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Phylogenetic analysis of neuraminidase gene of H9N2 avian influenza viruses isolated from chicken in Iran during 2010-2011

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ABSTRACT

Backgrounds and Objectives: Classified as low pathogenic avian influenza (LPAI) viruses, the H9N2 subtype causes severe respiratory disease in poultry farms and occasional respiratory disease in humans. In this study, the neuraminidase (NA) gene of three Avian Influenza (AI) H9N2 strains isolated from poultry farms in Iran during 2010-11, as well as other reported Iranian H9N2 isolates, were genetically analyzed and their nucleotide changes were evaluated.

Materials and Methods: The NA gene of three AIVs were sequenced and evaluated for genetic characteristics and phylogenetic relationship.

Results: One new potential glycosylation site (PGS) at amino acid position 306 was observed in one of the studied isolates (A/Chicken/Iran/N102/2011). Antigenic sites of NA in Iranian H9N2 isolates have varied in a yearly manner. The Iranian isolates can be divided into 2 main subgroups; 11-T like subgroup viruses isolated mainly during 1998-2004 and second subgroup viruses isolated during 2004-2009. Interestingly, the three studied isolates fell into a third subgroup. The nucleotide sequences of the three studied isolates showed high identity to recent Pakistani H9N2 isolates (94.5-97%) compared to former Iranian AIV isolates (89-94%).

Conclusion: High frequency of substitutions in the NA gene of studied isolates in recent years and effects of those substitutions on the pathogenicity of AI virus highlight the need to continue surveillance of genetic characteristics of AIV H9N2 in Iran.

Keywords: Avian influenza, H9N2, Iran, Neuraminidase, Phylogenetic analysis

INTRODUCTION

Influenza A virus is a member of the *Orthomyxoviridae* family. The surface of the virion is covered by 2 types of glycoprotein: Hemagglutinin (HA) and Neuraminidase (NA). Facilitating the budding of progeny virions from cells, the NA removes sialic acid from cell surface glycoconjugates and newly synthesized viral proteins (1). Several studies have implicated the NA as an important protein in the pathogenicity of AIV (3- 6). The surface NA glycoprotein of influenza viruses are an important target for host neutralizing antibodies. Point mutations in the antigenic domains of the NA protein are considered as a way to escape host immune system for viruses (7).

Avian influenza (AI) viruses naturally circulate in wild aquatic birds (2). They transmit the viruses to domestic birds. Some adaptive changes needed to happen in these viruses, to generate increased transmissibility and pathogenicity of the viruses for domestic poultry (8). AIV H9N2 isolates are classified as 3 major lineages; North American, human and swine, and European–Asian. The last lineage was also subdivided into 3 sublineages represented by prototypes A/Duck/Hong Kong/Y280/97(Y280), A/Quail/ Hong Kong/G1/97 (G1) and A/ Chicken/ Korea/38349-p96323/96 (Korean) (15).

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Since the late 1990s, outbreak of AIV H9N2 subtype in poultry farms in many Asian countries, including the Middle East as well as Iran, has been reported (9). The H9N2 subtype is classified as low pathogenic avian influenza (LPAI). However, severe respiratory distress accompanied by high morbidity and mortality rate and marked reductions in egg production in poultry farms has been reported, particularly in combination with other respiratory pathogens (10, 11). The H9N2 viruses have caused occasional respiratory disease in humans and became a global concern for human societies (12, 13). Interestingly, it is proposed that H9N2 viruses donated the internal genes to human H5N1 viruses in Hong Kong in 1997 (14).

In this study, the neuraminidase gene of three AI H9N2 strains isolated from poultry farms in different regions of Iran during 2010-11, as well as, selected Iranian H9N2 isolates were genetically analyzed and their nucleotide changes were discussed.

MATERIALS AND METHODS

Viruses. The strains of A/Chicken/Iran/N101/2011 (H9N2), A/Chicken/Iran/N102/2011(H9N2) and A/Chicken/Iran/EBGV-88/2010(H9N2), were isolated from industrial poultry farms with high mortality rate affected by respiratory disease complex. The most frequent signs of affected flocks included severe respiratory signs such as coughing, rales, excessive lacrimation and gasping. In addition, non characteristic generalized clinical signs including huddling, ruffled feathers, depression, decreased activity and diarrhea were seen.

