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Original Article

Nucleoprotein (NP) Gene Based Phylogenetic Analysis of Iranian H9N2 Avian Influenza Isolates during 1998-2011

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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 Eurasia. In Iran, the H9N2 virus was first isolated from broiler chickens in 1998 in Ghazvin province and it is the most prevalent subtype of influenza virus in poultry industry in Iran at the present time.

Materials and Methods: In this study, we sequenced and analyzed Nucleoprotein (NP) gene of six AI H9N2 isolates from broiler farms of different parts of Iran from 1998 to 2011 to show probable changes since first advent.

Results: Results indicate that nucleotide homology among these isolates with NP genes is between 91.8% to 98.8%. The divergences between isolates have significantly been increased since 2007.Iranian AI H9N2 Isolates based on NP gene divided in two distinct clusters according to their isolation year. Group 1 is located in Y-439 clade and Group 2 is located in G1 Clade. Iranian H9N2 isolates of avian influenza virus show more amino acid substitutions Compare to those found in human H9N2 isolates.

Conclusion: The results shown here that further gene reassortment has occurred subsequent to the emergence of viruses in the Middle East highlights the potential for viruses to evolve rapidly.

Keywords: Avian Influenza; H9N2; NP; Phylogenetic Study; Iran

Introduction

The genome of influenza A virus, a member of the Orthomyxoviridae, is composed of eight separate segments of single-stranded, negative-sense RNA (1, 2). 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. Avian influenza viruses bearing 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes, which those have been identified and isolated from birds (1, 2). The AIV H9N2 subtype was first reported to infect turkeys in the United States in 1966 and has been panzootic in Eurasia and has been isolated from terrestrial poultry worldwide (3). Generally, the affected

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chickens show mild to severe respiratory signs, edema of the head and the face (head and face edema), and decreased egg production accompanied with soft-shelled or misshaped eggs. The mortality is usually about 5–30% depending on the type of husbandry (4) . The Eurasian lineage can be further divided into three major sub lineages; the G1 lineage, represented by A/Quail/Hong Kong/G1/97 (G1-like); theY280 lineage, represented by three prototype viruses A/duck/Hong Kong/Y280/97 (Y280-like), A/Chicken/Beijing/1/94 (BJ94-like), and A/Chicken/Hong Kong/G9/97 (G9-like) and the Korean lineage, represented by A/chicken/Korea/38349-p96323/96 (Korean-like) and A/duck/Hong Kong/Y439/97 (Y439-like) (3, 5, 6). Influenza virus nucleoprotein (NP) is a structural protein which encapsidates the negative strand viral RNA. NP is one of the main determinants of species specificity. The question of how far the NP gene can cross the species barrier by reassortment and become adapted by mutation to the new host has been discussed (7). In Iran, the H9N2 virus was first isolated from chickens in 1998 in Ghazvin province and it is the most prevalent subtype of influenza virus in poultry industry in Iran at the present time. The mortality rates in some broiler farms in Iran have been up to 65%. (8). The first report of HPAI H5N1 incidence in Iran came from swans in north of Iran in 2006. Since then there is no official report on HPAI occurrences from commercial poultry farms in the country (9). Although Fereidoni S *et al* reported the detection of some other avian influenza virus subtypes from migratory birds in Iran (10). Up to now, there is just three submitted NP nucleotide sequence of H9N2 Iranian isolate in Genbank (11). In present study, we sequenced and analyzed NP gene of six AI H9N2 isolates from broiler farms of different parts of Iran from 1998 to 2011 to show probable changes since first advent.

Methods

Viruses

Six H9N2 viruses tested in this study have been isolated from 1998 to 2011 from different outbreaks of broiler farms in Iran. The virus subtype identification was determined by standard PCR tests. Initial isolation was performed in 10-day-old specific pathogen free (SPF) embryonated chicken eggs and stored at -70°C for use in all the experiments described here in (12).

RNA Extraction

Viral RNA was extracted from 200 µl/l virus-positive allantoic fluid using using *ExiPrep*™ Viral DNA/RNA Kit from Bioneer, Korea according to the manufacturer's instructions. The RNA (50ul) was stored at -70°C until use.

