Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd

Document heading doi: 10.1016/S2222-1808(14)60651-4

Sequencing and genetic analysis of hemagglutinin gene of H9 avian influenza viruses from Iran (2008 to 2011)

Masoud Soltanialvar[1*], Ali Bagherpour[1], Hassan Norozian[2]

[1]*Department of Veterinary Medicine, Faculty of Agriculture, Islamic Azad University, Shoushtar Branch, Khozestan, Iran*

[2]*Department of Veterinary Medicine, Faculty of Agriculture, Lorestan University, Lorestan, Iran*

PEER REVIEW

Peer reviewer

Dr. Farshad Akbarnejad, Scientific Director, Floor 2, No 35, Ostad Nejatollahi St., East Saremi Ave, Research Department. Dr Jahangir Pharmaceutical & Hygienic Co. Tehran, Iran.
Tel: +9821-88948885
E-mail: farshad.borom@gmail.com

Comments

This is good study in which the authors studied the genetic analysis and nucleotide sequence of HA genes of Iranian H9N2 isolates that were found that H9N2 viruses of Iran had acquired human virus receptor, as did Hong Kong H9N2 viruses. It is proposed that poultry might be a potential carrier for transmission form waterfowl to human and also Iranian poultry industry has been affected by avian influenza virus, subtype H9N2.
Details on Page 193

ABSTRACT

Objective: To survey on molecular characterization of Hemagglutinin(HA) gene in H9N2 viruses and determine genetic relationship between Iranian H9N2 viruses and other Asian viruses.
Methods: Ten H9N2 viruses were isolated from commercial broiler chickens in Iran during 2008-2011 and their HA genes were analyzed by reverse transcription-polymerase chain reaction and sequencing. Meanwhile, nucleotide sequences (open reading frame) of the HA genes were used for phylogenetic tree construction.
Results: All the isolates possessed the same amino acid motif -R-S-S-R/G-L- at the cleavage site of HA and possessed H at amino acid position 183, A and T at position 190, S at position 138, whereas the representative strains of the other sublineage (except CK/HK/G1/97-like) of H9N2 viruses had N, E, and A at these positions respectively. Phylogenetic analysis of HA genes showed that it shared a common ancestor Qa/HK/G1/97 isolate which had contributed internal genes of human H5N1 viruses.
Conclusions: The present results indicated the HA genes of H9N2 influenza viruses circulating in Iran were well conserved in the past years and the earliest Iranian isolates may be considered to represent such a progenitor.

KEYWORDS

Influenza virus, Genetic analysis, Haemagglutinins, Iran.

1. Introduction

Avian influenza virus genome consists of eight separate segments[1]. Hemagglutinin (HA) is the most important protein of avian influenza virus encoded by segment 4 of avian influenza virus gene[2]. The first step in viral infection is the attachment of the viral HA protein to the host cell receptor sialic acid. The HA gene is the primary determinant of high pathogenicity in chickens[3]. The cleavage of the HA into the HA1 and HA2 proteins is essential for the virus to be infectious and produce multiple replication cycles. It seems that the receptor- binding specificity of the HA is transformed early after the transmission of an avian virus to humans and pigs[4]. Since 1998, Avian virus serotype H9N2 has been influenced Iranian poultry industry[5]. In a previous study, Rahimian *et al.* (2008) detected anti-H9N2 antibody in human sera in Iran[6]. In present study, we performed genetic analyses of HA gene of ten H9N2 viruses

*Corresponding author: Masoud Soltanialvar, Department of Veterinary Medicine, Faculty of Agriculture, Islamic Azad University, Shoushtar Branch, Khozestan, Iran.
Tel: +98 09169684173
E-mail: soltanialvar@gmail.com
Foundation Project: Supported by the Islamic Azad University, Shoushtar Branch (Grant No. 149).

