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The sequence analysis of M2 gene for identification of amantadine resistance in avian influenza virus (H9N2 subtype), detected from broiler chickens with respiratory syndrome during 2016-2018, in Isfahan-Iran

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ABSTRACT. Amantadine and rimantadine are used for prevention and treatment of influenza A virus (IAV) infection. The rates of resistant IAVs have been increasing globally. However, amino acid substitutions in the M2 transmembrane channel lead to amantadine resistance. The residues of 26, 27, 30, 31 or 34 are marker of amantadine resistance in IAVs. In this study, 15 pooled tracheal samples collected from 15 chicken farms with severe respiratory sign and mortality in 2016-2018. After identification of influenza A and H9 subtype, the 1027 bp fragment of M gene was sequenced for molecular evaluation of amantadine resistance in AIV strains. Results showed 12 out of 15 pooled samples were positive for IAV and H9 subtype. Based on M2 gene analysis, 8 out of 12 (66.66%) were resistance to amantadine. Four out of 8 (50%) showed S31N substitution (serine to asparagine) and four out of 8 (50%) have V27A substitution (valine to alanine). There was no dual amantadine resistance mutation in any specimens. In conclusion, the emergence of amantadine resistance variants of AIV in Iran, can raise concerns about controlling of the seasonal and the future pandemic influenza. Therefore, greater caution is needed in the use of adamantanes.

Keywords: Avian influenza; M gene; amantadine resistance.

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Introduction

Avian influenza virus (AIV) belongs to the *orthomyxovirus* family. The virus has a negative single-stranded RNA with eight gene segments, namely PB2, PB1, PA, HA, NP, NA, M, and NS that comprise 12 genes. One of the potential characteristics of the virus is the mutation and recombination that can occur slowly by continuous genomic and antigenic changes that have led to the formation of different subtypes (Suarez & Sims, 2013). Considering the significant threat that influenza A viruses pose to global economy and human health, preparedness for a potential influenza pandemic is a global priority (Dong et al., 2015). Influenza A viruses cause epidemics and pandemics by antigenic drift and antigenic shift, respectively. Antigenic drift is due to an accumulation of point mutations leading to minor and gradual antigenic changes. Antigenic shift involves major antigenic changes by introduction of new subtypes into the human population (Kayal, Brundha, & Sivaswamy, 2021). These mutation high rates occur because of the lack of proofreading ability in RNA viruses and it is common in several of these viruses. Unlike antigenic drift, common in several viruses, influenza A viruses (IAV) display a rare ability to undergo antigenic shift. This process, also known as reassortment, occurs occasionally when segments of RNAs from two different strains of IAV infect the same cell allowing their rearrangement into a new viral strain. Since the majority of humans do not have immunity to such novel subtypes, the morbidity and mortality impacts of pandemic influenza can be much higher than those of seasonal influenza (Rosário-Ferreira, Preto, Melo, Moreira, & Brito, 2020).

Avian influenza (AI) is a serious viral respiratory disease in the poultry, which causes annual economic losses all over the world. The influenza A can cause acute respiratory disease in birds and in some mammals, such as humans. Based on biological components, subtypes are classified into two highly pathogenic and low

pathogenic AIVs (Arab, Gholami-Ahangaran, & Jafarian-Dehkordi, 2017). The H9N2 strain of avian influenza virus has spread to Iran since June 1998. Although the virus according to biological test and sequencing of the surface antigen of hemagglutinin was classified to low pathogenic AIV (Vasfi Marandi, Bozorg Mehrifard, & Hashemzadeh, 2010), since entrance to Iran caused the high mortality and economic losses to the poultry industry (Toroghi & Momayez, 2006).

