 or 34 in the transmembrane region of M2 protein (Shobugawa *et al.*, 2008; De Clercq *et al.*, 2004; Holsinger *et al.*, 1994; Wang *et al.*, 1993).A mutation at position 31 (Ser to Asn) is the most frequent in amantadine-resistant IAVs (H3N2) (Kitahori *et al.*, 2006).

In this study, we investigated the frequency of amantadine-resistant IAVs (H3N2) in Iran in the years 2005–2008. For this purpose, we performed RT-PCR and sequencing to detect mutations in M2 gene related to the amantadine resistance.

Materials and Methods

Specimens. All clinical samples were collected from individuals diagnosed with influenza-like disease from January 2005 to October 2008. The specimens were provided by Iran Ministry of Health and sent to the National Influenza Centre in Tehran University of Medical Sciences.

RT-PCRs. We performed RNA extraction from the collected samples and used RT-PCR and real-time PCR to detect IAVs. The MDCK cells were inoculated with clinical samples for virus iso-

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Abbreviations: IAV = Influenza A virus; WHO = World Health Organization

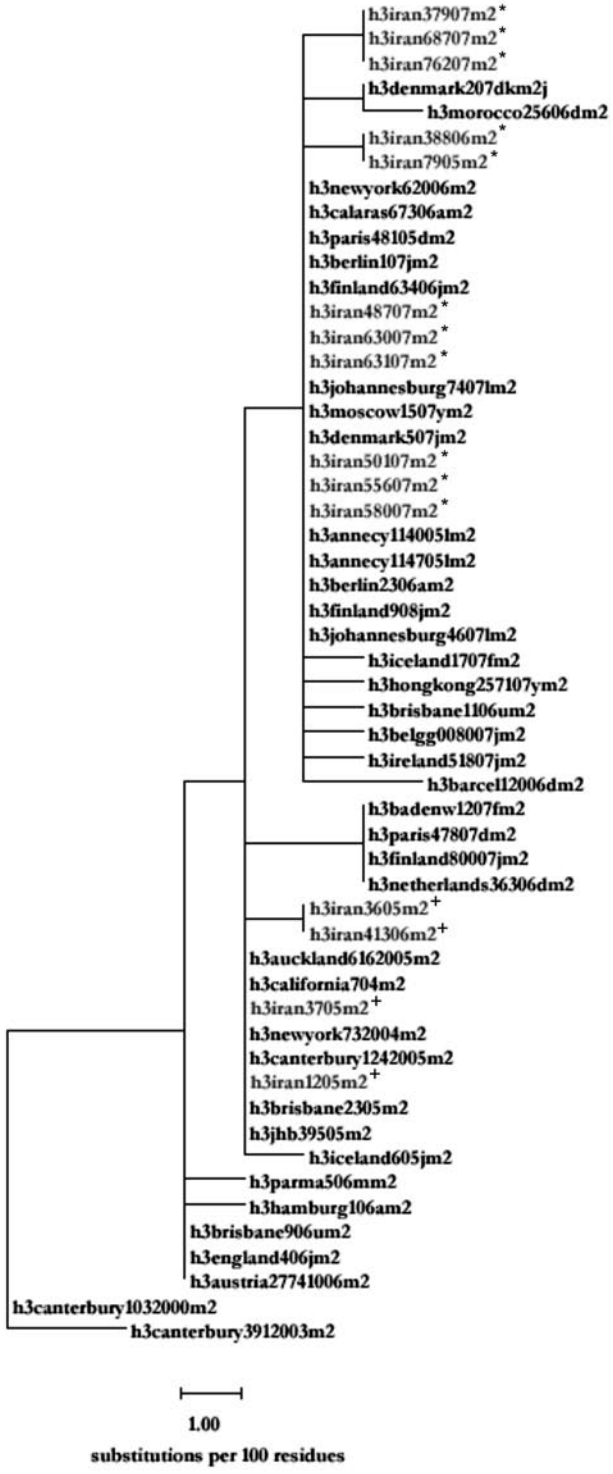

Fig. 1

M2 gene-based phylogenetic tree of Iranian IAVs (H3N2)

Resistant IAVs (*), sensitive IAVs (+).

