Iranian Journal of Virology 2011;5(3): 1-6

Original Article

Full Length Characterization of PA Gene of H9N2 Isolated from Broilers During 1998 to 2009

Tayefeh-Fazel-Khalkhali F[1], Soleimani M[2], Majidzadeh-A K[3*], Ghalyanchi-Langeroudi A[4], Zolfaghari MR[5], Karimi V[6]

1. Science and Research Branch, Islamic Azad University, Tehran, Iran.
2. TASNIM Biotechnology Research Center, AJA University of Medical Sciences, Tehran, Iran.
3. Iranian Centers for Breast Cancer (ICBC); Academic Center for Education, Culture and Research (ACECR), Tehran, Iran.
4. Departments of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
5. Department of Microbiology, Qom Branch, Islamic Azad University, Qom, Iran.
6. Departments of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Abstract

Background and Aims: Avian Influenza (AI) H9N2 subtype was first reported to infect turkeys in the United States in 1966 and has been panzootic in Europe and Asia. The impact of avian influenza caused by H9N2 viruses in Iran is now significantly more severe than in previous years.

Methods: Sequence analysis and phylogenetic study of the complete coding region Polymerase A (PA) gene of H9N2 subtype of avian influenza virus isolates (outbreaks of 1999-2009) in Iran were studied.

Results: Sequence and phylogenetic analysis revealed a large number of similar substitution mutations and close evolutionary relation among sequences of PA. Based on analysis of AminoAcid sequences Iranian H9N2 isolates have some substitution that found in human and mouse adapted isolates. It is raised concern that H9N2 Isolates has trend to infect mammalian host. In Phylogenetic analysis Iranian isolates cluster in unknown Indian-Middle East cluster. However, the early isolate of H9N2 (11T strain) in Iran classified in DK-1 lineage.

Conclusion: The results show, Iranian avian influenza H9N2 isolates have undergone extensive genetic reassortment which led to emergence of a new genotype. Interestingly, continuous monitoring of viral genetic changes throughout the years is warranted to monitor variations of Influenza viruses in the field.

Keywords: Avian Influenza (AI) H9N2 subtype; Iran; Phylogenetic Study

Introduction

Avian Influenza virus (AIV) is a member of the family Orthomyxo-viridae; containing negative sense single stranded RNA. The AIV genome consists of eight segments of negative-sense, single-stranded RNA that encode at least ten proteins including two surface glycoproteins [haemagglutinin (HA) and neuraminidase (NA)], nucleoprotein (NP), three polymerase proteins [polymerase basic (PB) PB2, PB1 and polymeraseacidic (PA)], two matrix (M1 and M2) proteins and two Non-structural (NS1 and NS2) proteins (1, 2). The polymerase proteins also play important roles in the pathogenicity of AIVs in different species (3). H9N2 subtype avian influenza viruses (AIV) have widely

***Corresponding author:** Keivan Majidzadeh-A, PhD. Iranian Centers for Breast Cancer (ICBC); Academic Center for Education, Culture and Research (ACECR), Tehran, Iran.
Tel: (+98) 21 88 33 79 28;
Email: kmajidzadeh@razi.tums.ac.ir

[DOI: 10.21859/isv.5.3.1]

circulated in the world since its first detection from turkeys in Wisconsin in 1966 (4). These viruses produce significant disease problems in poultry resulting in great economic losses due to reduced egg production or high mortality with co-infection with other opportunistic pathogens such as infectious bronchitis viruses (5, 6). H9N2 influenza viruses are considered to be one candidate for the next pandemic (7). Two distinct lineages of H9N2 influenza viruses have been defined, the North American lineage and the Eurasian lineages which consists of at least three sub lineages represented by their prototype strains: A/quail/Hong Kong/G1/97 (G1-like), A/duck/HongKong/Y280/9(Y280-like), A/Chicken/Beijing/1/94 (BJ94-like), and A/chicken/Korea/38349-P96323/96 (Korean-like) (4).

