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Amantadine resistance in relation to the evolution of influenza A(H3N2) viruses in Iran

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ABSTRACT

The aminoadamantanes, amantadine and rimantadine, have been used to prevent and treat influenza A virus infections for many years. Several reports have shown an increased level of resistance to these drugs, particularly among influenza A(H3N2) subtype viruses, during recent years. We observed an increase in amantadine resistance, due to a Ser31Asn mutation in the M2 channel protein, among A(H3N2) viruses circulating in Iran during 2005–2007. Sequence analyses of the haemagglutinin and neuraminidase genes as well as the M gene of these viruses revealed that the emergence of resistance was in general consistent with the progressive worldwide evolution of H3N2 viruses.

1. Introduction

Influenza A viruses cause annual epidemics of disease resulting in high levels of morbidity and mortality worldwide (Nicholson et al., 2003). Thus, prevention and treatment is a high priority. Besides annual vaccination for preventing influenza infections, antiviral drug therapy is an effective means of preventing and treating influenza infections, particularly during the early stages of an emergent pandemic. The aminoadamantanes, amantadine and rimantadine, have been licensed in many countries for use in prevention and treatment of influenza A infections (Hayden, 2006; Hayden and Pavia, 2006). They block the M2 ion channel protein, a minor component of the virus membrane, which is encoded by a spliced mRNA derived from genome segment 7, and inhibit pH changes necessary for the uncoating of the virus and the release of viral RNA into the cytoplasm of infected cells (Hay, 1992; Pinto and Lamb, 2007). They are economic and chemically stable, but more frequent emergence of resistance has become a matter of serious concern (Bright et al., 2005; Saito et al., 2007; Suzuki et al., 2003). When infections caused by resistant and sensitive viruses have been compared, they have been observed to cause similar symptoms and illness duration (Shobugawa et al., 2008; Sweet et al., 1991).

The molecular basis of resistance to amantadine and rimantadine is well understood and involves mutations in the M2 ion channel protein (Belshe et al., 1988; Hay et al., 1985). Single amino acid substitutions at positions 26, 27, 30, 31 or 34 in the transmembrane region of the M2 proton channel cause loss of inhibition of proton flux, either by preventing binding of the drug or by changing the structure of the channel, thereby abrogating inhibition of virus replication (Astrahan et al., 2004; Cady et al., 2010; Chizhmakov et al., 1996; Wang et al., 1993).

Mutation causing substitution of serine by asparagine at position 31 (Ser31Asn) has most frequently been responsible for amantadine resistance of A(H3N2) viruses (Furuse et al., 2009b; Nelson et al., 2009). Another mechanism whereby drug resistance may emerge is genetic reassortment between sensitive and resistant viruses.

The reduction in the proportion of resistant viruses during 2006–2007 was associated with the emergence of drug-sensitive variants, which acquired, by genetic reassortment, sensitive M genes, close to those of amantadine-sensitive variants previously prominent during 2005–2006 (Furuse et al., 2009a; Hay et al., 2008). Alternatively, drug resistance mutations in the M2 gene may occur spontaneously, as previously reported for some earlier influenza A (H1N1) viruses, such as A/WSN/33 and A/PR/8/34, which were resistant to amantadine before the drug was developed. Influenza A viruses have two prominent surface glycoproteins, the haemagglutinin (HA) and neuraminidase (NA), which are important for attachment, infection, pathogenesis and spread of the viruses. Some previous studies have suggested a relationship between amantadine resistance and changes in the haemagglutinin associated with antigenic drift (Inoue et al., 2007).

A preliminary report (Yavarian et al., 2009) described the detection of amantadine-resistant influenza A(H3N2) viruses isolated in Iran from 2005 to 2007, despite the lack of use of amantadine for