lation. RNA was extracted from the volume 140 µl of cultivation medium or original specimen using the QIAGEN viral RNA Mini Kit. The cultivation medium was used for samples that multiplied in the cell culture and original specimen was used in the case of samples that did not grow in the cell culture. Then, we performed an RT-PCR with the QIAGEN One Step RT-PCR Kit using the following primers for M gene: Forward primer: 5'-TATTCGTCT CAGGG-3' and reverse primer: 3'-ATATCGTCTCGTATT-5' with 40 amplification cycles consisting of cDNA synthesis (60˚C for 1 min, 50˚C for 30 mins and 95˚C for 15 mins) and DNA amplification (94˚C for 30 secs, 50˚C for 30 secs, and 72˚C for 1 min) with a final extension at 72˚C for 10 mins. PCR products were purified with the GFX PCR DNA and Gel Band Purification Kit.

Sequence and phylogenetic analysis. We carried out sequencing reactions using BigDye Terminator V1.1 cycle sequencing kit with the following program: 25 cycles with denaturation at 96˚C for 30 secs, primer annealing at 50˚C for 15 secs, and extension at 60˚C for 4 mins. The primers used for the sequencing were: 1/ 5'-TATTCGTCTCAGGG-3'forward, 3'-GGCAAGTGCAC CAGCAGAATA-5'reverse and 2/ 5'-GCTAAGGCTATGGAG CAAATGGCT-3'forward, 3'-ATATCGTCTCGTATT-5'reverse. Then, after ethanol precipitation the reactions were resolved on the MEGA BACE sequencing machine at the National Institute for Medical Research, Mill Hill, London, and the results were edited with Staden package. The M2 part of the gene was aligned using the GCG package. A phylogenetic tree was constructed for the M2 gene by means of the neighbor-joining method using the GCG package. The lengths of the horizontal lines are proportional to the numbers of nucleotide differences as indicated by the bar (Fig. 1). All sequences have been deposited in the GenBank database under Acc. Nos. FJ664618 to FJ664632.

Results

Four hundred specimens were collected from January 2005 to October 2008. Fifty specimens (12.5%) were identified as influenza B, 35 specimens (8.75%) as IAV (H1N1) and 15 specimens (3.75%) as IAV (H3N2). In recent years, there has been a high frequency of IAVs (H1N1) and influenza B virus circulation rather than IAVs (H3N2) worldwide. According to the report in WHO Influenza Center in London, from 700 influenza viruses isolated in 30 countries from October 2007 to February 2008, the majority was IAV (H1N1)(77%) and only a few of them (2%) were IAV (H3N2) (Hay *et al.*, 2008). This analysis was based on the M gene sequence.

We found a high frequency of amantadine-resistant strains isolated in recent years. We studied 15 isolated IAVs (H3N2) and 11 of them were resistant and 4 were sensitive to amantadine. All viruses isolated in 2007 were resistant, while one virus from 2006 was resistant and one was sensitive. Viruses obtained in 2005 were mostly sensitive, but surprisingly one strain was resistant. For this resistant virus (A/Tehran/79/05/ H3N2), we had no data about the patient, who might have used amantadine as a treatment for influenza or Parkinson's

Table 1. Amantadine resistance in IAVs (H3N2) isolated in Iran in 2005–2007

Viruses	Date of collection	Amantadine-resistance (R) or sensitivity (S)	aa 31 in M2 protein
A/Tabriz/12/05	25/02/2005	S	Ser
A/Babol/36/05	12/02/2005	S	Ser
A/Babol/37/05	13/02/2005	S	Ser
A/Tehran/79/05	04/01/2005	R	Asn
A/Esfahan/413/06	15/02/2006	S	Ser
A/Boshehr/388/06	15/01/2006	R	Asn
A/Tehran/631/07	02/02/2007	R	Asn
A/Tehran/630/07	02/02/2007	R	Asn
A/Tehran/379/07	12/01/2007	R	Asn
A/Kermanshah/580/07	27/01/2007	R	Asn
A/Kermanshah/687/07	06/02/2007	R	Asn
A/Tehran/487/07	21/01/2007	R	Asn
A/Esfahan/501/07	19/01/2007	R	Asn
A/Tehran/762/07	11/02/2007	R	Asn
A/Tehran/556/07	26/02/2007	R	Asn

Ser = Serine; Asn = Asparagine.