Article history:
Received 15 Dec 2013
Received in revised form 5 Jan, 2nd revised form 14 Jan, 3rd revised form 21 Jan 2014
Accepted 15 Mar 2014
Available online 10 Aug 2014

that were isolated from poultry in Iran during 2008 to 2011. The full-length genes of these isolates were obtained by means of reverse transcription-polymerase chain reaction. Sequence analysis and phylogenetic study was conducted by comparing the full length of each isolate with sequences available in Genbank. The HA is the major antigen for neutralizing antibodies and is involved in binding of virus particles to receptors on host cells[2]. However, the recent research extensively focuses on nucleotide sequences and deduced amino acid of HA genes of H9N2 influenza viruses.

2. Materials and methods

2.1. Sampling and virus isolation

The study is carrying out in order to determination of the genetic relationship between Iranian H9N2 and other Asian viruses. Samples collected from different parts of the country. Sample collection was performed according to the standard protocol[7]. During the period 2008 to 2011, lung and trachea samples were transported to the National Reference Laboratory. The samples were frozen at −70 °C immediately until they were used. They were treated with 2× phosphate buffer solution (pH=7.4) containing antibiotics and antimycotic (penicillin 10 000 unit/mL, streptomycin 10 000 unit/mL and nystatin 20 000 unit/mL). Initial viral isolation was performed by using ten-day-old specific pathogen free fertilized chicken eggs. Eggs candled daily, and embryos dying within 24 h post inoculation were discarded. Infected allantoic fluids were extracted from the eggs and the presence of viruses was confirmed by haemagglutination assay. Standard haemagglutination-inhibition and neuraminidase-inhibition assays were used for subtype identification of the viruses[8]. The H9N2 virus isolates used in the present study are given in Table 1.

Amplification and sequencing of the HA gene: the viral RNA was obtained directly from the allantoic fluid by applying the high pure viral nucleic acid kit (Roche Germany). Purified genomic RNA was used to generate cDNA clones (reverse transcription-polymerase chain reaction) according to the standard protocol. The specific primers were used for genes amplification as following: Forward primer (1 720 bp): 5′-GCA AAA GCA GGA GTG AAA ATG-3′; Reverse primer (1 720 bp): 5′-AGT CCT GAG CAC AAA TAA CTG G-3′.

High pure product purification kit (Roche Germany) was used for the polymerase chain reaction products purification and then purified products used for direct sequencing (MWG Co., Germany). Nucleotide and deduced amino acid sequences of the HA genes were edited with the Editseq (DNASTER Software package Version 5.2). Nucleotide and deduced amino acid sequences were aligned by ClustalW, Version 1.4. Megalign programs and BLAST software were used to analyze the sequence similarity of the HA genes from Iranian isolates.

Table 1

Cleavage site of H9N2 viruses and right and left edges of binding pocket.

