Molecular evaluation of M2 protein of Iranian avian influenza viruses of H9N2 subtype in order to find mutations of adamantane drug resistance

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Abstract:

BACKGROUND: The H9N2 subtype of influenza A viruses is considered to be widespread in poultry industry. Adamantane is a group of antiviral agents which is effective both in prevention and treatment of influenza A virus infections. These drugs inhibit M2 protein ion channel which has role on viral replication. **OBJECTIVES:** The main objective of this study is to evaluate M gene of avian influenza viruses (AIVs) of H9N2 subtype in order to find adamantane drug resistance mutations. **METHODS:** Over 100 suspected samples were collected from different geographical regions of Iran during 2012-2013. Samples were injected via allantoic sac of 9-11 day-old chicken embryos. A total of 11 out of 100 were AIV. The H9N2 subtype was confirmed by specific RT-PCR. The RT-PCR was conducted for full length M gene. PCR amplified products were purified and then conducted for commercial direct sequencing. Finally, sequences were checked for possible sites of adamantane resistance mutations. **RESULTS:** Overall, 8 out of 11 viruses harbored the adamantane resistance-associated mutations. Of which, four viruses were isolated in 2013 and four viruses in 2012. Two different resistance-associated mutations were observed during different years. **CONCLUSIONS:** The present study provided clear evidence concerning resistance AIVs of H9N2 subtype that were circulating in Iranian poultry sector. This concern is always present as M segment might be introduced into human influenza viruses by reassortment phenomenon.

Introduction

Disease of poultry caused by type A influenza viruses in the family Orthomyxoviridae has been recognized since the late 19th century, causing mainly sporadic but serious outbreaks in poultry industry (Swayne et al. 2013). These viruses are divided into two subtypes based on their two surface glycoproteins including hemagglutinin (HA) and neuraminidase (NA) (Zambon, 1999). Sixteen HA subtypes (H1-16) and nine NA subtypes (N1-9) are recognized among avian influenza viruses (AIVs) (Swayne et al. 2013). From late on, H9N2 subtype of influenza A viruses have been considered to

be widespread in poultry industry (Peiris et al. 1999). The H9N2 subtype is known to be a cause of subclinical and clinical diseases with mild to severe manifestations in respiratory system associated with egg decline, diarrhea and renal disorders (Swayne et al. 2013; Davison et al., 1999; Shalaby et al., 1994).

First isolation and characterization of H9N2 subtype of influenza A in Iran was conducted by Vasfi Marandi and Bozorgmehri Fard in 1998 (Vasfi Marandi et al., 2002). Nowadays, several researchers are working on different aspects of H9N2 subtype because of its importance both in public health and poultry sector (Bashashati et al., 2013; Emadi Chashmi et al., 2013; Nili and Asasi, 2003; Homayounimehr et al., 2010; Karimi et al., 2004).

Type A viruses consist of 8 segmented negative strand genome (Lamb et al. 2001; Swayne et al. 2013). Both matrix and membrane proteins encode by M gene of 7th segment. This gene has 1027 bp in which nucleotide positions of 26-784 encode M1 and positions of 26-51 and 740-1007 encode M2 protein. The M2 is a tetramer membrane protein where locates intra the viral envelope and projects from the surface of the virus. The M2 protein comprises 3 parts of extracellular, transmembrane and cytoplasmic domain (97 amino acids) (Furuse et al., 2009). Transmembrane domain of M2 is a proton channel which balances pH of viral membrane during cell entry and also during viral maturation, and equilibrates pH across the trans-Golgi membrane of infected cells.

Adamantane is a group of antiviral agents which is effective both in prevention and in treatment of influenza A virus infections (Saito et al., 2002). During early stage of infection, these drugs inhibit virus replication by blocking proton flow through M2 ion channel and suppressing viral ribonucleoprotein complex release into cytoplasm of infected cells (Iwahashi et al., 2001; Kitahori et al., 2006). In general they prevent virus replication within the infected cell.