disease. Also, the resistance of this virus may have been acquired by spontaneous mutation or the strain may have been a resistant variant transmitted to the patient. In our study, the predominant mutation was the amino acid change Ser31Asn in the M2 protein. During those three years (2005–2007), we have seen the drug resistance growing rapidly. The increased level of amantadine resistance in IAVs (H3N2) has been reported also in other countries such as China, Hong Kong, and United States (Barr *et al.*, 2007). In the phylogenetic tree, we found that our resistant and sensitive strains were compatible with the other resistant and sensitive viruses worldwide (Fig. 1). The M2 gene from 4 amantadine-sensitive strains fell within the A/California/7/04 sequence that is typical for the majority of viruses circulating in other countries. All of the examined amantadine-resistant viruses grouped into a clade represented by viruses that have been recognized as almost exclusively amantadine-resistant.

Discussion

Amantadine inhibits IAV replication by interfering with the M2 protein ion channel activity (Kitahori *et al.*, 2006; Belshe *et al.*, 1998). Viruses become resistant to amantadine through a single amino acid substitution at positions 26, 27, 30, 31 or 34 in the transmembrane region of M2 protein (Bright *et al.*, 2006; Kitahori *et al.*, 2006; Satio *et al.*, 2002; Holsinger *et al.*, 1994; Wang *et al.*, 1993). A mutation at position 31 (Ser to Asn) is the most frequent in amantadine-resistant IAVs (H3N2) (Holsinger *et al.*, 1994). Resistance to adamantanes develops rapidly, often within the first three days of therapy in approximately one third of patients (De Clercq *et al.*, 2004). In this study, all of the most recently obtained viruses were resistant to these drugs, and all of them had the single amino acid change Ser31Asn. There were no dual point mutations in the M2 gene. The emergence of resistance usually occurs following treatment with amantadine. The striking rise of patients with circulating amantadine-resistant IAVs (H3N2) was reported in Japan, United States, Canada, and various Asian countries (Satio *et al.*, 2008; Bright *et al.*, 2006). The Advisory Committee on Immunization Practices in the Centers for Disease Control and Prevention recommended that neither amantadine nor rimantadine be used for treatment or prophylaxis of IAV in the United States for 2006–2007 (Satio *et al.*, 2002).

The present study provides clear evidence that resistant IAVs are circulating in Iran. In the years 2005, 2006, and 2007 our data indicated that 25%, 50%, and 100% of isolated strains, respectively, were amantadine-resistant. The reason for this rapid increase of resistance is not clear, because amantadine is not routinely used for the prevention or treatment of seasonal influenza in Iran. Thus, further investigations are needed to clarify the genetic characteristics of amantadine-resistance in IAV (H3N2) in Iran.

In the following years, recommendations for adamantane antivirals will be based on the resistance patterns documented in strains circulating during those influenza seasons.

The increase of resistant IAVs incidence is a major problem worldwide and we have to increase a surveillance to find suitable option for the influenza control. Intense planning for the possibility of an influenza pandemic with a virulent strain of H5N1 or another influenza subtype is ongoing at

international, national, and local levels. It is important to analyze the circulating viruses and examine their M genes together with their resistance to adamantanes. In this way we can get the best decision for the prevention and treatment of influenza in the next possible pandemic. Antiviral resistance monitoring should be intensified and maintained for rapid feedback into treatment strategies and selection of alternative therapeutic agents.

This study is the first attempt to evaluate the frequency of amantadine-resistant viruses in field isolates from Iran. Although the body of examined samples was rather small, our results suggested that an increasing rate of resistant strains is a serious problem. In order to control this trend, we strongly support the limitation of amantadine use for the next few years to allow resistant strains to be replaced by the non-resistant IAVs (Barr *et al.*, 2007).

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References

Barr IG, Hurt AC, Iannello P, Tomasov C, Deed N, Komadina N (2007): Increased adamantine resistance in Influenza A(H3) viruses in Australia and neighbouring countries in 2005. Antiviral Res.73, 112–117. doi:10.1016/j.antiviral.2006.08.002 PMid:16963130

Belshe RB, Smith MH, Hall CB, Betts R, Hay AJ (1988): Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J. Virol. 62, 1508–1512.