Viruses	Right-edge of binding pocket	Left-edge of binding pocket	cleavage site
A-chicken-Iran-B11A-2005	GTSKS	NGLIGR	RSSR/G
A-chicken-Iran-B76-2004	GTSKS	NGLIGR	RSSR/G
A-chicken-Iran-B99-2005	GTSKA	NGLIGR	RSSR/G
A-chicken-Iran-B308A-2004	GTSKS	NGLQGR	RSSR/G
A-chicken-Iran-B308B-2004	GTSKS	NGLIGR	RSSR/G
A-chicken-Iran-B326-2005	GTSKS	NGLIGR	RSSR/G
A-chicken-Iran-L248-2003	GTSKS	NGLIGR	RSSR/G
A/chicken/Iran/RZ37/2008	GTSKS	NGLIGR	RSSR/G
A/chicken/Iran/RZ42/2008	GTSKS	NGLIGR	RSSR/G
A/chicken/Iran/RZ53/2008	GTSKS	NGLIGR	RSSR/G
A/chicken/Iran/RZ28/2008	GTSKS	NGLIGR	RSSR/G
A/chicken/Iran/RZ71/2009	GTSKS	NGLIGR	RSSR/G
A/chicken/Iran/RZ75/2009	GTSKS	NGLIGR	RSSR/G
A/chicken/Iran/RZ77/2009	GTSKS	NGLIGR	RSSR/G
A/chicken/Iran/RZ80/2010	GTSKS	NGQIGR	RSSR/G
A/chicken/Iran/RZ81/2011	GTSKS	NGQIGR	RSSR/G
A/chicken/Iran/RZ37/2011	GTSKS	NGLIGR	RSSR/G
A-Hong Kong-1073-99	GTSRA	NGLQGR	RSSR/G
A/chicken/Hong kong/G9/97	GTSKA	NGLIGR	RSSR/G
A-chicken-Fujian-FB22- 2007	GTSKA	NGLQGR	RSSR/G
A-chicken-Fujian-FG13-2007	GTSKA	NGLQGR	RSSR/G
A-chicken-Guangdong-SS-94	GTSKA	NGQQGR	GSSR/G
A-duck-Hong Kong-Y280-97	GTSKA	NGLQGR	RSSR/G
A-chicken-Korea-MS96-96	GTSKA	NGPTGR	ASYR/G
A-chicken-Korea-S15-03	GTSKA	NGQQGR	ASGR/G
A-chicken-Korea-S27-04	GTSKA	NGQQGR	RSSR/G
A-chicken-Korea-S5-2003	GTSKA	NGQQGR	RSSR/G
A-chicken-bejing-1-95	GTSKA	NGQQGR	RSSR/G
A-chicken-bejing-2-97	GTSKA	NGLQGR	RSSR/G
A/chicken/Hong kong/G1/97	GISRA	NDLQGR	RSSR/G

3. Results

3.1. Molecular characterization:

In this study 1 720 base pairs of the HA genes were sequenced. This protein region consists of a complete receptor-binding pocket and cleavage site. The analysis of deduced amino acid indicated that the Iranian isolates did not show insertions or deletions within HA gene with compared to the prototype, A/turkey/wisconsin/66, but rather numerous point mutations were registered. Sequence analysis showed these isolates were very closely related (96.7-99.6%) and shared a homology of (92.0-96.0%) with H9N2 isolates of A/Qa/HK/G1/97 lineage and the 2 human isolates A/Hong Kong/1073/99 and A/Hong Kong/1074/99.

Comparison of the receptor-binding site with the HA of the H9N2 viruses (numbered according to H3), indicated its potential to infect humans. Analysis of protein sequences showed that the motif of cleavage site was RSSR/GLF which is similar to those of H9N2 viruses isolated from the Middle East and Indian sub-continent between 2000 and 2008 (Table 1).

Table 2
Amino acid at the receptor-binding site.

Viruses	Receptor-binding site							
	98	153	155	183	190	194	195	226
A-chicken-Iran-B76-2004	N	W	T	H	A	L	Y	L
A-chicken-Iran-B99-2005	N	W	T	H	T	L	Y	L
A-chicken-Iran-B308A-2004	N	W	T	H	A	L	Y	L
A-chicken-Iran-B308B-2004	N	W	T	H	A	L	Y	L
A/chicken/Iran/RZ37/2008	N	W	T	H	A	L	Y	L
A/chicken/Iran/RZ42/2008	N	W	T	H	A	L	Y	L
A/chicken/Iran/RZ53/2008	N	W	T	H	A	L	Y	Q
A/chicken/Iran/RZ28/2008	N	W	T	H	A	L	Y	L
A/chicken/Iran/RZ71/2009	N	W	T	H	A	L	Y	L
A/chicken/Iran/RZ75/2009	N	W	T	H	A	L	Y	L
A/chicken/Iran/RZ77/2009	N	W	T	H	A	L	Y	L
A/chicken/Iran/RZ80/2010	N	W	T	H	A	L	Y	L
A/chicken/Iran/RZ81/2011	N	W	T	H	A	L	Y	Q
A/chicken/Iran/RZ37/2011	N	W	T	H	A	L	Y	L
A-Hong Kong-1073-99	N	W	T	H	E	L	Y	L
A/chicken/Hong kong/G9/97	N	W	T	H	E	L	Y	L
A-chicken-Fujian-FB22- 2007	N	W	T	H	A	L	Y	L
A-chicken-Fujian-FG13-2007	N	W	T	N	A	L	Y	L
A-chicken-Guangdong-SS-94	N	W	T	N	A	L	Y	L
A-duck-Hong Kong-Y280-97	N	W	T	N	A	L	Y	Q
A-chicken-Korea-MS96-96	N	W	T	N	T	L	Y	L
A-chicken-Korea-S15-03	N	W	T	H	E	L	Y	Q
A-chicken-Korea-S27-04	N	W	T	H	E	L	Y	Q
A-chicken-Korea-S5-2003	N	W	T	H	E	L	Y	Q
A-chicken-bejing-2-97	N	W	T	N	A	L	Y	L