Mutation which causes substitution of Aspargine to Serine at position 31in M2 protein is responsible for all resistant viruses (Iwahashi et al., 2001; Bright et al. 2006). Furthermore, single amino acid substitution of residue 26, 27, 30, 31, 34 or 38 over helical turns of M2 transmembrane domain lead to drug resistance (Lee et al. 2008; Ilyushina et al., 2005; Schnell et al., 2008; Saito et al., 2007; Shiraishi et al., 2003).

The objective of present study was evaluation of AIVs of H9N2 subtype isolated from Iranian poultry sector during 2012 and 2013 to identify single amino acid mutations responsible for adamantane drug resistance.

Material and Methods

Virus isolation: Over one-hundred tissue samples were collected from different geographical regions of Iran during 2012-2013 and stored at -70˚C. These samples include of trachea, lung and cecal tonsil of susceptible birds to avian influenza.

Samples were prepared by procedure described by Swayne, et al.'s 2008 method. Four 9-11 days-old chicken embryos were used for each sample. Samples were injected via allantoic sac. Inoculated embryos were incubated at 35-37˚C for 5 days. Eggs were candled daily and died embryos were collected. After 5 days all live embryos were killed by chilling up to 4˚C. Amnioallantoic fluids of dead embryos were collected for hemagglutinating activity (HA) test. The HA positive samples were confirmed by

Hemagglutinin and Nuaraminidase specific primers of H9 and N2 subtypes with RT-PCR (Lee et al., 2001; Qiu et al., 2009). A total of eleven positive AIVs of H9N2 Subtype samples were detected for further tests.

Viral RNA extraction and cDNA synthesis: Viral RNA was extracted by RNX-PlusTM solution (Cinaclon® ,Iran) as indicated on manufacturer's instructions. cDNA was prepared with the following method: 10 µl of prepared RNA was mixed with 2 µl of 20 pmol oligonucleotide influenza universal primer,Uni12 (5′-AGCRAAAGCAGG-3′). The mixture was incubated at 65˚C for 5 min. Then, mixture was put on ice and 2 µl Reaction buffer 10X, 1 µl ribonuclease inhibitor (Fermantase®, Canada), 2 µl dNTP 10 mM and 1 µl M-MULV reverse transcriptase enzyme (Fermantase®, Canada) were added. The reaction was carried at 42˚C for 60 min and was terminated by heating at 70˚C for 5 min.

Polymerase chaise reaction (PCR) amplification: Amplification of M gene was performed by universal primers as follows: Bm-M-1F: 5′- TATTCGTCTCAGGGAGCAAAAGCAGGTAG-3′ and Bm-M-1027 R: 3′-ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT-5′. Mentioned primers amplified complete M gene segment of avian influenza with length of 1027 bp (Hoffmann et al., 2001).

A 50 µl total volume of master mix contained: 5 µl PCR buffer 10X, 20 pmol of each forward and reverse primer (2 µl), 2 µl dNTP 10 mM, 4 µl MgCl2 50 mM, 0.5 µl Pfu DNA polymerase (Fermentase, Canada) and 2 µl cDNA samples. The amplification protocol was: one step denaturation at 95˚C for 3 min, 35 cycle of 94˚C for 15 sec, 58˚C for 30 Sec, 72˚C for 1 min and final extension at 72˚C for 5 min (Hoffmann et al., 2001). A volume of 10µ of PCR products were electrophoresed on 1% agarose gel in 1x TBE buffer near 100bp Marker, and visualized by ethidium bromide staining. After ethidium bromide staining, the gels were photographed under UV light with a Gel Doc system (VilberLourmat Inc. Cedex, France).