Initial isolation was performed in 10-day-old embryonated chicken eggs (ECE) according to the standard method (16). Briefly, homogenized tissue samples (lung, trachea, intestine) treated with antibiotic and antimycotic solutions (penicillin 10,000 IU/ml, streptomycin 2 mg/ml, nystatin 20,000 U/ml) for 30 min at 37˚C and centrifuged at 1500×g for 10 min. Then, the homogenate were inoculated through allantoic routes. The allantoic fluid was harvested after incubation at 37˚C for 3–4 days and clarified by centrifugation at 1500 × g for 15 min at 4˚C. HA test negative samples were given two more passages and tested again before being declared negative.

Subtype identification of the virus was determined by standard hemagglutination-inhibition (HI) using standard chicken antisera (17) and specific PCR assay for HA and NA. Amplification of the H9 gene was carried out by PCR using one pair of diagnostic primers H9F: 5'- TTG CAC CAC ACA GAG AAT-3', H9R: 5'- TGA TGT ATG CCC CAC ATG AA-3' (18). Amplification of the N2 gene was performed by a pair of primers mentioned later in the RT-PCR protocol. Allantoic fluid was harvested from ECEs for RNA extraction.

RNA extraction and RT-PCR protocol. Viral RNA was obtained from allantoic fluid by RNX plus kit (Cinnagen, Iran), according to the manufacturer instruction. Reverse transcription was done by using influenza universal primer, Uni12: 5'-AGC AAA AGC AGG-3'. Amplification of the neuraminidase full-length gene was carried out by PCR assay (19). The length of amplified fragment was 1447 bp. The sequences of specific primers were as follows:

NA-F: 5'-AGC AAA AGC AGG AGT GAA AAT GAA-3' and NA-R: 5'-TTC TAA AAT TGC GAA AGC TTA TAT-3'.

Gene sequences. PCR products were subjected to electrophoresis in a 1% (w/v) agarose gel. DNA fragments of the expected length were extracted and purified with GeneJET™ Gel Extraction kit (Fermentas, Canada, catalogue#k0691). The purified DNA fragments were cloned into pTZ57R/T cloning vector (Fermentas, Canada, catalog#k1213). Three clones of each fragment were sequenced using M13 forward and M13 reverse (Promega, USA) at Sequetech Co. Ltd., USA. All the other H9N2 sequences of neuraminidase genes used in this study were available in the GenBank database.

Sequence analysis and phylogenetic study. Pairwise sequence alignments were performed with the Clustal W alignment algorithm. Sequence similarity and phylogenetic relationship of different H9N2 subtype viruses was performed with MegAlign program (DNASTAR Inc., Madison, WI, USA).

RESULTS

Analysis of the neuraminidase sequence. Full coding segment of the NA gene of the three isolates (A/Chicken/Iran/N101/2011(H9N2), A/Chicken/Iran/N102/2011(H9N2) and A/Chicken/Iran/EBGV-88/2010(H9N2)) were sequenced. The sequences determined in this study are available in the GenBank under accession numbers: JQ307201, JQ307202 and

JX465625.1.

Amino acid sequences of those isolates were deduced from the nucleotide sequences. The length of coding sequence (CDS) of NA gene is 1410 base pairs. The AI virus (H9N2) strains sequenced in this study had no deletion of amino acid in the stalk of the NA protein.

Amino acids of hemadsorbing (HB) sites at position 366-373 of these isolates were –IKKDSRAG-, amino acids at position 399-404 were –DSDNRS- and amino acids at position 431-433 were –PQE- (Table 1).

Almost other isolates (H9N2) from Iran as well as many other G1-like viruses including A/Quail/HK/G1/1997 had seven potential glycosilation site (PGS) with N-X-T/S motif, in which X may be any amino acid except proline (positions 61, 69, 86, 146, 200, 234, 402). Three viruses sequenced in this study and many viruses (H9N2) isolated after 2009 have one more PGS at position 44 compared to previous isolates due to substitution 45P→S. Sequence of one isolate (A/Chicken/Iran/N102/2011) has possessed a new PGS at position 306 compared to Qa/HK/G1/97 virus due to substitution 308A→T (Table 1).