The amino acids of the right edge of binding pocket (134-138) were -G-T-S-K-S-, but other representative strains had A not S at amino acid 138. The left edge of binding pocket possessed -N-G-L-I-G-R motif (224-229) (Table 1).

All Iranian isolates, Qa/HK/G1/97, and Hong Kong/1073/99 possessed H (histidine) at amino acid position 183, whereas Ck/Bei-like viruses, CK/HK/G9/97 and DK/HK/Y280/97 had N (asparagine) at this position. At position 190, A (alanine) was registered in the all isolates, and surprisingly one isolate (A/chicken/Iran/B99/2005) showed T (threonine) at position 190 (Table 2). Analysis of HA protein sequences revealed that all Iranian H9N2 viruses have seven glycosylation sites. Five of these sites were located in the HA1 region and two in HA2 region.

3.2. Phylogenetic analysis

The phylogenetic relationships between the HA genes of these Iranian isolates and other countries showed that all the Iranian isolates belonged to G1 lineage (Figure 1).

The Iranian isolates can be classified into three main subgroups according to the time of their isolation. The first subgroup closely related to A/HK/1073/99 and A/ HK/1074/99, isolated from human. The Iranian viruses isolated during 2003 to 2005 fall into the second subgroup. However, most HA genes of the H9N2 viruses isolated during 2008-2011 were similar with the Pakistani H9N2 viruses.

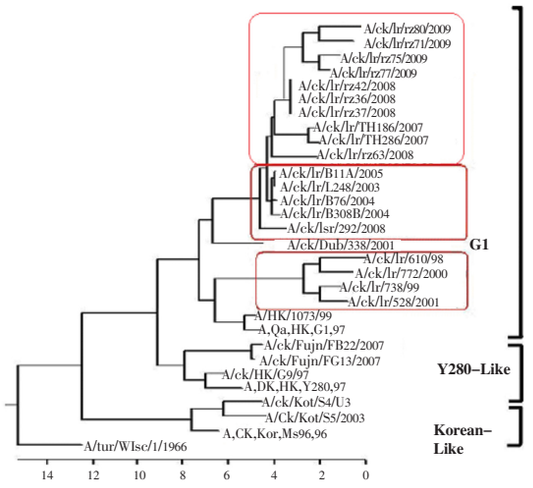

Figure 1. All the Iranian isolates fall into a special group, related to the G1 sublineage, and distributed among three HA gene subgroups associated with the time of their isolation.

4. Discussion

In recent years, there has been an increase in outbreaks of avian influenza in various countries[9-12].

