

Pathogenicity and haemagglutinin gene sequence analysis of Iranian avian influenza H9N2 viruses isolated during (1998–2001)

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Summary

Sixteen avian influenza (AI) H9N2 viruses were isolated from disease outbreaks in different parts of Iran during (1998–2001). These AI isolates were used for pathogenicity, haemagglutinin (HA) gene variation and phylogenetic analysis. Results in both pathogenicity tests and HA gene cleavage site sequence detection represented a non-highly pathogenic feature for all Iranian AI isolates studied. The cleavage site motif (R-S-S-R) of all AI isolates however, indicated that they had capability of becoming highly pathogenic viruses following 2 nucleotide substitutions at this region. Based on 450 nucleotides region obtained for local isolates and those for referenced viruses available in Gene Bank database used in phylogenetic analysis, all viruses placed on 3 distinct groups, 2 for Iranian and 1 for reference viruses. Among the reference AI viruses, isolates from Pakistan, Saudi Arabia and 1 from Germany showed less differences with Iranian AI isolates. Results also revealed that the circulating viruses in neighbouring provinces have been remained with less mutation for about 2 years.

Key words: Influenza H9N2 virus, Iranian, Pathogenicity, Phylogenetic

Introduction

Avian influenza (AI) viruses belong to the influenza virus A genus of the Orthomyxoviridae family and are negative-stranded, segmented RNA viruses. Each segment encodes at least one protein; two of these proteins, haemagglutinin (HA) and neuraminidase (NA) project through the viral envelope and are available for interaction with cellular molecules (Hughes *et al.*, 2001). Each virus has one H and one N antigen. So far, 16 H subtypes (H1-H16) and 9 N subtypes (N1-N9) have been recognized (Fouchier *et al.*, 2005). The majority of possible combinations of H and N antigens have been isolated from avian species (Banks *et al.*, 2000). Influenza A viruses are divided into two extremes of virulence for chickens. Highly pathogenic avian influenza (HPAI) isolates cause rapid mortality up to 100% while other viruses may cause mild respiratory disease with low

mortality (Naeem *et al.*, 1999). To date, all HPAI viruses have been of H5 or H7 subtypes (Banks *et al.*, 2000). Based on the pathogenicity definition of HPAI virus, any influenza A virus that kills more than 75% of 4–6-week-old chickens within 10 days following intravenous injection with 0.2 ml of 1/10 dilution of virus, is considered as HPAI (Alexander, 1986). After application of polymerase chain reaction (PCR) technique in avian influenza virus characterization, the effect of amino acid sequence at HA protein cleavage site, on virulence of virus has been determined. Molecular-based studies revealed that all HPAI viruses have multiple basic amino acids at this region causing systemic infection while non-HPAI viruses have only a pair of basic amino acids led to restricted infection (Hongqi *et al.*, 2003). Therefore, this study was performed to determine conventional and molecular basis of the pathogenicity among 16 Iranian AI viruses

isolated throughout the country during (1998–2001).

Materials and Methods

Virus isolates

In this study 16 AI viruses isolated at the Department of Research and Diagnosis of Avian Diseases, Razi Vaccine and Serum Research Institute, Karadj, Iran from suspected flocks submitted during disease outbreaks in different parts of the country were used. One passage for each isolate was conducted in specific pathogen-free (SPF) chicken embryonated eggs (Lohmann, Germany). Data of the isolated viruses are shown in Table 1.

Table 1: Data on Iranian avian influenza virus isolates

Isolates	Isolation date	Province
IR/353	Aug. 1998	Markazi
IR/430	Sep. 1998	Isfahan
IR/414	Oct. 1998	Ghazvin
IR/685	Nov. 1999	Ilam
IR/694	Dec. 1999	Tehran
IR/738	Dec. 1999	Ghazvin
IR/26	Apr. 2000	Ilam
IR/222	Jul. 2000	Mazandaran
IR/327	Aug. 2000	Gillan
IR/675	Jan. 2001	Khorassan
IR/796	Mar. 2001	Ghazvin
IR/08	Mar. 2001	Tehran
IR/221	Jun. 2001	Ghazvin
IR/261	Sep. 2001	Mazandaran
IR/577	Oct. 2001	Tehran
IR/610	Dec. 2001	Tehran

SPF chickens

Four- to six-week-old SPF chickens oriented from the same source of SPF eggs. Intravenous pathogenicity index (IVPI) tests were carried out by intravenous injection of 200 µl of 1/10 dilution of refreshed virus as described earlier (CEC, 1992). The chickens were kept under secure condition for 10 days and monitored daily.