The genome of the influenza virus is fragmented and consists of eight distinct monocular RNA molecules with negative sense (Suarez et al., 2013). In Iran, studies on the antigenic diversity of this virus have focused on shifted antigen changes in both the 4 and 6 RNA sequences that code the surface antigens of hemagglutinin and neuraminidase (Bashashat, Vasfi-Marandi, & Saboury, 2013). There is little information about the antigenic diversity of other RNAs encoding other proteins. The matrix gene (M) of the influenza virus consists of 1027 nucleotides, which include two subunits M1 (nucleotide sequences 26 to 784) and M2 (26 to 51 and 740 to 1007). These components contain 252 and 97 amino acids, respectively. The M gene is intriguing because it encodes both matrix and membrane proteins, and has multiple functions (Gholami-Ahangaran, Salehi, Karimi-Dehkordi, & Azizi, 2020). M1 plays a vital role in assembly by recruiting the viral components to the site of assembly and essential role in the budding process including formation of viral particles. Extracellular domain of M2 is recognized by hosts' immune system. Transmembrane domain of M2 has ion channel activity, which involved in un-coating process of the virus in cell. Amantadine inhibits virus replication by blocking the acid-activated ion channel. The cytoplasmic domain of M2 interacts with M1 and is required for genome packaging and formation of virus particles (Furuse, Suzuki, Kamigaki, & Oshitani, 2009). By this fact that M protein involves (coded by genomic segment 7) in the mechanism of immunization and pathogenicity of AIV (Pielak & Chou, 2011; Tang, Zaitseva, Lamb, & Pinto, 2002), the evaluation of molecular characteristics can draw a new horizon for future genomic studies. In this regard, the rapid and sensitive RT-PCR method was utilized to detect AIV infection based on M gene amplification. In this study, by determining the M gene sequence of AI H9N2 strains, their molecular characteristics were compared with other reference strains in world gene bank.

Material and methods

Sampling

The tracheal samples were collected from 15 chicken farms with mortality above 1% in day for at least 5 days with severe respiratory signs. Totally, 80 tracheal samples were collected from 15 chicken farms (at least 5 samples from each farm). The samples preserved in -70ºC until experiment.

DNA extraction

The samples that collected from each farm pooled in one sample. The DNA was extracted from tracheal samples with AccuPowe® DNA extraction kit (BioNeer corporation, South Korea), according to manufacture instruction.

cDNA preparation

the cDNA was synthesized using AccuPowe® RT PreMix kit (BioNeer corporation, South Korea) according to instruction of manufacturer. Briefly, 5µL of total RNA and 20 pmol of each specific primer was used for cDNA preparation.

RT-PCR

To identify avian influenza virus by RT-PCR, we used specific primers based on conserved sequences of the NP gene of viruses of avian, human, swine and equine origin as described previously (Lee, Chang, Shien, Cheng, & Shieh, 2001) to amplify 330 bp fragment of the gene of the influenza A virus.

The NP-specific primers, we used in this study are NP 1200 (forward): 5- CAG(A/G)TACTGGGC(A/T/C)ATAAG(A/G)AC-3, NP1529 (reverse): 5- GCATTGTCTCCGAAGAAATAAG-3

For identification of H9 subtype in AI positive samples, we used one pair of primers based on conserved sequence of H9 AI subtype as described previously (Lee et al., 2001), to amplify 488 bp fragment of the H9 gene of the influenza A virus.

For PCR reaction, the AccuPowe® PCR PreMix kit (BioNeer corporation, South Korea) was used with a 20 µL reaction mixture containing 1.5 mM Mgcl2, 30 mM KCl, 10 mM tris-HCl, 250 µM (each) dNTP and 1 U DNA Polymerase (BioNeer corporation, South Korea). Five µL of cDNA and 10 pmol of each primer were used in each reaction.

The PCR condition for amplification of NP gene was 45°C for 45 min. (reverse transcriptase), 94°C for 3 min., 35 cycles of 94°C for 60 sec. (denaturation), 55°C for 40 sec. (annealing) and 72°C for 40 sec. (extension), followed by 72°C for 10 min. (final extension). The PCR condition for H9 amplification was the same as above, except that the annealing temperature was reduced to 53°C. The characterization of primer for detection of H9 subtype (Lee et al., 2001) was presented in Table 1.

The PCR products were resolved in 1% (w/v) agarose gel containing ethidium bromide and visualized under UV light.

After identification of H9N2 subtype of AIV, the RT-PCR reaction was performed to amplify a fragment of the AIV M gene. The primer used in this study is described in Table 2. The length of the amplified fragment is about 1027 bp of the M gene (Hoffmann, Stech, Guan, Webster, & Prezz, 2001).

Table 1. The characteristics of the primer used to amplify the NP and H9 genes of avian influenza.

Gene	Specify	Primer Sequence (5-3)	Length of fragment (bp)
NP	Influenza A	CAG(A/G)TACTGGGC(A/T/C)ATAAG(A/G)AC GCATTGTCTCCGAAGAAATAAG	330
H9	H9 subtype	CT(C/T)CACACAGA(A/G)CACAATGG GTCACACTTGTTGTTGT(A/G)TC	488

Table 2. The characteristics of the primer used to amplify the M gene of avian influenza.