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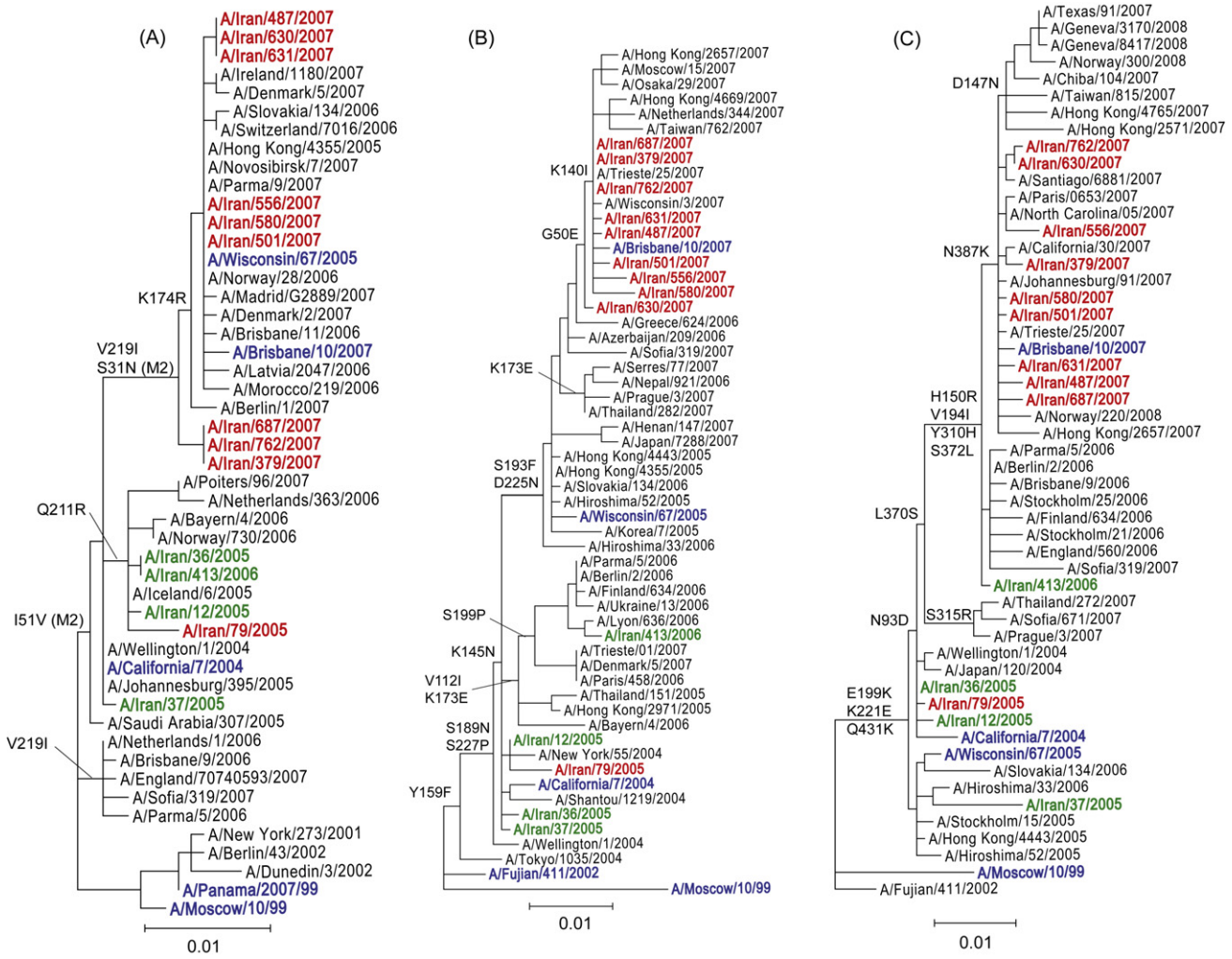

Fig. 1. Phylogenetic comparisons of M (A, nucleotides 298–915), HA (B, nucleotides 175–1030) and NA (C, nucleotides 43–1386) genes of Iranian H3N2 viruses with those of viruses circulating in other parts of the world. Iranian amantadine-sensitive and -resistant viruses are highlighted in bold italics or green and bold or red, respectively, and prototype vaccine strains are in capitals or blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

treatment or prophylaxis in the Iranian population. To understand how they may have arisen, in this study we have compared the sequences of the HA and NA genes, as well as the M genes, to investigate the relationships between changes in the Iranian isolates and the evolution of contemporary H3N2 viruses.

2. Materials and methods

2.1. Virus detection and isolation

Throat swabs or wash specimens were collected from patients with influenza-like illness who were admitted to state health care units and hospitals in all parts of Iran, and sent under the national surveillance system to the WHO Influenza Centre in the Tehran University of Medical Sciences. Virus-positive samples were identified by RNA extraction and real-time PCR and inoculated onto MDCK cells for virus isolation.

2.2. RNA extraction and RT-PCR

RNA from the positive A(H3N2) specimens was extracted from 140 μl of each sample by using a QIAGEN viral RNA Mini Kit. We used either the supernatant of cell cultures in which the isolates grew, or the clinical sample, if no virus was isolated.

RT-PCR was done with a QIAGEN One Step RT-PCR Kit using primers for M, HA or NA genes: M forward primer, 5′-TATTCGTCTCAGGG-3′ and M reverse primer, 3′-ATATCGTCTCGTATT-5′ with 40 amplication cycles consisting of cDNA synthesis (60 °C for 1 min, 50 °C for 30 min and 95 °C for 15 min) and DNA amplification (94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min) and final extension at 72 °C for 10 min. Using these primers 7 samples were positive, but 8 were negative. We then did hemi-nested RT-PCR on the PCR reactions from the negative samples. We used the previous reverse primer with a new internal forward primer: 5′-GCTAAGGCTATGGAGCAAATGGCT-3′ and Platinum-PFX enzyme, with 40 amplification cycles consisting of denaturation at 95 °C for 5 min, primer annealing at 55 °C for 30 s, extension at 72 °C for 2 min and a final extension step at 68 °C for 10 min. All reactions gave products.