Viral RNA extraction and RT-PCR

Viral RNA was extracted from virus samples using Tripure RNA isolation solution as instructed by the manufacturer (Roche, Germany). For cDNA synthesis a reaction mixture (20 µl) containing reverse transcription (RT) enzyme buffer, 1 µg of

Uni-12 oligonucleotide, 25 mmol dNTPs, 2.5 U of Moloney murine leukemia virus reverse transcriptase (Roche, Germany), 10 U of RNase inhibitor and 10 µl of viral RNA (100 ng/ml) was used. The RT conditions were 37°C for 10 min, 42°C for 40 min and 94°C for 5 min. The cDNA products were amplified during 37 cycles of PCR with HA gene specific primers, Fai55 and Rai55, (sequences are available on request) and 2.5 U Taq DNA polymerase (Roche, Germany). The PCR conditions were 95°C for 3 min, 94°C for 1 min, 61°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were purified using PCR clean up kit (Roche, Germany) and used for automated fluorescent sequencing.

Sequence and phylogenetic analysis

The sequencing process was carried out by MWG Biotech Company (Germany). All sequence data were identified by Blast search in NCBI database with registered AI isolates in Gene Bank. The Gene Bank accession numbers of reference sequences used in phylogenetic analysis are as follows: IT94 (AF218098), HK94 (AF156379), GER90/95 (AF218099), GER45/98 (AF218107), SA224/98 (AF218109), SA532/99 (AF218119), PAK1/99 (AF218114), PAK2/99 (AF218115), PAK4/99 (AF218117). The BioEdit software was used for initial alignment, translation and phylogenetic tree construction.

Accession numbers

The genomic data of Iranian AI viruses were deposited in Gene Bank with accession numbers AY345925-40.

Results

Pathogenicity

Clinically, among the infected groups, only depression, ruffled feather and diarrhoea were observed on third day post-inoculation. No mortality was recorded during the experiment for each isolate. Chickens in the control group did not show any clinical signs.

Genetic analysis

A 460-nucleotide region of HA gene including cleavage site area was amplified for all AI virus isolates. A segment of 447-

bp (nt577-1024, position of nucleotides was numbered according to HA sequence of H9 subtype A/chicken/HK/WF120/03) of each sequence was used for initial alignment, translation and phylogenetic tree construction. Some nucleotide substitutions among the local AI isolates resulted in 18 amino acids changes. The amino acid sequence at cleavage site (P-A-R-S-S-R-G-L) in all local isolates was similar and contained only 1 pair arginine (Fig. 1).

Analysis of the HA gene of H9N2 viruses revealed six glycosylation sites with the N-X-T/S motif (X may be any amino acid except proline); five in HA1 and one in HA2 (Kornfeld and Kornfeld, 1985). Based on the amino acid sequences of Iranian AI isolates, one of the three glycosylation sites in this segment, Asn218, which is conserved in most other AI isolates, was not found. This was due to a substitution (Thr → Ile/Val) at this area (Fig. 2).