Butt *et al.,* (2011) determined epitopes of NA proteins of Pakistani AIV H9N2 isolates by the Cytotoxic T Lymphocyte (CTL) epitope prediction method (20). Epitope (SCHDGRAWL) from the position 182-190 that was absent before 2004, appeared in Iranian H9N2 viruses in 2004 and seems to be stable in all Iranian H9N2 isolates, including three isolates sequenced here, since 2008. Three epitopes (FSKDNSIRL, SCYPRYPEV and GSNRPVLYI at position 100-108, 279-287 and 297-305, respectively) were conserved in three studied and other Iranian H9N2 isolates (Table1).

Phylogenetic analysis. All studied Iranian H9N2 isolates fell into the same main group (G1 sub-lineage) as the viruses isolated in Pakistan. The Iranian isolates can be divided into 2 main subgroups (Fig. 1). The first subgroup (11-T like sub-lineage) included viruses that were isolated between 1998, first AI outbreak reported in Iran, and 2004. The former Pakistani isolates (A/chicken/Pakistan/4/99) were placed in a close relationship with this subgroup of Iranian isolates. The second subgroup comprised the strains isolated from 2004 to 2008. However, some exceptions were seen in both subgroups. Interestingly, 3 viruses isolated in this study on 2010-2011 didn't place with other Iranian isolates in the second subgroup, but placed in a new subgroup in a close relationship with some recent Pakistani H9N2 isolates (A/chicken/Pakistan/UDL-01/2006) (Fig. 1).

Analysis of the nucleotide sequences of the studied Iranian isolates available in Genbank showed that the difference between the studied isolates and first subgroup isolates was relatively high compared to the second subgroup (Table 2). The viruses belonging to the first subgroup exhibited 89.3-93% (average 91.3%) identity with the studied isolates, whereas the viruses belonging to the second subgroup exhibited 92.2-94.3% (average 92.7%) identity with the studied isolates. The nucleotide sequences of the three Iran isolates showed high identity (99.4%) to each other. Interestingly, three studied isolates showed higher identity to new Pakistan isolates (94.5-97%) than to the identity with the first and second subgroup of Iranian H9N2 isolates (91.3-92.7%). The percent of homology of the three studied isolates with one of the first Iranian isolates (A/chicken/Iran/ZMT-101/98), the prototype isolate of G1-like sub-lineage (A/Quail/Hong Kong/G1/97) and human H9N2 isolates (A/HongKong/1074/99) were 93%, 92.5% and 92.5%, respectively.

DISCUSSION

The importance of NA activity in influenza infection is well-known and its role in infection is more complex than just destroying viral receptors. Many roles for NA in the pathogenicity of AI virus have been proposed (3- 6).

Amino acids at the HB site are conserved among aquatic bird viruses, while those of human and swine viruses are easily substituted and can result in the decreased hemadsorption capacity (22). Matrosovich *et al.* (2001) have shown the HB site in the NA of Asian H9N2 viruses to be under positive selection pressure for mutations, which result in compatible combinations of HA and NA (23). Poultry adapted H9N2 viruses, including isolates sequenced in this study and other Iranian isolates, contained substitutions in HB sites similar to those detected in both A/Qa/HK/G1/97 and A/Dk/HK/Y280/97 (Table 1). Such substitutions were similar to those H9N2 viruses isolated from Pakistan and the United Arab Emirates (24, 25). At residue 403 in H9N2 viruses, amino acids W, L, S or R was observed. The biological significance of any of these substitutions in the HB site is unknown.

Table 1. Amino acid substitutions in HB sites, antigenic sites and PGS of NAs of Iranian H9N2 isolates.

Virus	HB sites		antigenic sites							PGS	
	366	399	431	2-10	100-108	182-190	248-256	279-287	297-305	461-469	44	306
A/Ck/Ir/EBGV-88/10	IRKDSRAG	DSDNLSGY	PQE PQE	-[a]	+[b]	+	+	+	+	-	+	-
A/Ck/Ir/N101/11	IKKDSRAG	DSDNRSGY	PQE	-	+	+	+	+	+	-	+	-
A/Ck/Ir/N102/11	IKKDSRAG	DSDNRSGY	PQE	-	+	+	+	+	+	-	+	+

[a]: absence of antigenic site or PGS
[b]: presence of antigenic site or PGS

The NA protein of almost all G1-like viruses including A/Quail/HK/G1/1997 have 7 potential glycosilation site (PGS) (15). Three viruses sequenced in this study and many viruses isolated after 2009 have one more PGS at position 44 compared to previous isolates due to substitution 45P→S. One isolate (A/Chicken/Iran/N102/2011) has possessed a new PGS at position 306 compared to Qa/HK/G1/97 virus due to substitution 308A→T (Table 1). Such changes in the PGS pattern of NA protein may affect function of the protein and consequently the virus pathogenicity (19).