In 1998, H9N2 subtype influenza A virus of low pathogenicity has been reported in the industrial poultry populations of Iran and thereafter it has caused outbreaks in commercial broiler chickens in Iran (8). During previous years some reports were published that indicated H5N1 and other serotypes have been circulating in non-industrial birds in Iran (9, 10).

Till now, just one complete sequence of PA gene of Iranian H9N2 isolate has been characterized (11T).

In this study, we present full length characterization and phylogenetic analysis of PA gene of H9N2 influenza virus isolated from broiler in Iran during 13 years.

Methods

Viruses

Tree H9N2 viruses tested in this study have been isolated from 1998 to 2011 from different outbreaks in Iran. The virus subtype identification was determined by standard RT-PCR tests. Initial isolation was performed in 10 day old embryonated chicken eggs and stored at - 70 C for use (11).

RNA Extraction & RT - PCR reaction

Viral RNA was extracted from 200 µl/l virus-positive allantoic fluid using *ExiPrep*™ Viral DNA/RNA Kit from Bioneer, Korea according to the manufacturer's instructions. Reverse transcription was done by using oligonucleotide influenza universal primer uni12: 5-AGC AAA AGC AGG-3 with Revert Aid first strand cDNA synthesis Kit (Fermentas, Canada).Amplification of the PA gene was carried out by PCR as described by using two pairs of specific primers. Primer sequences are available upon request. The reaction mixture (50 µl) contained 5 µl of cDNA, 15 pmoles of forward and reverse primers (4 µl), and Cinnagen master mix (Cinnagen, Iran). The amplification protocol was : One step of denaturation at 94°C for 3 mins, 35 cycles of 94°C /45 sec, 58°C /45 sec, and 72°C /60 secs, and final extension at 72°C for 10 mins (12).

TA cloning & Phylogenetic Analysis

Purified PCR products for sequencing were cloned into plasmid for TA cloning with *Ins T/A cloning* kit (Fermentas ,Canada) according to the manufacturer's instruction .Plasmid extraction from positive clone was carried out by QIA miniperep plasmid extraction kit (Qiagen, Valencia, CA, USA) (12). The nucleotide sequences were analyzed by plasmid sequencing on an automated 3700 DNA sequencer (Applied Biosystems, Foster city, CA). A BLAST analysis was initially performed using PA sequence of mentioned study to establish its identity to GenBank accessions. A comparative analysis of PA sequences was performed using the CLUSTAL W Multiple Sequence Alignment Program, version 1.83 (13). The tree was constructed using the neighbor-joining program with 100 bootstrap replicates with MEGA5 Software program. Sequences used for comparison or phylogenetic analysis in this study were obtained from the GenBank database (14).

Accession Numbers

The nucleotide sequences obtained in this study have been submitted to the GenBank database and are available under accession numbers.

Results

The lengths of PA gene were obtained for the three isolates. BLAST software and MEGA5

[DOI: 10.21859/isv.5.3.1]