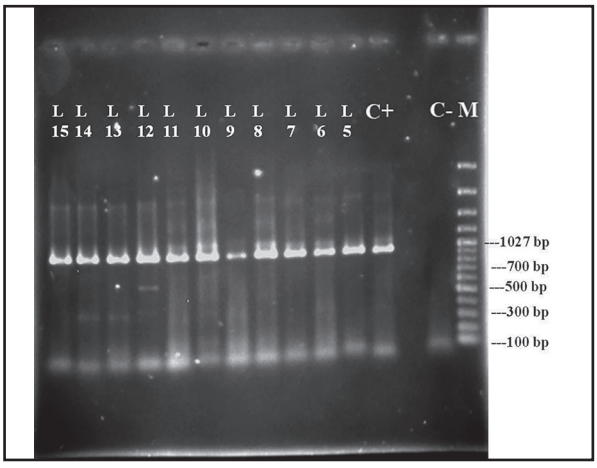

Figure 1. Illustration of gel electrophoresis result of amplified M gene fragments of Iranian avian influenza H9N2 subtype isolates . M=Marker (Ladder) 100bp, L=Lanes, C=Control.

The DNA sequencing: PCR amplified products were purified by QIA quick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and then conducted for commercial direct sequencing service by Takapouzist Co. (Bioneer, Republic of Korea).

Bioinformatics analysis: The obtained sequences were analyzed and trimmed by FinchTV software (http://www.geospiza.com/finchtv) and then registered in NCBI. Nucleotide sequences were translated to protein sequences, comparatively aligned and their variations evaluated by taking advantage of Bioedit version 7.7.9 software. Finally, sequences were checked for possible sites of adamantane resistance mutations including L26F, A30T, A30S, A30V,S31N, G34E and L38F.

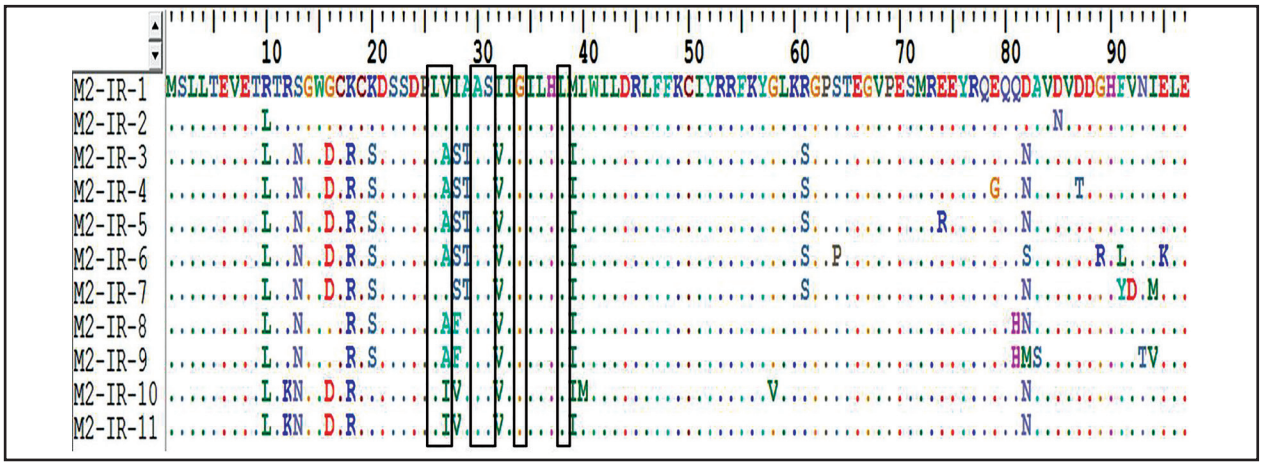

Figure 2. Demonstration of complete M2 amino acid sequences of avian influenza H9N2 subtype by multiple sequence alignment (MSA). Results are illustrated and edited by Bioedit 7.7.9 . Conserve, semi-conserved and variable regions along M2 protein could be seen in this figure. All residues that are identical to the top sequences in an alignment as a dot ('.') used. Rectangular shape showing sequences with possible sites of adamantane resistance mutations.