A study on evolution of AIVs in Middle East and Indian sub-continent showed variability in antigenic sites of HA during 1994-2009 (26). Antigenic sites for NA (Table 1) also showed a great deal of variation, sometimes in a yearly manner. This finding is in agreement with Xu *et al.* (2007) who demonstrated that antigenic diversity in H9N2 viruses corresponds with phylogenetic relationships (27). NA epitopes could have evolved due to antigenic drift and reassortment or antigenic shift. Antigenic diversity in H9N2 isolates also has a great effect on vaccine effectiveness (28).

In Iran, Pakistan and China, under national control program for AI, most poultry flocks vaccinate against AI (H9N2 subtype), using vaccines made of early isolates at the beginning of the outbreak (21, 25, 28). It is revealed that vaccines could induce faster rates of antigenic drift in human and avian influenza viruses possibly due to inducing a positive selective pressure in the host society (29, 30). Sun *et al.* (2010) found that commercial inactivated Ck/SD/6/96 vaccine cannot protect chickens against some of heterologous H9N2 viruses in cases of virus shedding or, even, appearing of clinical signs. There is a concern about that the antigenic differences between the vaccine strain and prevailing viruses might worsen the continued antigenic drift of H9 avian viruses (31).

Table 2. Degree of homology of the nucleotide sequences of NA of Iranian isolates within and between different subgroups and isolates.

subgroup	Similarity between viruses (%)		
	Three studied isolates		
	Max	Min	Average
First Subgroup	93	89.3	91.3
Second Subgroup	94.3	92.2	92.7
Three studied isolates		99.4	

Four mutations (E119G, E119A, E119D and R292K) and two mutations (E119V and N294S) in the NA gene have been identified as a source of Zanamivir and Oseltamivir resistance in influenza A viruses of N2 subtype, respectively (32). None of the Iranian isolates were found to have mutations that are known to cause resistance to NA inhibitors (NAI) (data not shown).

The NA protein has been related to the potential of AIVs to pass species barrier and adaptation to human and mice (6). Zhang *et al.* (2011) reported that two amino acid substitutions located on the head of NA, Q88R and D356N, may be important in increased pathogenicity of the virus for mice (6). These substitutions were not seen in studies on Iranian H9N2 isolates (data not shown).

Previous studies on phylogenetic analysis of NA gene have determined that all Iranian H9N2 viruses fell into a special group and suggested that they came from a single progenitor (33-35). It seems that H9N2 isolates from Pakistan, UAE, Palestine, India and Iran showed very close relationships and represent a single sub-lineage, G1-like, indicating they might have originated from a common source. However, there are some ecological barriers to virus movement based around geographic location, with isolates grouped according to country of isolation (26). The Iranian H9N2 isolates can be further divided into 2 main subgroups (Fig. 1). The first subgroup (11-T like subgroup) viruses isolated mainly between 1998 and 2004 and the second subgroup mainly from 2005