For HA and NA we did One Step RT-PCR in 2 pieces. For HA we used primers: (F6)5′-AAGCAGGGGATAATTCTATTAACC-3′ and (R1075) 3′-GTTTCTCTGGTACATTCCGC-5′ for one fragment and (F567) 5′-CTGAACGTGACTATGCCAAACAAT-3′ and (R1765) 3′-AACCGTACCAACCRTCCACCATTC-5′ for the second fragment.

For NA, primers (F1) 5′-AGCAAAAGCAGGAGTGAAAATGAA-3′ and (R1104) 3′-ATCCACACGTCATTTCCATCGTCA-5′ were used for the first fragment and for the second, (F387) 5′-CATGCGATCCTGACAAGTGTTATC-3′ and (R1447) 3′-

TTCTAAAATTGCGAAAGCTTATAT-5′. For HA and NA, the amplification cycles were the same as for the M gene and good products were obtained for all samples. PCR products were purified with GFX PCR DNA and Gel Bond Purification Kit. (In the primer names, F and R stand for forward and reverse and the numbers indicate the first nucleotide of the primer sequence.)

2.3. Sequencing

Sequencing reactions were done using a BigDye Terminator V1.1 Cycle Sequencing Kit with the following program: 25 cycles of denaturation at 96 °C for 30 s, primer annealing at 50 °C for 15 s and extension at 60 °C for 4 min. The primers used for sequencing were: for One-step RT-PCR M gene products: (1) 5′-TATTCGTCTCAGGG-3′, (2) 3′-GGCAAGTGCACCAGCAGAATA-5′, (3) 5′-GCTAAGGCTATGGAGCAAATGGCT-3′, and (4) 3′-ATATCGTCTCGTATT-5′; for hemi-nested M gene PCR products: 5′-GCTAAGGCTATGGAGCAAATGGCT-3′, 3′-AGAAACAAGGTAGTTTTTTACTC-5′ and 3′-ATATCGTCTCGTATT-5′.

The primers used for HA & NA sequencing were the same as those used to generate the PCR products. After ethanol precipitation, the reactions were resolved on a MegaBACE 1000 DNA sequencer. M (M1 and M2), HA and NA gene sequences were edited and analysed using the Wisconsin Sequence Analysis package (GCG), version 10. All sequences have been deposited in the GenBank database under Acc. Nos. FJ664618–FJ664632 and FJ769866–FJ769915.

2.4. Phylogenetic analysis

Phylogenetic trees of M, HA & NA genes (Fig. 1) were constructed using maximum parsimony (PAUP, version 4.0; Sinauer Associates, Sunderland, MA).

3. Results and discussion

Of 14 influenza A(H3N2) viruses, isolated from 2005 to 2007, 10 possessed an amantadine resistance mutation, all of which resulted in the amino acid substitution Ser31Asn in the M2 protein (Table 1), that has been identified most frequently in recent amantadine-resistant A(H3N2) viruses. Most of the viruses isolated in 2005–2006 lacked such a mutation; only one of the four viruses isolated in 2005 was resistant, while the only H3N2 virus detected in 2006 lacked any amantadine resistance mutation. All nine viruses from 2007 were amantadine-resistant. This increase in the frequency of amantadine resistance of H3N2 viruses during these 3 years corresponds to that reported in other countries, such as China, Hong Kong, and the United States (Barr et al., 2007; Deyde et al., 2007).

Table 1
Amino acid changes observed in the M1 and M2 proteins of influenza A(H3N2) amantadine-resistant and -sensitive viruses.

Virus	Date of collection	Amino acid change			
		M1			M2
		174	211	219	31
A/Iran/12/05	25/02/2005	K	R	V	S
A/Iran/36/05	12/02/2005	K	R	V	S
A/Iran/37/05	13/02/2005	K	Q	V	S
A/Iran/79/05	04/01/2005	K	R	V	N
A/Iran/413/06	15/02/2006	K	R	V	S
A/Iran/631/07	02/02/2007	R	Q	I	N
A/Iran/630/07	02/02/2007	R	Q	I	N
A/Iran/379/07	12/01/2007	K	Q	I	N
A/Iran/580/07	27/01/2007	R	Q	I	N
A/Iran/687/07	06/02/2007	K	Q	I	N
A/Iran/566/07	26/02/2007	R	Q	I	N
A/Iran/501/07	19/01/2007	R	Q	I	N
A/Iran/762/07	11/02/2007	K	Q	I	N
A/Iran/487/07	21/01/2007	R	Q	I	N