Results

RT-PCR analysis and sequencing results: Complete M gene was amplified by PCR and electrophoresis results depicted in Fig. 1. The RT-PCR products of H9N2 isolates showed the same length (approximately 1027bp) at electrophoresis. Briefly, lane M is Molecular weight marker (100 bp) and lanes 5-15 are specific molecular sized 1027 bp amplicons representing M gene obtained from Iranian avian influenza H9N2 subtype.

Alignment and comparison of sequencing results: After sequencing, M gene sequences chromatograms were analyzed by FintchTV software for quality checking and editing. Nucleotide sequences of complete M2 gene were translated to protein sequences, and comparatively aligned. Results (Fig.2) revealed limited variations of M2 gene of Iranian avian influenza H9N2 subtype and these limited variations are due to the conserve nature of M gene.

Then, analysis across 6 possible mutation sites related to adamantane resistance were shown by rectangular shape/marking. Details will be described later in Table 1.

Adamantane resistance mutations analysis: The M2 sequence of 11avian influenza (H9N2) isolates collected during 2012-2013 in Iran were obtained. A total of 6 out of 11 (54.5%) harbored the adamantane resistance-associated V27A mutation and 2 (18.2%) harbored V27I. Of these, 4 (66.6%) viruses showed V27A were isolated in 2013 and 2 (40%) viruses of V27A in 2012. All mutation V27I belonged to 2012 isolates . No mutations were observed in all other adamantine resistance associated sites including L26F, A30T, A30S, A30V,S31N, G34E and L38F (Fig. 2 and Table 1). Also, there were significant variations in amino acid 28 and 82 (Table 2).

Discussion

Current study aimed to evaluate M2 protein of Iranian avian influenza H9N2 subtype in order to find mutation of adamantane drug resistance. Under field conditions, sequenced isolates caused considerable complexities in affected poultry farms such as significant respiratory complications.

Sequence analysis of the M gene of these

Table 1. Observed Adamantane Resistance Mutations in M2 protein of Iranian avian influenza H9N2 subtype during 2012 and 2013. 1- L26F; 2- [a]V27A, [b]V27I; 3- A30T, A30S, A30V; 4- S31N; 5- G34E; 6- L38F.

Year	2013							2012				
Isolated Virus	M2-IR-1	M2-IR-2	M2-IR-3	M2-IR-4	M2-IR-5	M2-IR-6	M2-IR-7	M2-IR-8	M2-IR-9	M2-IR-10	M2-IR-11	Reference
AA Code												
26 1	-	-	-	-	-	-	-	-	-	-	-	Iwahashi et al., 2001; Shiraishi et al., 2003; Saito et al., 2007; Ilyushina et al., 2005; Lee et al., 2008; Schmidke et al., 2006
27 2	-	-	+a	+ a	+ a	+ a	-	+ a	+ a	+ b	+ b	Iwahashi et al., 2001; Shiraishi et al., 2003; Saito et al., 2007; Ilyushina et al., 2005; Lee et al., 2008; Schmidke et al., 2006; Cheung et al., 2006
30 3	-	-	-	-	-	-	-	-	-	-	-	Iwahashi et al., 2001; Shiraishi et al., 2003; Saito et al., 2007; Ilyushina et al., 2005; Lee et al., 2008; Schmidke et al., 2006
31 4	-	-	-	-	-	-	-	-	-	-	-	Iwahashi et al., 2001; Shiraishi et al., 2003; Saito et al., 2007; Ilyushina et al., 2005; Lee et al., 2008; Schmidke et al., 2006
34 5	-	-	-	-	-	-	-	-	-	-	-	Iwahashi et al., 2001; Ilyushina et al., 2005; Lee et al., 2008; Schmidke et al., 2006
38 6	-	-	-	-	-	-	-	-	-	-	-	Lee et al., 2008; Schmidke et al., 2006

Table 2. Amino acid variation at different sites of M2 protein in H9N2 subtype viruses isolated in 2012-2013.

