Neuraminidase gene sequence analysis of avian influenza H9N2 viruses isolated from Iran (1998-2001)

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ABSTRACT

Influenza A viruses possesses two virion surface glycoproteins including haemagglutinin (HA) and neuraminidase (NA). The NA plays an important role in viral replication and promotes virus release from infected cells and facilitates virus spread throughout the body. To find out any genomic changes that might be occurred on NA gene of avian influenza circulating viruses, we have genetically analyzed the neuraminidase gene of six Avian Influenza (AI) viruses H9N2 subtype isolated from different parts of Iran. A comparison of deduced amino acid sequences, showed some amino acid substitutions among the local AI isolates. However no insertions/deletions or shortening in the stalk region of the genes were observed. Mutation in Glu 119 as a marker for enzyme sensitivity to the antiviral drugs was not observed. Phylogenitic analysis revealed three distinct groups among the isolates of Iran, Hong Kong, and Pakistan/Japan/Saudi Arabia respectively. Based on the results, no significant mutations in NA genes of the viruses isolated during the period of the study occurred and our findings are in agreement with results of previous study of the viruses indicated a low pathogen character for the isolates on the basis of amino acid sequence of HA cleavage site and experimental infection.

Keywords: Avian influenza, H9N2, Neuramindase gene

INTRODUCTION

Avian influenza (AI) viruses can cause serious diseases in a wide variety of birds and mammals, but its natural host ranges are wild ducks, gulls and shorebirds (Suarez and Schultz-cherry 2000). The causative agent is a segmented, negative- sense, single stranded RNA virus of the family *Orthomyoviridae* and is divided into types A, B and C on the basis of the antigenic character of their internal nucleoprotein and matrix proteins (Naeem *et al* 1999). All avian influenza viruses blong to type A viruses. Influenza A viruses are further categorized into different subtypes based on the antigenicity of surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). There are 16 different HA antigens (H1 to H16) and nine different NA antigens (N1 to N9) among

influenza viruses (Fouchier *et al* 2005). While the majority of avian influenza viruses are avirulent, viruses of a limited number of subtypes cause severe disease in birds. This difference in pathogenicity among avian influenza viruses is primarily determined by the amino acid sequence at the viral haemagglutinin cleavage site. The influenza receptor binding site (RBS) is a pocket of conserved amino acids on the globular head of the HA surrounded by varying antigenic regions. Changes in HA receptor binding are concomitant with changes in the viral NA because of the requirement for a functional balance between receptor binding activity and the receptor destroying activity of NA (Thompson *et al* 2004).

NA is a tetramer protein composed of a cytoplasmic tail, a transmembrane domain, a stalk region and a globular head (Gubareva *et al* 2000).The main function of NA is the promotion of virus release by removing sialic acid from newly synthesized HAs and NAs which are sialisylated by cellular enzymes. NA has also been considered a suitable target for antiviral drugs, since it possesses an active site whose amino acid sequence is conserved among all types and subtypes of influenza virus (Bantia *et al* 1998). The H9N2 virus subtype was first isolated from turkey in North America in 1966. Following long-term surveillance in Hong Kong from 1975 to1985 detected H9N2 viruses in apparently healthy ducks but not in chickens (Guan *et al* 1999). Since the early 1990s, H9N2 influenza viruses have become widespread in domestic chickens in Asia. Isolation of two H9N2 viruses from human for the first time in Honk Kong in 1999 drew attention of public health authorities worldwide from the view pandemic preparedness. After the first outbreak of H9N2 avian influenza in Iran in early 1998 that has imposed great economical losses to the poultry industry, some general and molecular-based studies have been conducted mostly on HA protein to evaluate its probable significant mutations related to varied

pathogenicity features of the local AI isolates (Pourbakhsh *et al* 2000, Toroghi *et al* 2006). Because of different roles of NA gene in pathogenicity, host rang and antiviral properties, any genetically mutations can affect the virus behavior in mentioned area. This study was carried out for the first time to determine genetically changes in NA gene of circulating H9N2 influenza viruses, if any, in Iran

MATERIALS AND METHODS

AI isolates. In this study six AI viruses isolated from different parts of the country were obtained from   Research & Diagnosis of Avian Diseases Department of Razi Vaccine & Serum Research Institute, Karadj, Iran. Data of the viruses are shown in Table 1.

Table 1: Iranian H9N2 virus isolates

Virus	Isolation region	Isolation date
IR430	Isfahan	1998
IR738	Ghazvin	1999
IR496	E.Azarbayjan	1999
IR327	Gillan	2000
IR261	Mazandaran	2001
IR675	Khorassan	2001

Virus propagation. To gain better RNA yield, AI isolates comprising IR430, IR738, IR496, IR327, IR261, IR675 were inoculated into the embryonated SPF eggs.