Avian influenza is a highly contagious disease with significant potential to harm poultry industry often resulting in extensive losses. Most of the affected birds showed typical signs of influenza, such as a pronounced drop in egg production, decrease in food consumption and severe tracheitis[13]. However, experimental studies indicated no or low mortality rates with the current outbreak of H9N2 in Iran[14,15]. In this study, we observed overt clinical signs in chickens at affected farms (acute respiratory signs included: tracheitis, respiratory congestion) and 1-12% mortality rates during the outbreak. It seems that co-infection with concurrent bacterial infections enhances the replication of the H9N2 virus, resulting in exacerbation of the disease[16,17]. A number of amino acids within the receptor-binding site which are involved directly in binding sialic acid are highly conserved among the different HA subtypes[1]. Previous studies have shown that all of influenza viruses isolated from avian species possess H (histidine), E (glutamic acid) and Q (glutamine) at amino acid positions 183, 190, and 226 respectively at the receptor binding site[18]. HAs of H9N2 viruses isolated in Korea and North America maintained the specificity while most of HAs of H9N2 belonged to Qa/HK/G1/97 and Dk/HK/Y280/97 sublineage had different type. Here, distinct sequences of the receptor binding site were found between the H9N2 viruses isolated in Iran and the other representative strains. At position 190, all isolates possessed A (alanine) or T (threonine). Nine of the isolates possessed L (leucine) at position 226 while the rest had Q (glutamine). Leucine at position 226 of these viruses is typical of the sequence found in human H2 and H3 strains but not in avian viruses. These observations indicate that the H9N2

viruses of Iran have acquired human virus-like receptor specificity, as did Hong Kong H9N2 viruses. Furthermore it suggested that chicken might be a potential intermediate host for transmission of viruses from wild waterfowl to human[19]. As reported by Matrosovich *et al* (2001), our results also showed that HA genes of H9N2 viruses formed different sub lineages including G1-like, Ck/Beijing-like (or Y280-like) and Korean-like viruses[20]. The HA genes of H9N2 viruses isolated from poultry farms in Iran during 1998-2011 were closely related only to G1 group-like H9N2 viruses and differed from the HAs of H9N2 viruses isolated in southeastern China and Korea[21-23]. In contrast, we recently reported that the internal genes of these viruses represented at least 2 genotypes and some were closely related to those of viruses isolated from aquatic birds in southeastern China[24]. This is probably related to accumulation of mutations occurrences among Iranian H9N2 viruses within poultry reservoir. Also, phylogenetic analyses suggest that Iranian H9N2 viruses′ were co-circulated with other H7N3 and H9N2 viruses isolates and had re-assorted their internal genes[24]. Our previous studies have shown that one of the factors driving the evolution of low-pathogenic influenza viruses is the immunological pressure, which increases in the case of the ongoing vaccination[25-27]. It was found in the present study= that the HAs of the Iranian isolated from chickens in Iran since 1998 had genetically close relationships with that of Qa/HK/G1/97 and were distinct from those of Dk/HK/Y280/97 (Y280-like) and A/Chicken/Korea/38349-p96323/96(Korean-like). Based on the finding of this research, it is very critical to identify any variation of poultry H9N2 isolates. Thus, continuous surveillance would enhance our understanding of the role of different avian hosts in ecology of H9N2 viruses. In order to identify the source of the Iranian H9N2 outbreaks it is necessary to investigate the phylogenic analysis on nucleotide sequence of the HA gene or other genes of other Iranian H9N2 isolates.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was supported by Grant No. 149 from the Islamic Azad University, Shoushtar Branch. The authors thank Dr Reza Goodarzi for their scientific guidance.

Comments

Background

Avian influenza is a highly contagious disease with significant potential to harm poultry industry often resulting in extensive losses. Since 1998, avian virus serotype H9N2 has been influenced Iranian poultry industry. Most of affected poultry indicated typical signs of respiratory symptoms such as tracheitis, respiratory congestion. On the other hand, according to published reports for high similarity of the poultry H9N2 viruses and the human H5N1 isolates, it is important to consider Iranian avian influenza viruses may be as potential threat to infect human.

Research frontiers

The study is carrying out in order to determin the genetic relationship between Iranian H9N2 and other Asian viruses. The lung and trachea samples were collected from commercial broiler chickens during 2008 to 2011 in Iran. The ten of H9N2 virus isolate and their HA genes were analysed by reverse transcription-polymerase chain reaction and sequencing. Sequence analysis and phylogenetic was conducted by comparison the full length of each isolate with sequences available Genbank.