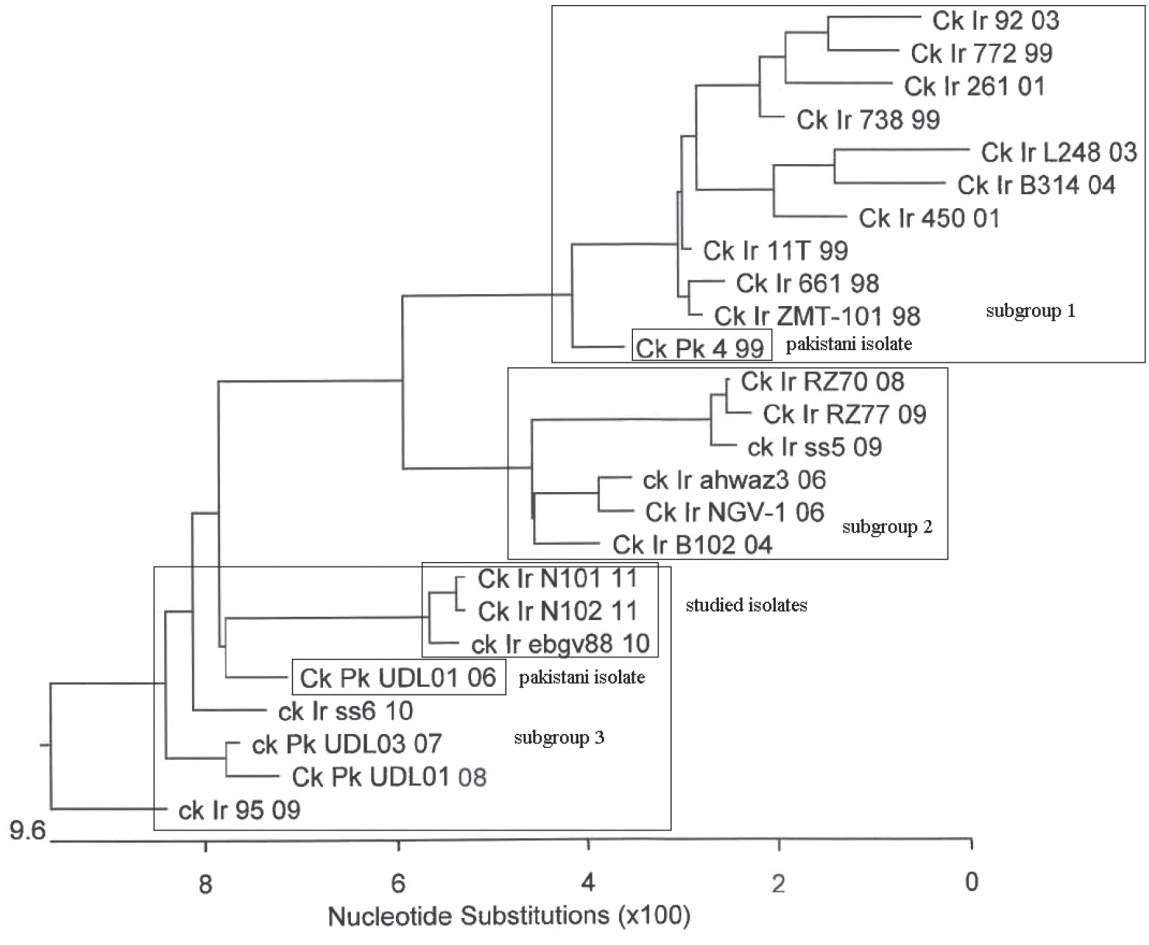

Fig. 1. Phylogenetic relationships of NA gene of some influenza A viruses isolated in Iran and Pakistan. The lengths of horizontal lines are proportional to the minimum number of nucleotide differences required to join nodes. Vertical lines are for spacing branches and labels. ck, chicken; pk, pakistan; Ir, Iran.

to 2009, with some exceptions in both groups. The 3 studied isolates did not fall within any of these subgroups, but they formed a third subgroup (Fig. 1). This fact shows high degree of difference between new studied Iranian isolates and previous isolates.

These results affirmed that for each subgroup of Iranian H9N2 viruses, probably there is one common progenitor, but viruses in the first and third subgroups had a close relationship to viruses isolated from Pakistan at the same times (Fig. 1). So, we cannot easily conclude that isolates from these two neighbor countries are different in the progenitor. The three isolates sequenced in this study had more homology to new Pakistan isolates (2005-2008) compared to former Iranian isolates and in the phylogenetic tree placed closer to recently isolated Pakistani isolates than other Iranian isolates (Fig. 1).

In conclusion, high difference between the NA gene of three studied isolates and previous Iranian H9N2 isolates, on one hand, and high similarity between three isolates and recent Pakistani H9N2 isolates, on the other hand, suggest that the origin of the studied isolates, now circulating in poultry farms of Iran, may be from Pakistan. High frequency of substitutions in the NA gene of studied isolates in recent years and effects of those substitutions on the pathogenicity of AI virus highlights the need to continue surveillance of genetic characteristics of AIV H9N2 in Iran.

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