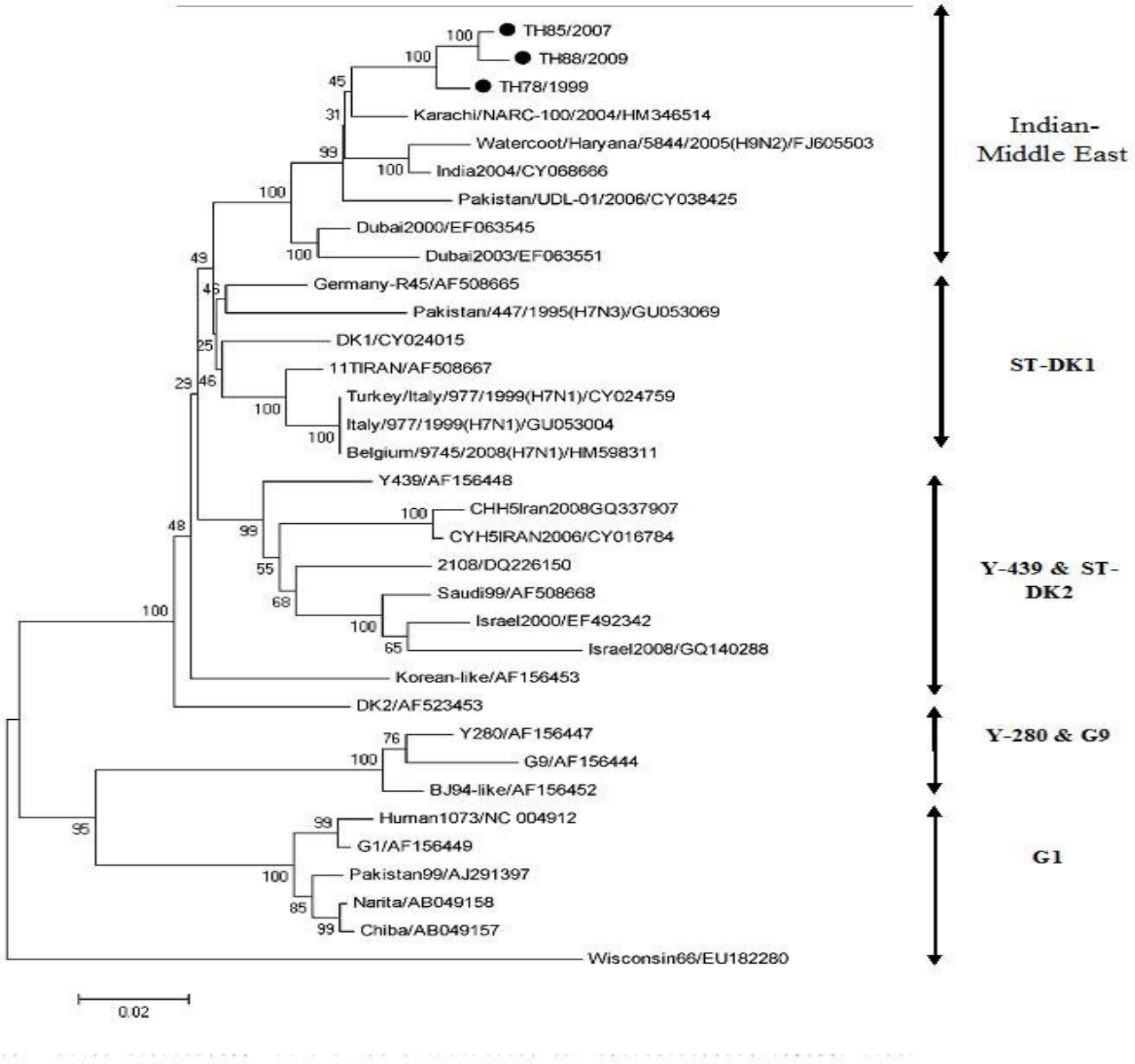

Fig. 1. Nucleotide Based Phylogenetic relationships of PA gene of H9N2 Avian Influenza isolated from Iran. The Phylogenetic tree was generated using Neighboring Joining model with MEGA (version 5.1 beta). Numbers below branches indicate bootstrap value percentages from 1000 replicates, bootstrap values. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes. The vertical lines are for spacing branches and labels. Analysis was based on complete open reading frames of all gene segments. The scale bar represents the distance unit between sequence pairs. The viruses characterized in this report are indicated as Black Circle. The sequences obtained from Gene Bank.

programs were used to determine the sequence similarity of the eight genes from the threeisolates. All of Iranian H9N2 isolates contains 2148 nucleotide acid & 716 amino acid. They did not exhibit insertion or deletion within PA gene as compare with their prototype Turkey/Wisconsin/66. The similarity between this work H9N2 isolates were between (85.6%-99.3%) and (94.6%-98.7%) based on nucleotide and amino acid respectively. Recent Iranian H9N2 isolates show high similarities with Pakistani and Indian (Almost of them had been isolated after 2000) H9N2 based on PA gene. Iranian H9N2 isolates that submitted in gene bank are cluster in two groups based on PA gene (CHINA-SD1

[DOI: 10.21859/isv.5.3.1]

Like and Indian-Middle East Lineage). More detailed results of phylogenetic finding will be discussed. The phylogenetic relation between isolates is represented in figure 1.