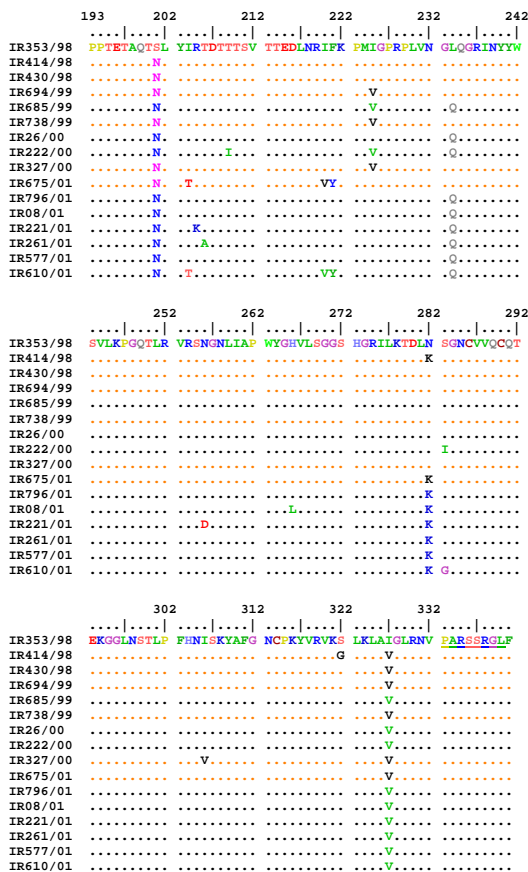


Fig. 1: Comparison of amino acids (residues 193-341) of the Iranian H9N2 viruses (position of amino acids was numbered according to HA sequence of H9 subtype A/chicken/HK/WF120/03). Cleavage site area is underlined

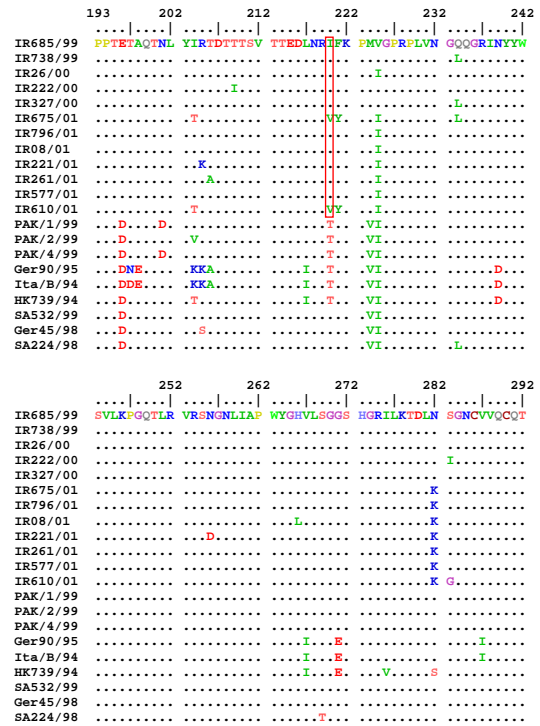


Fig. 2: Comparison of amino acids (residues 193-341) among the Iranian and references H9N2 viruses (position of amino acid was numbered according to HA sequence of H9 subtype A/chicken/HK/WF120/03). The missed and the 2 other glycosylation sites are boxed and underlined, respectively

The phylogenetic tree was constructed using BioEdit software based on Clustal method (Hongqi *et al.*, 2003). This analysis resulted in forming 3 groups; 2 for local AI isolates and 1 for reference isolates (Fig. 3). AI isolates from Pakistan, Saudi Arabia and one from Germany, (published data on Gene Bank), were closer to the Iranian isolates than others. However, they belonged to a separate group.

Discussion

The remarked feature of the influenza

viruses is variability resulted from lack of proof-reading mechanism during the viral

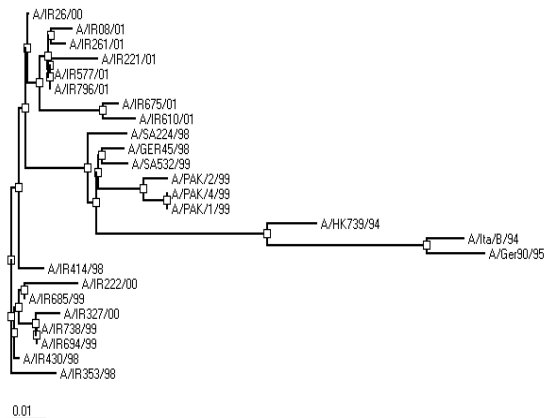


Fig. 3: Phylogenetic analysis of partial amino acid sequences (residue 193-341) of Iranian AI isolates HA gene (position of amino acid was numbered according to HA sequence of H9 subtype A/chicken/HK/WF120/03). The branch lengths are proportional to the amino acid differences between the sequences