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 (30 µl) containing reverse transcription (RT) enzyme buffer, 1 µg of Uni-12 oligonuclotide, 25 mmol dNTPs, 2.5 U of Moloney murine lekemia virus reverse transcriptase (Roche, Germany),10 U of RNAse inhibitor and 5µl of viral RNA (100 ng/ml) was used. The RT conditions were 37 ^{0}C for 10 min,

42 ^{0}C for 40 min and 94 ^{0}C for 5 min. The cDNA products were used to amplify the NA gene in 2 segments with expected size826 and 711 nt (135 nt overlap) using 2 pairs designated primers (sequences are available on request). Amplification was done during 37 cycles using following PCR profile 95 ^{0}C for 3 min, 94 ^{0}C for 1 min, 61 ^{0}C for1 min, 72 ^{0}C for 1 min and final extension at 72 ^{0}C for 10 min.

Sequence and phylogenetic analysis. Purification of PCR products was performed using the High Pure PCR Product Purification Kit (Roche, Germany). 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 GenBank. The GenBank accession numbers of reference sequences used in phylogenetic analysis are shown in Table 2.

**Table 2:** AI H9N2 isolates compared in this study.

Virus	Source	Accession No
HK/G9/97	Genbank	AF156391
Chiba/1/97	Genbank	AB049163
Narita/92A	Genbank	AB049164
Pak/5/99	Genbank	AF508577
Pak/4/99	Genbank	AF508576
SA/532/99	Genbank	AF508580
IT/11T/99	Genbank	AF508579
HK/NT16/99	Genbank	AF222654
HK/KC12/99	Genbank	AF222658
HK/SF2/99	Genbank	AF222660
HK/1073/99	Genbank	NC-004909

The BioEdit and ExPasy softwares were used for initial alignment and translation. The phylogenetic tree was constructed using BioEdit software based on Clustal method.

RESULTS

PCR Products. The entire NA gene in two segments for each AI isolate was amplified. The expected size of PCR products were confirmed by gel electrophoresis.

Sequencing analysis. Following compilation, 1402 bp PCR products of NA gene of each AI isolate was aligned. Sequencing analysis revealed some amino acid changes among the Iranian AI isolates with no insertion or deletion in NA gene. Deduced amino acid comparison on 462 amino acids long region among the Iranian and some neighboring and regional countries isolates showed difference in amino acid sequences. All the Iranian AI isolates possessed 7 glycosylation sites (N-Link) as most H9N2 isolates. Comparing the reference AI isolates used in this study, HK/G9 showed 2 extra N-link (N44, N309). Further more, HK/SF2 and HK/KC12 with 3 amino acids deletion in the stalk region, missed one N-link (N61) and isolate SA532/99 missed N-link 146 due to amino acid substitution at this position, while Iranian isolates did not show any insertion or deletion in mentioned area (Figure 1).
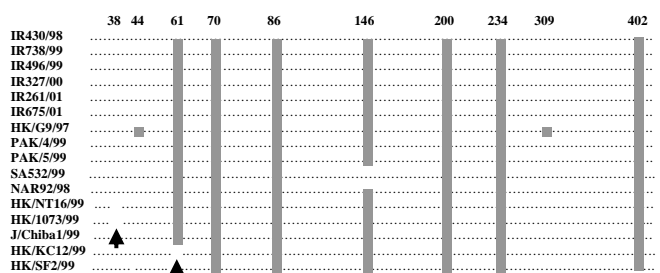
Figure 1. The potential glycozylation sites of NA protein of H9N2 influenza viruses. Arrows show deletion N-link sites.

Phylogenetic analysis. Phylogenetic analysis was performed using amino acid data of Iranian and 11 other isolates from the neighbouring and regional countries isolates (data from GenBank) on 462 amino acids long region. Based on the results, all the AI viruses used in this analysis were classified

in three distinct groups (Figure 2). Comparing six Iranian isolates, 2 isolates including IR430 and IR675 that showed more changes placed separately from the other 4 isolates which placed in group one. Isolates from Honk Kong classified in group two. Group three contained isolates from Japan, Pakistan and Saudi Arabia. Two AI isolate from Pakistan and isolates from Japan showed less distance from the root compared to the Saudi Arabia and Hong Kong isolates. The sequencing data of Iranian AI isolates were deposited in GenBank with accession number: AY785752-7.
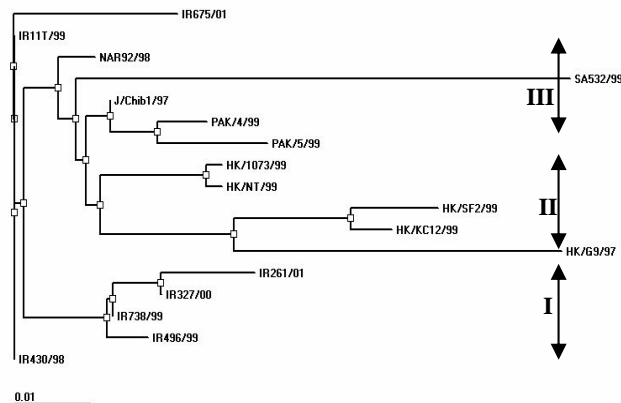
Figure 4. Phylogenetic tree based on 462 amino acids of NA gene. Group I: Iranian Isolates, Group II: Honk Kong Isolates, Group III: Japan/ Saudi Arabia isolates.