Discussion

In recent years, AI has caused major economic harms in Iranian poultry industry. The latest Iranian H9N2 isolate has been reported to be low pathogenic for SPF chickens (Data has not shown). However, recent H9N2 outbreaks have caused up to 65% mortality rate and are the only influenza subtype in the chicken population in Iran. Compared with the HA and NA genes of the H9N2 viruses isolated in Iran, only two sequences of the PA gene have been submitted.[15] Growing evidences suggested that the influenza viral trimeric polymerase complex formed by polymerase basic 2 (PB2), polymerase basic 1 (PB1), and polymerase acid (PA) might be associated with viral virulence and/or interspecies transmission. Previous studies revealed that the viral polymerase subunits from human isolates might not be fully compatible to those isolated from avian strains. Others further identified that some residues in these polymerase subunits might control host-restriction (16, 17).

In this study, the full length PA gene of three H9N2 avian influenza viruses isolated between 1998 to 2011 from different farms in Iran were phylogenetically analyzed.

The early H9N2 isolate 11T that isolate in 1998 is clustered in SD-like lineages whereas polymerase A genes (PA) of H9N2 isolates that characterized in this study were from an unknown source that was just to be defined in Pakistan and India previously (Indian-Middle East Lineage). Genetic reassortment is one of the mechanisms of influenza A virus evolution. Evidence of reassortment between H9N2 and the highly pathogenic avian influenza virus H5N1 subtype was reported in 2000. In subsequent years, multiple reassortant variant genotypes of H9N2 avian influenza viruses from domestic poultry in Iran have been identified and well characterized. Based on the genetic similarities, our findings suggest that the precursors of the Iranian viruses had undergone genetic reassortment with other influenza subtypes. This feature suggests the pandemic potential of the H9N2 avian influenza virus and emphasizes the need for continuous surveillance. Several studies have been carried out on PA gene of H9N2 isolate in different countries. Research on H9N2 Influenza A Viruses Isolated from Poultry in Pakistan by Iqbal et al indicates that the 12 H9N2 viruses retained PA from a lineage of viruses from the Indian sub-continent and the Middle-East (18). Sequence and phylogenetic analysis of H7N3 avian influenza viruses isolated from poultry in Pakistan 1995-2004 indicate that Among the PA genes from the Pakistani H7N3 isolates the nt sequence identity was above 99.5% with the exception of 100/Karachi-04 which had around 92.0% nt identity with the other H7N3 isolates.[19] Amir et al in study on Emirate H9N2 isolates reported that the "internal" genes PB1, PA, M, and NP are also similar to those of other Middle and Far Eastern strains, which can be ultimately traced back to the same G1-like lineage. Also, they indicate that Iranian 11T strain of H9N2 was belonging to G1 lineage. Our results don't coordinate with their works (20).

Phylogenetic analysis of the entire coding regions of the PA genes showed that the parakeet (Narita & Chiba) H9N2 viruses cluster with the H9N2 human and G1 quail viruses as well as human H5N1 Hong Kong viruses (21). As determined in previous national and international researches on HA, NA, NS and M genes of Iranian AI H9N2 isolates, these isolates are located in G1 clade base on motioned genes and have high similarity with Chiba and Narita isolates (10, 22, 23) . In another study, PA genes of the viruses from domestic poultry in Mainland China belong to the Eurasian lineage. They form three sub-lineages represented by the CK/BJ /1/94, G1, and CK/SH/ F/98 viruses, respectively. The genes share 94 – 100 % homology within the sublineages, but 84 – 92% homology between the sub-lineages (24). Also, PA gene of H5N1 viruses are linked with G1/97 virus, indicating that they either share a common precursor or transmitted the gene

[DOI: 10.21859/isv.5.3.1]

complex from one to from one to the other (25).