Related reports

The data about HA genes H9N2 viruses showed that these viruses were formed from different sublineages are in agreement with Mastrosovich *et al*. (2001). But the HA genes H9N2 viruses isolated from poultry farm in Iran during 1998-2011 were close relationship with G1 group like H9N2 viruses and were different from the HAs of H9N2 viruses isolated in south-eastern China and Korea (2007, 2010 and 2012). In contrast, we recently reported that the internal genes of these viruses represented at least 2 genotypes and some were closely related to those of viruses isolated from aquatic birds in southeastern China (2012). This is probably related to accumulation of mutations occurrences among Iranian H9N2 viruses within poultry reservoir. Also, phylogenetic analyses suggest that Iranian H9N2 viruses′ were co-circulated with other H9N2 viruses isolates and have re-assorted their internal genes.

Innovations & breakthroughs

The finding of this study indicate that HAs of the Iranian H9N2 isolated from commercial chickens since 1998 had closely genetic relationship with Qa/HA/G1/97 and distinctly from those Dk/HK/Y280/97(Y280-like) and A/chicken/Korea/38349-p96323/96 (Korean-like).

Applications

Based on the finding of this research, it is very critical to identify any variation of poultry H9N2 isolates. Thus, continuous surveillance would enhance our understandings of the role of different avian hosts in ecology of H9N2 viruses. In order to identify the source of the Iranian H9N2 outbreaks it is necessary to investigate the phylogenic analysis on

nucleotide sequence of the HA gene or other genes of other Iranian H9N2 isolates.

Peer review

This is good study in which the authors studied the genetic analysis and nucleotide sequence of HA genes of Iranian H9N2 isolates that were found that H9N2 viruses of Iran had acquired human virus receptor, as did Hong Kong H9N2 viruses. It is proposed that poultry might be a potential carrier for transmission form waterfowl to human and also Iranian poultry industry has been affected by avian influenza virus, subtype H9N2.

References

[1] Bouvier NM, Palese P. The biology of influenza viruses. *Vaccine* 2008; **26**(Suppl 4): D49-53.

[2] Lee CW, Saif YM. Avian influenza virus. *Comp Immunol Microbiol Infect Dis* 2009; **32**: 301-310.

[3] Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, et al. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J Virol* 2000; **74**(18): 8502-8512.

[4] Kongchanagul A, Suptawiwat O, Kanrai P, Uiprasertkul M, Puthavathana P, Auewarakul P. Positive selection at the receptor-binding site of haemagglutinin H5 in viral sequences derived from human tissues. *J Gen Virol* 2008; **89**: 1805-1810.

[5] Vasfi Marandi M, Bozorgmehrifard MH. An outbreak of non-highly pathogenic avian influenza in chicken in Iran [CD-ROM]. France: Proc. 61st meeting of Word Veterinary Association; 1999.

[6] Rahimian AE, Shoshtari AA, Pourbakhsh SA, Momayez R, Rahimi LE, Mehrabanpour MJ. Serological and molecular survey of avian influenza H9N2 in human poultry farm industries. *Med J Mashhad Univ Med Sci* 2009; **52**: 133-140.

[7] Swayne DE. *A Laboratory manual for the isolation and identification of avian pathogens*. 4th ed. USA: American Association of Avian Pathologist; 1998, P. 311.

[8] Alexander DJ, Spackman D. Characterization of influenza A viruses isolated from turkeys in England during March-May 1979. *Avia Pathol* 1981; **10**: 281-293.

[9] Okamatsu M, Tanaka T, Yamamoto N, Sakoda Y, Sasaki T, Tsuda Y, et al. Antigenic, genetic, and pathogenic characterization of H5N1 highly pathogenic avian influenza viruses isolated from dead whooper swans (*Cygnus cygnus*) found in northern Japan in 2008. *Virus Genes* 2010; **41**: 351-357.