replication and the high rate of reassortment due to segmented genome (Capua and Marangon, 2003). After the first outbreak of AI in Iran in late (1997) varying mortality rate (20–65%) from infected poultry farms were reported (Nili and Asasi, 2002). The poultry industry in Pakistan (eastern neighbouring country to Iran) had experienced a severe H7N3 outbreak of AI in (1995) (Naeem *et al.*, 1999). It was therefore necessary to determine the pathogenicity of the circulating viruses in Iran and also to determine whether a genomic variation had been occurred. In pathogenicity tests, none of the local AI isolates could fulfill the early EU/OIE definitions for HPAI viruses. Generally, infections of chicken with viruses of low virulence result in mild respiratory signs and egg production losses. However, co-infections and environmental stresses exacerbate the influenza infection resulting in severe form of the disease (Banks *et al.*, 2000). At the peak of the disease prevalence in Iran, other viral and bacterial agents like NDV, IBV and *E. coli* were also isolated from the sick and dead chickens (Kianizadeh and Pourbakhsh, unpublished data). Based on the Kawaoka and Webster (1988), the HAs of H9 viruses possess two different motifs at the C-terminus of HA1. One motif

of non-pathogenic influenza viruses consist of X-X-X-R, where X and R stand for non-basic and basic amino acids, respectively. The second motif is R-S-S-R, which is similar to the motif (R-X-R/K-R) required for highly pathogenic viruses of the H5 and H7 subtypes. In the second motif, the presence of serine amino acid has made it a fragile motif because only 1 nucleotide substitution (C to A or G) at 2 positions is enough to convert this motif to a virulent sequence. Based on the amino acid sequences, all the Iranian AI isolates possess the second amino acid motif at their cleavage site.

Like other HA subtypes, the HA of H9N2 viruses contains six potential glycosylation sites at Asn_(s) 29, 141, 218, 298, 305 and 492, of which five are located in HA1 portion and one in HA2 portion of the HA (Kornfeld and Kornfeld, 1985). All Iranian AI isolates and three of reference viruses (SA532, SA224, GER45R) used in this study lack the Asn218 glycosylation site, which is conserved in most other HA subtypes (Fig. 2). Although the Iranian AI isolates did not meet the criteria for a HPAI virus in IVPI tests and despite of the presence of basic amino acid residues at the cleavage site of HA gene, one reason for different mortality rates observed in IVPI tests and farm conditions can be attributed to the second motif (RSSR) allowing possible bacterial protease cleavage of HA, as was demonstrated previously (Callan *et al.*, 1997). Genetic relationship among the HA gene of recently H9N2 isolates from Pakistan, Germany, Iran and Saudi Arabia, based on 380 nucleotides, showing high homology with HA gene of G1 H9N2 isolated in Hong Kong (Cameron *et al.*, 2000). Based on amino acid sequence (residue 193-341) of HAs used in phylogenetic analysis, the local and reference viruses, isolated from Pakistan, Saudi Arabia, Germany, Italy and Hong Kong were grouped differently, though the AI isolates from the region (Pakistan and Saudi Arabia) and one from Germany showed less differences with Iranian isolates. Placing the local H9N2 viruses in two groups indicates that viruses in each group have been in circulation and accumulating point mutations after about

two years. Further molecular-based studies should be performed on other genome segments to find out more precise relation and possible common source between the local and neighbouring countries H9N2 isolates. Furthermore, from a public health point of view, because of the transmission to humans by this subtype (Peiris *et al.*, 1999), circulation of H9 viruses in poultry population has posed a potential threat for human health as well. To understand any transmission and genetic changes, regular molecular monitoring of the circulating human and AI isolates is needed.

Acknowledgements

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