Molecular analyses of the haemagglutinin and neuraminidase genes of the resistant and sensitive viruses were therefore done to compare the Iranian isolates with contemporary A(H3N2) viruses isolated in other countries. The phylogenetic relationships of the HA, NA and M genes were compared to understand more clearly the evolutionary relationships between the viruses (Fig. 1). In addition, the amino acid sequences of the proteins were compared, particularly in terms of differences in HA and NA (Table 2) which distinguish major antigenic/genetic variants of H3N2 viruses circulating during that period (Lin et al., 2010). The HAs of the four 2005 isolates fell within the A/California/7/2004 clade, typical of the majority of A/California/7/2004-like viruses circulating in other countries at that time. The NA and M genes of three of the viruses were also similar to each other and closely related to those of A/California/7/2004, although the M genes fell within a subgroup characterized by the amino acid substitution Gln211Arg (Q211R), while the NA and M genes of A/Iran/37/05 were somewhat more distantly related. The amantadine-resistant A/Iran/79/05 was similar in all three genes to the two Iranian amantadine-sensitive viruses. Since other similar contemporary viruses were in general sensitive, resistance of A/Iran/79/05 may have been acquired by spontaneous mutation rather than representative of a minor population of circulating resistant variants. The HA of A/Iran/413/06

Table 2
Amino acid changes observed in the HA and NA of influenza A(H3N2) amantadine-resistant and -sensitive viruses.

Virus	Amino acid change											
	HA						NA					
	50	140	193	225	375	450	150	194	310	370	372	387
A/Iran/12/05	G	K	S	D	N	R	H	V	Y	L	S	N
A/Iran/36/05	G	K	S	D	N	R	H	V	Y	L	S	N
A/Iran/37/05	G	K	S	D	N	R	H	V	Y	L	S	N
A/Iran/79/05	G	K	S	D	N	R	H	V	Y	L	S	N
A/Iran/413/06	G	K	S	D	D	R	R	I	H	S	L	N
A/Iran/631/07	E	I	F	N	D	K	R	I	H	S	L	K
A/Iran/630/07	E	K	F	N	D	K	R	I	H	S	L	K
A/Iran/379/07	E	I	F	N	D	K	R	I	H	S	L	K
A/Iran/580/07	E	I	F	N	D	K	R	I	H	S	L	K
A/Iran/687/07	E	I	F	N	D	K	R	I	H	S	L	K
A/Iran/566/07	E	I	F	N	D	K	R	I	H	S	L	K
A/Iran/501/07	E	I	F	N	D	K	R	I	H	S	L	K
A/Iran/762/07	E	I	F	N	D	K	R	I	H	S	L	K
A/Iran/487/07	E	I	F	N	D	K	R	I	H	S	L	K

was closely related to a variant group of viruses, represented by A/Berlin/2/06, prevalent in some other countries and which were in general sensitive to amantadine (Lin et al., 2010). The NA, like that of A/Berlin/2/06, was more closely related to those of the later 2007 isolates, whereas the M gene was similar to those of the 2005 isolates, reflecting the genetic diversity, due to genetic reassortment, among different variants. None of the viruses fell within the amantadine-resistant A/Wisconsin/67/05 clade, prevalent in other parts of the world during 2005 and 2006, possibly due to the time of isolation and small sample size of Iranian viruses.

The three genes of the 2007 amantadine-resistant viruses grouped into the clades represented by A/Brisbane/10/07, which have been observed to be comprised almost exclusively of amantadine-resistant viruses; with the exception of A/Iran/630/07, the HAs possessed the clade-defining amino acid change K140I.

Although these observations agree in general with the suggestion of Inoue et al. (2007) that the two amino acid substitutions in the HA of A/Wisconsin/67/2005-like viruses, Ser193Phe (S193F) and Asp225Asn (D225N), may have contributed to the increased frequency of resistance among these viruses, the emergence of sensitive viruses during 2006-7, particularly within the A/Nepal/921/2006 clade, illustrates the potential importance of genetic reassortment in determining drug resistance.

This study was the first attempt to evaluate the frequency of amantadine-resistant viruses among field isolates in Iran and their relationship to contemporary viruses circulating in other countries. Although the sample size was small, the results indicate that, with the possible exception of A/Iran/79/2005, the spectrum of amantadine susceptibility of Iranian viruses circulating between 2005 and 2007 was similar to that observed among H3N2 viruses in other parts of the world. With the recent emergence of amantadine-resistant pandemic H1N1 viruses and the lack of effect against influenza B viruses, it is evident that amantadine and rimantadine currently have little role in combating influenza in the human population.

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