Phylogenetic Analysis of Eight Genes of H9N2 Subtype Influenza Virus , Mainland China Strain, showed that four genes (HA, NA, M an d NS genes) of Ck/SH/F/98 were incorporated into the sublineage represented by the early mainland China strain, Ck/BJ/1/94. However, the other four of RNP genes of Ck/ SH/F/ 98 did not show close relationship with those of the three know nsubli neages' viruses (26). Kim et al (2006) reported that H9N2 influenza viruses isolated from poultry in Korean live bird markets based on PA were clustered with those of the early Korean isolates of H9N2 influenza viruses (27). Genetic analysis of Indian H9N2 avian influenza viruses showed that that 50% of the genes (HA, NA, NP and M) were similar to G1-like viruses, In PB2, PB1, PA and NS gene trees, all the Indian viruses formed a separate cluster with high (100%) bootstrap values. The PA genes of this study are 93.8–94.3% similar to a H5N9 chicken isolate from Italy (Ck/Italy/9097/97) and formed a distinct sublineage with viruses of G1-, Y280- and Korean-like (86.2–93.2% similarity) (28).

Amino acid residues of the H9N2 viruses that correlate with mouse replication phenotype found some amino acid substitution in PA protein (24). In our study all H9N2 isolates (In this study) have some the same substations except 388 (I). In genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses, Some positions have been discussed. One of these positions that has been changed in two Iranian H9N2 isolates (TH85, TH89), was (S409N) (29). In another work the mouse-adapted Ck/Js-MA virus was found to share specific amino acid identities with these representative H9N2 viruses. Common residues were found in A (35S, 16 2A and 347N) (30) that were not present in the Iranian H9N2 strains examined but are have the similar AA with H9N2 that cause AI in human. The highly pathogenic (HP) influenza viruses H5 and H7 are usually nonpathogenic in mallard ducks. However, the currently circulating HP H5N1 viruses acquired a different phenotype and are able to cause mortality in mallards. Results of research work by Hulse-Post etal (2007) show that the PA and PB1 genes of HP H5N1 influenza viruses are associated with lethality in ducks. This mutation in PA is (T515A).All H9N2 isolate in this work possess T that indicate don't lethal for ducks molecularly base (17). Also, when comparing the avian SC35 strain (H7N7) with its mouse-adapted variant SC35M, researchers previously identified six mutations in SC35M responsible for the increased virulence in mice (L13P and S678N in PB1, D701N and S714R in PB2, K615N in PA, and N319K in NP) (31). H9N2 Isolates that have been sequenced, have K in 615 positions. Data shows mentioned Iranians H9N2 isolate trend to replicate in mammalian hosts based on mutations in PA. It is not yet known which substitutions, or combinations of substitutions, are necessary for adaptation to the new host. In summary, our study provides useful and Valuable information that were not available previously about Iranian H9N2 isolates. Therefore, urgent attention should be paid to the control of H9N2 influenza viruses in animals and to human's influenza pandemic preparedness. Considering these new mutations, the molecular bas is of tropism, host responses and enhanced virulence will be defined and studied. Otherwise, Continuous monitoring of viral genetic changes throughout the year is warranted to monitor variations of Influenza viruses in the field.

Acknowledgments

Authors gratefully acknowledge extensive technical support (Especially from Mr A. Morvvati and Mr AH Mohseni) of TASINIM Biotechnology Research Center, AJA University of Medical Sciences.

References

1. Alexander DJ. A review of avian influenza in different bird species. Vet Microbiol. 2000;74(1-2):3-13.

2. Fields BN, DM Knipe, PM Howley. Fields virology. 5th ed. 2007, Philadelphia ; London:

[DOI: 10.21859/isv.5.3.1]