Reference	Target gene	Amplified fraqment length (bp)	Primer sequence	Primer name
Hoffmann et al., 2001	M	1027	F:5'AGCAAAAGCAGGTAG3' R:5'AGTAGAAACAAGGTAGTTTTT3'	Bm-M-1 (F) & Bm-M-1027R (R)

The RT-PCR reaction was utilized using 10 µL RT-PCR reaction (with magnesium chloride), DTT 2.5 µL, dNTPs 1 µL, forward primer 2 µL, reverse primer 2 µL (10 pmol each primer), 1 µL enzyme mix, 4 µL template RNA, sterilized distilled water (27.5 µL) in final volume of 50 µL.

The PCR reaction was followed by reverse transcription of RNA. The cDNA synthesis was done at 45°C for 45 min., primary denaturation at 94°C for 3 min., denaturation at 94°C for 60 sec., annealing at 48°C for 60 sec., extension at 68°C for 60 sec. and final extension at 68°C for 10 min. In this study, the process of denaturation, annealing, and extension was repeated for 35 cycles.

In this study, a 100 bp marker was prepared from Fermentas company (Germany) and the final amplified product was detected and analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide.

The PCR product belonging to H9N2 strains of AIV was sequenced to analyze the nucleotide and amino acid sequences of the M gene. For this, the PCR products were purified with a commercial DNA purification kits (Roche, Germany) from the agarose gel according to the company's instruction. The extracted product together with the general primers of the AIV and the specific M gene used in PCR, were sent to MWG-Biotech company (Germany). Strain sequencing was done directly (DACS) and was done bilaterally (Gholami-Ahangaran, Shoushtari, & Charkhkar, 2008).

To phylogenetic analyzing, the nucleotide sequence of the M gene was first compared to the sequence of other viruses registered in the gene bank using the BLAST search in the EMBL / GenBank gene (WWW.NCBI.nlm.nih.gov/BLAST). After identifying the sequence similarity of sequences with other gene bank viruses, reference strains were selected for further analysis. The sequence of the strains was analyzed with the DNAStar software package (DNAStar Inc., Madison, WI, USA). The sequencing of the strains edited with the Edit Sequence software to match the length of the sequences of all strains. Then, the nucleotide sequences translate into the corresponding amino acid sequence for increasing the validity. The nucleotide and amino acid sequences were aligned and analyzed by MegAlign software (version 5) using the Clustal W method (Gholami-Ahangaran, Salehi, Karimi-Dehkordi, & Azizi, 2020).

Results

From 15 pooled tracheal samples that collected from 15 chicken farms, 12 pooled samples were belonged to the A group of AI and H9N2 subtype. The 1027 bp fragment of M gene was amplified in all samples (Figure 1). Comparison of nucleotide and amino acid sequences of recent H9N2 AIV strains indicates that recent strains have most homology with previous Iranian AIV strains, which in each case is more than 90%.

Comparing the amino acid sequence of the recent strains in positions 26, 27, 30, 31 and 34 of the M2 protein (determining the resistance to amantadine) showed that in 8 from 12 pooled samples possessed genomic mutation that represented amantadine resistance (66.66%). 4 strains (50%) at position 31 have S31N replacement (serine to asparagine substitution) and 4 strains (50%) at the position of 27 have V27A replacement (valine to alanine substitution). There was no dual amantadine resistance mutation in any samples. There was no mutation in other positions on M2 gene that represent amantadine resistance.

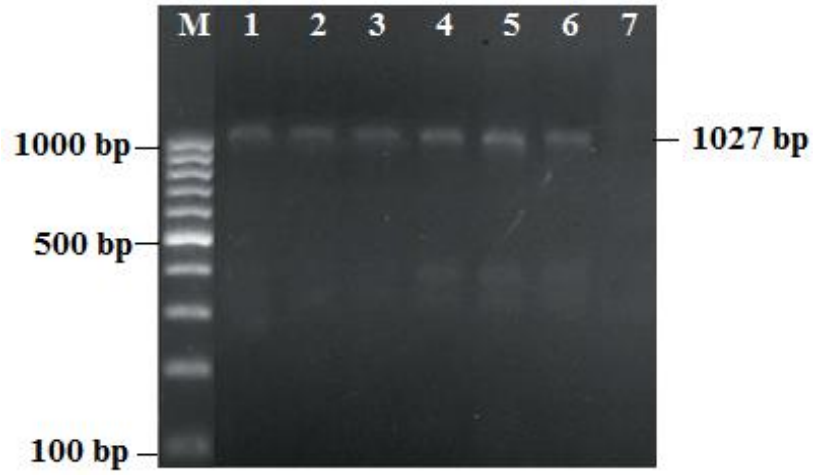

Figure 1. The electrophoresis of PCR product of 1027 bp fragment of M gene (H9N2, AIV). (Column M; Marker 100bp, Column 1-5: Positive samples, Column 6: Positive control, Column 7: Negative control)