[10] Tombari W, Nsiri J, Larbi I, Guerin JL, Ghram A. Genetic evolution of low pathogenecity H9N2 avian influenza viruses in Tunisia: acquisition of new mutations. *Virol J* 2011; **8**: 467.

[11] Kalthoff D, Globig A, Beer M. (Highly pathogenic) avian influenza as a zoonotic agent. *Vet Microbiol* 2010; **140**: 237-245.

[12] Bi Y, Lu L, Li J, Yin Y, Zhang Y, Gao H, et al. Novel genetic reassortants in H9N2 influenza A viruses and their diverse pathogenicity to mice. *Virol J* 2011; **8**: 505.

[13] Aamir UB, Wernery U, Ilyushina N, Webster RG. Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. *Virology* 2007; **361**: 45-55.

[14] Nili H, Asasi K. Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. *Avian Pathol* 2002; **31**: 247-252.

[15] Keshtkar M, Nili H, Dadras H, Asasi K. Experimental assessment of the pathogenicity of avian influenza virus H9N2 subtype in Japanese Quail-Coturnix Coturnix Japanica. *Arch Razi Institute* 2010; **65**: 59-65.

[16] Haghighat-Jahromi M, Asasi K, Nili H, Dadras H, Shoushtari AH. Coinfection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. *Arch Virol* 2008; **153**: 651-655.

[17] Mosleh N, Dadras H, Mohammadi A. Molecular quantitation of H9N2 avian influenza virus in various organs of broiler chickens using TaqMan real time PCR. *J Mol Genet Med* 2009; **3**: 152-157.

[18] Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y, Nakajima K. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. *Virology* 1991; **182**: 475-485.

[19] Cardona CJ, Xing Z, Sandrock CE, Davis CE. Avian influenza in birds and mammals. *Comp Immunol Microbiol Infect Dis* 2009; **32**: 255-273.

[20] Matrosovich MN, Krauss S, Webster RG. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. *Virology* 2001; **281**: 156-162.

[21] Xu KM, Smith GJ, Bahl J, Duan L, Tai H, Vijaykrishna D, et al. The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. *J Virol* 2007; **81**: 10389-10401.

[22] Sun Y, Pu J, Jiang Z, Guan T, Xia Y, Xu Q, et al. Genotypic evolution and antigenic drift of H9N2 influenza viruses in China from 1994 to 2008. *Vet Microbiol* 2010; **146**: 215-225.

[23] Zhou JP, Ge FF, Liu J, Ju HB, Yang DQ, Wang J, et al. Epidemiological survey and genetic evolution of H9 subtype influenza viruses in Shanghai China, from 2006 to 2010. *Arch Virol* 2012; **157**(6): 1193-1198.

[25] Soltanialvar M, Goodarzi R, Akbarnejad F. Genetic analysis of polymerase complex (PA, PB1 and PB2) genes of H9N2 avian influenza viruses from Iran (1999 to 2009). *Asian Pac J Trop Biomed* 2012; **2**: 858-862.

[26] Soltanialvar M, Shoushtari H, Bozorgmehrifard M, Charkhkar S, Eshratabadi F. Molecular characterization of hemagglutinin and neuraminidase genes of H9N2 avian influenza viruses isolated from commercial broiler chicken in Iran. *J Biol Sci* 2010; **10**: 145-150.

[27] Soltani M, Shoushtari A, Morovati M, Gharoni M, Dalirannia A, Akbarnejad F. Sequence and phylogenetic analysis of nucleoprotein gene in Iranian H9N2 avian influenza viruses. *Modar J Med Sci Path* 2011; **13**: 43-51.

[28] Soltanialvar M, Shoshtari H, Bozorgmehrifard M, Charkhkar S, Akbarnejad F. Sequence and phylogenetic analysis of neuraminidase genes of H9N2 avian influenza viruses isolated from commercial broiler chicken in Iran (2008 and 2009). *Trop Anim Health Prod* 2012: **44**; 419-425.