Site Number	Amino acid Variation
16	Glycine, Aspartic acid
20	Serine, Lysine
28	Isoleusine, Serine, Phenyalanine, Valine
29	Alanine, Threonine
61	Serine, Argenine
82	Aspartic acid, Aspargine, Methionine, Serine

viruses revealed that six out of eleven isolates harbored the adamantane resistance associated V27A and two harbored V27I mutations. Also, there were significant variations in amino acids 28 and 82 among analyzed sequences.

Yavarian et al., in two different studies in 2009 and 2010, reported an increase in amantadine resistance, due to a S31N mutation in the M2 protein, among influenza A H3N2 isolates circulating in Iran during 2005-2007 and 2005-2008 (Yavarian et al., 2009 and 2010). Ilyushina et al., 2005 by analysis of north America and Asian isolates showed diversity of adamantane resistant mutants among AIVs in different positions of V27A, A30S, S31N (Ilyushina et al., 2005). Saito et al., 2003 found that the overall frequencies of amantadine-resistant strains were 29.6% (24 of 81) during the 1999-2000 influenza season and 23.3% (7 of 30) during the 2000-2001 influenza season. They also found dominant amino acid substitution residues differ significantly with the subtype: namely, V27A in H1N1 strains and S31N in H3N2 strains (Saito, et al., 2003). Barr et al., 2007 investigated 102 (H3) and 37 (H1) influenza viruses isolated at WHO Reference Research Center of In-

fluenza in Melbourne. 43% of H3 and 0% of H1 subtypes showed S31N resistance mutation. They concluded, despite the introduction of neuraminidase inhibitors, amantadine and rimantadine continue to be used for the treatment or prevention of influenza A, mainly due to their low cost (Barr et al., 2007). Higgins et al., 2009 found that from 49 H3N2 subtype isolates 46 carried a point mutation in the M gene coding for the M2 protein which would be associated with amantadine resistance (S31N and V27A) (Higgins et al., 2009). Schmidke et al., 2005 reported a total 3 of 12 isolates show S31N and V27A major substitution in resistant isolates of H1N1, H1N2 and H3N2 subtypes (Schmidke et al., 2006).

Antiviral resistance analysis of M2 protein sequences of H9N2 subtype from United Arab Emirates from 2000 to 2003 revealed that 6 viruses carried a V27A substitution at position 27 and only one virus possessed S31N at position 31. Then, testing resistance against amantadine quantitatively, by using serially increasing concentrations of the drug showed all the Dubai isolates had a high level of resistance to amantadine in cell culture (Aamir et al., 2007).

Therefore, the main substitution position of adamantane resistance of Iranian viruses circulating between 2012 and 2013 in present study is similar to that observed among H9N2 isolates reported before from the United Arab Emirates.

Kitahori et al., 2006 isolated H3N2 and reported 3.4% of isolates revealed adamantane resistance mutations at position 31 (S31N), 26 (L26F) and 27 (V27I) (Kitahori et al., 2006). Pabbaraju et al., 2008 conducted a study to find adamantane resistance in circulating human influenza from 1970 to 2003 in Canada. A total of 73 (23%) of the 317 influenza A H3 subtype viruses tested contained one or two amino acid substitutions in the M2 protein known to cause resistance to adamantanes. Most of the resistant isolates showed S31N mutation and few of them showed V27I substitution. No resistance was observed in the influenza A H1 subtype viruses tested. Their results showed a significant increase in adamantane resistance levels in circulating influenza A H3 viruses from December 2005 to March 2006 (Pabbaraju et al., 2006). Tang et al., 2008 reported overall 58 out of 281 H3N2 subtype isolates collected during 1997-2006 in Hong Kong harbored the adamantane resistance-associated S31N mutation. One of these S31N-containing isolates also harbored the V27I mutation (Tang et al., 2008). Here, in our results two isolates showed mutation on V27I which is compatible with their results.