Discussion

Amantadine is an antiviral agent that specifically inhibits influenza A virus replication. It is sufficiently proved that amantadine and amantadine-derived compounds can block the proton channel formed by M2 protein and prevents the pH changes required for the AIV un-coating process (Pielak & Chou, 2011). Resistance to amantadine occurs through a point mutation in each of the amino acid positions 26, 27, 30 and 31 or 34 of the M2 protein (Dong et al., 2015). Among the situations mentioned above, mutation at position 31 was reported more than other situations among resistant strains (Hata et al., 2007, Townsend et al., 2008). The analysis of recent AIV strains showed four strains from the eight strains (50%) have S31N substitution (serine to asparagine) and four strains from the eight strains (50%) have V27A substitution (valine to alanine). In other important determinants of resistance to amantadine, there is no change in the amino acid sequence of the strains have occurred. So, it seems that four strain from the 12 strains is sensitive to amantadine (33.33%) and the rest is resistant. Earlier, Yavarian et al. (2010) reported an increase in amantadine resistance, due to a S31N mutation in the M2 channel protein, among human influenza H3N2 strains, circulating in Iran during 2005-2007 (Yavarian et al., 2010). Saderi, Behzadian, Moasser, and Owlia (2017) evaluated the resistance to amantadine in eight strains of influenza A virus (H1N1 and H3N2 subtypes) in human respiratory swabs. The results of which showed that all strains possessed alternatives at position 31 (S31N). In poultry, Fanni, Barin, Moosakhani, and Ghalyanchi-Langeroudi (2011) reported the presence of resistance to amantadine in AIVs circulating in Iran during 2007- 2009, while the strains that detected in 1998, 1999, and 2006 did not have resistance alternatives (Fanni et al., 2011). Furthermore, Malekan et al. (2016) reported that 6 out of 11 (54.5%) detected AIV in 2012-2013 harbored the V27A amantadine resistance mutation and 2 strains (18.2%) harbored V27I. Also, Bashashati et al. (2013) studied the genetic diversity in H9N2 AIV that detected from poultry in early (1998) and recent (2010) years. They stated that analysis of the viral amino acid sequence of the M2 protein of the recent strain revealed a V27A mutation, which is associated with

amantadine resistance in avian influenza virus. However, in recent years, a significant worldwide increase in adamantane resistance has been reported for human H1N1 and H3N2 seasonal influenza viruses and H5N1 avian influenza viruses (Dong et al., 2015). It was reported that mutated viruses may either lose the ability to bind M2 ion channel blockers, as with the S31N or A30T amino acid substitutions (Astrahan, Kass, Cooper, & Arkin, 2004), or bind the blockers but retain M2 function, as with amino acid replacements L26F or V27A at residue 26 or 27 (Astrahan et al., 2004). Among all amantadine resistance mutations, the S31N is the most frequently reported in human, avian and swine (Dong et al., 2015). This fact suggests that that variants containing the S31N substitution might possess a significant advantage on viral replication or transmission, leading to more efficient circulation (Tang et al., 2002). Additionally, the high levels of the dual resistance mutations mainly detected in H5N1 variants obtained from Thailand, Vietnam, and Cambodia indicate that viruses carrying this dual motif are stably selected (Dong et al., 2015). Fortunately, in present study no dual amantadine resistance mutations were identified. However, the high frequency of adamantane-resistant variants indicates that continuous global surveillance and rapid identification of mutants are essential to monitor the emergence and spread of drug resistance, and to help with making informed decisions about antiviral usage in control of influenza virus infections. Our findings raise concerns about the increasing prevalence of adamantane-resistant influenza variants in Iran that can threat the controlling seasonal and pandemic influenza.

Conclusion

In conclusion, since the use of amantadine in Iranian poultry farms is prohibited and less used, it can be concluded that M2-associated amantadine resistant mutations can occur spontaneously before these drugs administered, implying that greater caution is needed in the use of adamantanes.

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