DISCUSSION

Avian influenza caused by H9N2 subtype hit the poultry industry in Iran in early 1998 and lifted heavily losses since then. Different comments have been revealed based on clinical and experimental infection features of the viruses regarding their pathogenicity (Banks *et al* 2000, Vasfi & Bozorgmehri 2001, Vasfi & Bozorgmehri 2002, Nili & Asasi 2002). Potential of antigenic variability is the main problem with AI viruses resulted from two main phenomenon, shift and drift. RNA viruses generally have very high mutation rates as they lack DNA polymerases which can find and fix mistakes,

and are therefore unable to conduct DNA repair of damaged genetic material. Reassortment rate in segmented genome viruses is high when individual cells are infected with two different influenza viruses (Capua & Marangon 2003). Among the Viral encoded proteins by the genome, the two outer glycoproteins, HA and NA have the major roles in antigenic feature and epidemiological behavior changes. The HA is synthesized as a polyprotein precursor (HA0) that is post translationally cleaved into two subunits. This cleavage step is necessary for virus infectivity. Studies of pathogenicity showed that the optimal combination of haemagglutinin (HA) and neuraminidase (NA) enabling the cleavage of HA was important (Webster *et al* 1971). Former study on HA protein of Iranian AI isolates revealed the motif amino acid at cleavage site (P-A- R-S-S-R-G-L) of isolates that contained only 1 pair arginine similar to all non highly pathogenic AI viruses (Kianizadeh *et al* 2006). Presence of serine amino acid at HA cleavage site of Iranian isolates has made it a fragile motif due to one nucleotide substitution (C to A or G) at two positions is enough to convert this motif to a virulent sequence.

Several studies indicate that the NA plays some role in pathogenicity (Goto *et al* 2001, Hulse, *et al* 2004). The NA protein facilitates the mobility of virions by removing sialic acid residues from the viral HA during entry and release from the cells (Els *et al* 1989). Virus particles with low NA activity cannot be efficiently released from infected cells. A balance in HA and NA activities is crucial: there must be enough HA activity to facilitate virus binding and enough NA activity to allow release of virus progeny (Itnaul *et al* 2000). It has been shown that greater NA activity results in higher HA cleavage in multiple organs, thereby enhancing virulence, specifically neurovirulence in mice (Li *et al* 1993). Results of a study indicated that NA mutation can contributed to the ability of influenza A virus to adapt to new environments (Hinshaw *et*

al 1983). Glycosylation can affect the structure, stability, localization and turn over of NA protein. Most of the H9N2 viruses contain 7 glycosylation sites, 3 in the stalk region and the rest on the head with the N-X-T/S motif (X may be any amino acid except proline). Variation in glycosylation site and stalk region length are main marker in pathogenicity role of NA. Some amino acid changes in NA gene of the Iranian AI isolates were observed of which some were more common such as amino acids 59, 67,144, 253,307 and 378. Substitutions in stalk region, 59 and 67, did not cause any changes in stalk region length or N-link sites and all the six Iranian AI isolates, possessed 7 potential glycosylation sites as most H9N2 isolates. Among the reference AI isolates used in this study, HK/G9 showed two extra N-link (N44, N309). Isolates HK/SF2 and HK/KC12 with three amino acids deletion in the stalk region, missed 1 N-link (N61). AI isolate from Saudi Arabia missed N-link 146 due to amino acid substitution at this position. The mechanism by which an additional glycozylation site in NA may affect pathogenicity is unknown, but it is most likely to be linked to the cleavage of HA in cells. For example, the highly glycozylated NA of the highly pathogenic virus may facilitate increased activation of the host proteases required to cleave the virus's HA, a mechanism suggested by Schulman. (Hulse *et al* 2004).

Since NA is a suitable target for antiviral drugs, any mutation may affect on enzyme sensitivity to antiviral drugs. The most common neuroaminidase mutation which arises under pressure of zanamivir is the conserved Glu 119, cause 100-fold reduction in the sensitivity of the enzyme to zanamivir (Tai *et al* 1998). All Iranian AI isolates showed conservation in residue Glu119. In phylogenitic relation three distinct groups were formed for isolates from Iran, Hong Kong, and Pakistan/Japan/Saudi Arabia respectively. Among the neighbouring countries isolates placed in separate groups, two AI isolate from Pakistan showed a less distance from the root than the Saudi Arabia isolate. Different position of Iranian isolates from the root in phylogenetic tree indicates that accumulating point mutations have been occurred during the circulation of the viruses since 1998 up to 2001. Grouping the Iranian AI isolates separately from the other isolates used in phylogenetic analysis, make it difficult to determine the precise origin of the Iranian isolates. Further molecular studies on different genes of AI isolates are needed to answer the question. Presence of two subtypes (H9, H7) in Pakistan has induced a critical condition for occurrence of an AI infection by a new subtype in Iran. Co-circulation of two AI subtype provides increased opportunities for re-assortment. From public health point of view, since both H9 and H7 subtypes have capability to infect human, to decrease human exposure and keep the disease under control it is important to have H9 and H7 viruses high on the list of potential candidate for next outbreak but with different features. So continues molecular monitoring on Iranian human and poultry AI isolates is recommended.

Acknowledgment

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