Wolters Kluwer Health / Lippincott Williams & Wilkins.
3. Thomas JK, Noppenberger J. Avian influenza: a review. Am J Health Syst Pharm. 2007;64(2):149-65.
4. Li JW, Yu KZ, Brown I, Shortridge KF, Pieris SM, GuanY. The evolution of H9N2 influenza A viruses isolated from Eurasian countries. 2004;2(1)3-9.
5. Peiris M. Influenza A H9N2: aspects of laboratory diagnosis. J Clin Microbiol. 1999;37(10):3426-7.
6. Saif YM, Barnes HJ. Diseases of poultry. 12th ed. 2008, Ames, Iowa: Blackwell Pub. Professional.
7. Saito T. Characterization of a human H9N2 influenza virus isolated in Hong Kong. Vaccine. 2001;20(1-2):125-33.
8. Nili H, Asasi K. Avian influenza (H9N2) outbreak in Iran. Avian Dis. 2003;47(3 Suppl):828-31.
9. Fereidouni SR. Avian influenza virus monitoring in wintering waterbirds in Iran. 2003-2007. Virology journal, 2010.7:43-43.
10. Ghalyanchi Langeroudi A. Nucleotide and Amino Acid Sequence Analysis of Hemagglutinin Protein in Cleavage Site Region of H9N2 Isolated from Broilers in Tehran Province during 1998-2007. Journal of Animal and Veterinary Advances, 2008;7(5):529-534.
11. Webster RG. WHO Manual on Animal Influenza Diagnosis and Surveillance. 2002: World Health Organization, Dept. of Communicable Disease Surveillance and Response.
12. Sambrook J, Russell DW. The condensed protocols from Molecular cloning: a laboratory manual. 2006, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. v, 800 p.
13. Larkin MA. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23(21):2947-8.
14. Tamura K. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24(8):1596-9.
15. Nili H, Asasi K. Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. Avian Pathol. 2002;31(3):247-52.
16. Li OT. Full factorial analysis of mammalian and avian influenza polymerase subunits suggests a role of an efficient polymerase for virus adaptation. PLoS ONE. 2009;4(5):5658.
17. Hulse-Post DJ. Molecular changes in the polymerase genes (PA and PB1) associated with high pathogenicity of H5N1 influenza virus in mallard ducks. J Virol. 2007;81(16):8515-24.
18. Iqbal M. Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 viruses. PLoS ONE. 2009;4(6):5788.
19. Abbas M.A. Sequence and phylogenetic analysis of H7N3 avian influenza viruses isolated from poultry in Pakistan 1995-2004. Virol J. 2010;7:137.
20. Aamir UB. Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. Virology. 2007;361(1):45-55.
21. Mase M. Imported parakeets harbor H9N2 influenza A viruses that are genetically closely related to those transmitted to humans in Hong Kong. J Virol. 2001;75(7):3490-4.
22. Kianizadeh M. Neuraminidase gene sequence analysis of avian influenza H9N2 viruses isolated from Iran. Archives of Razi. 2007;62(2):69-74.
23. Majidzadeh K. Phylogenetic Study on Nonstructural (NS) Gene of H9N2 Isolated from Broilers in Iran During 1998-2007. Pakistan Journal of Biological Sceince. 2011;14(17):838-843.
24. Li C. Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. Virology. 2005;340(1):70-83.
25. Guan Y. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci U S A. 1999;96(16):9363-7.
26. Lu JH. Phylogenetic analysis of eight genes of H9N2 subtype influenza virus: a mainland China strain possessing early isolates' genes that have been circulating. Virus Genes. 2005;31(2):163-9.
27. Kim JA. H9N2 influenza viruses isolated from poultry in Korean live bird markets continuously evolve and cause the severe clinical signs in layers. Vet Microbiol 2006;118(3-4):169-76.
28. Tosh C. Genetic analysis of H9N2 avian influenza viruses isolated from India. Archives of virology. 2008;153(8):1433-1439.
29. Naffakh N. Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses. J Gen Virol. 2000;81(5):1283-91.
30. Wu R. Multiple amino acid substitutions are involved in the adaptation of H9N2 avian influenza virus to mice. Vet Microbiol. 2009. 138(1-2):85-91.
31. Gabriel G. Differential polymerase activity in avian and mammalian cells determines host range of influenza virus. J Virol. 2007;81(17):9601-4.

[DOI: 10.21859/isv.5.3.1]