Bright RA, Shay DK, Shu B, Cox NJ (2006): Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA 295, 891–894. doi:10.1001/jama.295.8.joc60020 PMid:16456087

De Clercq E (2004): Antiviral drugs in current clinical use. J. Clin. Virol. 30, 115–133. doi:10.1016/j.jcv.2004.02.009 PMid:15125867

Fehlmann E, Le Corre N, Abarca K, Godoy P, Montecinos L, Veloz A, Ferrés M (2005): Search of amantadine-resistance in influenza A strains isolated in Santiago, Chile, 2001–2002. Rev. Chilena. Infectol. 22, 141–146.

Hata M, Tsuzuki M, Goto Y, Kumagai N, Harada M, Hashimoto M, Tanaka S, Sakae K, Kimura T, Minagawa H, Miyazaki Y (2007): 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. Dis. 60, 202–204.

Hay AJ, Daniels R, Lin YP, Zheng X, Gregory V, Bennett M, Whittaker L (2008): Characteristics of human influenza A/H1N1,A/H3N2 and B viruses isolated Sep 2007 to Feb 2008.WHO Report

Hayden FG (2004): Pandemic influenza: is an antiviral response realistic? Pediatr. Infect. Dis. J. 23 (Suppl.), S262–S269. doi:10.1098/rstb.2001.1007 PMCid:1088564

Holsinger LJ, Nichani D, Pinto LH, Lamb RA (1994): Influenza A virus M2 ion channel protein: a structure-function analysis. J. Virol. 68, 1551–1563.

Kitahori Y, Nakano M, Inoue Y (2006): Frequency of amantadine-resistant influenza A virus isolated from 2001–02 to 2004–05 in Nara Prefecture. Jpn. J. Infect. Dis. 59, 197–199.

Pinto LH, Lamb RA (2007): Controlling influenza virus replication by inhibiting its proton channel. Mol. Biosyst. 3, 18–23. doi:10.1039/b611613m PMid:17216051

Rahman M, Bright RA, Kieke BA, Donahue JG, Greenlee RT, Vandermause M, Balish A, Foust A, Cox NJ, Klimov AI, Shay DK, Belongia EA (2008): Adamantane-Resistant Influenza Infection During the 2004–05 Season Emerg. Infect. Dis. 14, 173-176.

Saito R, Oshitani H, Masuda H, Suzuki H (2002): Detection of amantadine-resistant influenza A virus strains in nursing homes by PCR- restriction fragment length polymorphism analysis with nasopharyngeal swabs. J. Clin. Microbiol. 40, 84–88. doi:10.1128/JCM.40.1.84-88.2002 PMid:11773097 PMCid:120099

Saito.R, Li D, Shimomura C, Masaki H, Le MQ, Nguyen HLK, Nguyen HT, Phan TV, Nguyen TTK, Sato M, Suzuki Y, Suzuki H (2006): An off-seasonal amantadine-resistant H3N2 influenza outbreak in Japan. Tohoku J. Exp. Med. 210, 21–27. doi:10.1620/tjem.210.21 PMid:16960341

Satio R, Li D, Suzuki H (2007): Amantadine-resistant influenza A(H3N2) virus in Japan,2005–2006. N. Eng. J. Med. 356, 312–313. doi:10.1056/NEJMc062989 PMid:17229966

Saito R, Suzuki Y, Li D, Zaraket H, Sato I, Masaki H, Kawashima T, Hibi S, Sano Y, Shobugawa Y, Oguma T, Suzuki H (2008): Increased Incidence of Adamantane-Resistant Influenza A (H1N1) and A (H3N2) Viruses During the 2006-2007 Influenza Season in Japan. J. Infect. Dis. 197, 630–632. doi:10.1086/525055 PMid:18275281

Shobugawa Y, Saito R, Sato I, Li D, Suzuki Y, Sasaki A, Sato M, Suzuki H (2008): Recurrence and persistence of fever in children who developed amantadine-resistant influenza viruses after treatment. Tohoku J. Exp. Med. 214, 29–138. doi:10.1620/tjem.214.129 PMid:18285670

Suzuki H, Saito R, Masuda H, Oshitani H, Sato M, Sato I (2003): Emergence of amantadine-resistant influenza A viruses: epidemiological study. J. Infect. Chemother. 9, 195–200. doi:10.1007/s10156-003-0262-6 PMid:14513385

Wang C, Takeuchi K, Pinto LH, Lamb RA (1993): Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. J. Virol. 67, 5585–5594.