In conclusion, the present study provided clear evidence that resistant AIVs of H9N2 subtype were circulating in Iranian poultry sector. We suggest adamantane resistance may arise due to selection pressure of illegal drug usage. A high frequency of adamantane drug resistance may cause a problem in environment and human health care system. This concern is always present as M segment may be introduced to human influenza virus by reassortment. This fact may cause special concern with regard to pandemic highly pathogenic influenza viruses.

Furthermore, results of present study help to make us awareness about resistance to adamantane drugs in Iranian poultry farms and to avoid the arbitrary use of adamantane to treat and prevent infections from avian influenza. Finally, it seems practical tests of the drug influence in-vivo and in-vitro, can ensure effective use of the drugs and their

probable resistance.

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ارزیابی مولکولی پروتئین M۲ ویروس‌های آنفلوانزای تحت تیپ H۹N۲ ایران به منظور ردیابی موتاسیون‌های مقاومت به داروهای گروه آدمانتان

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چکیده

زمینه مطالعه: ویروس‌های آنفلوانزای A تحت تیپ H۹N۲ دارای گسترش جهانی می‌باشند. آدمانتان‌ها گروهی از داروهای ضدویروس می‌باشند که هم در پیشگیری و هم درمان عفونت‌های ناشی از ویروس‌های آنفلوانزای تیپ A کاربرد دارند. این داروها کانال یونی پروتئین M۲ را مهار کرده و از این طریق بر تکثیر ویروس تأثیر می‌گذارند. **هدف:** هدف اصلی این مطالعه ارزیابی ژن M ویروس‌های آنفلوانزای پرندگان تحت تیپ H۹N۲ به منظور ردیابی موتاسیون‌های مقاومت به داروهای گروه آدمانتان می‌باشد. **روش کار:** در طول سال‌های ۲۰۱۲ تا ۲۰۱۳، بیش از ۱۰۰ نمونه مشکوک به آنفلوانزا از مناطق مختلف جغرافیایی ایران جمع‌آوری شد. نمونه‌ها پس از آماده سازی از طریق کیسه آلانتوئیک به جنین‌های ۱۱-۹ روزه ماکیان تلقیح شدند. ۱۱ نمونه ویروس آنفلوانزای پرندگان بودند. تحت تیپ H۹N۲ ویروس‌های جدا شده توسط تست RT-PCR اختصاصی تأیید شدند. سپس کل ژن M جدایه‌ها توسط RT-PCR اختصاصی تکثیر شده و برای تعیین توالی نوکلئوتیدی ارسال شدند. نهایتاً، سکانس‌های بدست آمده جهت ارزیابی وجود موتاسیون‌های مقاومت مورد بررسی قرار گرفتند. **نتایج:** ۸ نمونه از ۱۱ نمونه مورد بررسی دارای موتاسیون‌های مقاومت دارویی بودند. از این تعداد ۴ ویروس در سال ۲۰۱۳ و ۴ ویروس در سال ۲۰۱۲ جدا شده بودند. سایت‌های متفاوت موتاسیون در سال‌های مختلف مشاهده شد. **نتیجه‌گیری نهایی:** مطالعه اخیر شواهدی محکمی از حضور مقاومت دارویی در ویروس‌های آنفلوانزای تحت تیپ H۹N۲ در حال گردش در صنعت طیور ارائه نموده است. از اینرو نگرانی خطر بازآرایی ژنتیکی و انتقال قطعه M ژنوم ویروس‌های آنفلونزای طیوری و انسانی وجود دارد.

واژه‌های کلیدی: مقاومت به داروهای آدمانتان، ویروس آنفلوآنزای پرندگان،، پروتئینM۲، H۹N۲، طیور

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