RT-PCR Reaction

RT-PCR was carried out in a 50µl reaction mixture containing 10µl of 5× reaction buffer, 4 µl of mixed dNTPs (2.5 mM each), 1µl of AMV enzyme (Titan one tube RT-PCR system kit, Roche Diagnostic, Germany), 1µl of each primers (NPH9F: AGC RAA AGC AGG GTW GAT AAT CAC, NPH9R: AGT AGA AAC AAG GGT ATT TTT C) (10 ρmol each), 4 µl of RNA template, 2.5µl DTT, 3µl 25mM MgCl2, and 23.5µl of H2O. PCR program was 42°C for 30 min, 94؛C for 3 min, 30 cycles of 94°C for 30s, 56؛C for 30s, 72°C for 70s, followed by 72°C for 10 min.

Phylogenetic Study

The PCR product s were purified by the PCR AccuPrep® PCR Purification Kit (Bioneer Co., Korea) and Purified PCR products were used as a template for sequencing on an Applied Biosystems 373S automated DNA sequencer using dye terminator cycle sequencing chemistry (Perkin-Elmer/Applied Biosystems, Foster City, CA, U.S.A.). Purified PCR products were sequenced from both directions. A BLAST analysis was initially performed using NP sequence of mentioned study to establish its identity to GenBank accessions. A comparative analysis of NP 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).

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Accession Numbers

The nucleotide sequences obtained in this study have been submitted to the GenBank database and are available under accession numbers (JQ043382 to JQ043387).

Results

The lengths of NP gene were obtained for the six isolates. BLAST software and Megalign programs were used to determine the sequence similarity of the eight genes from the six isolates. CDs Length of all isolates is unique (1497 Pb). The Amino Acid (AA) lengths were 498. Results indicate that nucleotide homology among these isolates with NP genes is between 91.8% to 98.8%. The divergences between isolates have significantly been increased since 2007.The least similarities have been shown between TH 77, TH79 and TH90. Iranian H9N2 Isolates are divided in two groups: Group 1 (TH77, TH79, and TH80) and Group 2 (TH85, TH88, and TH90).In BLAST Search Isolates of Group 1 have 98-99% identities withA/Chicken/Iran/11T/99(H9N2), mallard /Sweden/86/2003 (H12N5) andmallard /Sweden/110/2002(H11N7)). The group 2 isolates have also 96% -97% identities chicken/Iran/AH-1/2006(H9N2), chicken/Pakistan/UDL-01/2005(H9N2) and A/chicken/Karachi/NARC-100/2004(H7N3) with isolates. More detailed results of phylogenetic finding will be discussed.

Discussion

Iranian poultry industry has been affected by by avian influenza virus H9N2 subtype since 1998. The association of high mortality and case report of H5N1 and H9N2 influenza virus in wild birds in recent years raised the specter of the notion of a new reassortant AI virus. If one considers the genes encoded by viruses with negative-sense RNA genomes, it becomes apparent that there is a core of three polypeptides common to all families: an integral membrane glycoprotein, an RNA-dependent RNA v polymerase and a single-strand RNA (ssRNA) binding protein, often referred to as a nucleoprotein (NP) (15). In simplistic terms, the glycoprotein gains the virus access to host cells, the RNA polymerase transcribes and replicates the virus genome, while the NP encapsidates the virus genome to form a ribonucleoprotein (RNP) particle for the purposes of transcription and packaging. This does not mean that this is the only function performed by NP (1) .

Compared with the HA and NA genes of the H9N2 viruses isolated in Iran, only a few sequences of the NP gene have been submitted. In this study, the NP gene of six H9N2 avian influenza viruses isolated between 1998 to 2011 from different farms in Iran were phylogenetically analyzed.

A number of residues in the polymerase proteins (PB1, PB2 and PA) and in the nucleoprotein (NP) are known to play a key role in the host range of AI viruses to increase virulence or replication in mammalian host (16). Nucleoprotein amino acids comparing of Iranian AI H9N2 viruses to Human H9N2 isolate from Hong Kong (A/Hong Kong/1073/99(H9N2) show a similar and unique substitution in following AA: Q (52),I(183),D(210),I(353),V(371),A(373),K(430) according to (A/turkey/Wisconsin/1/1966 (H9N2) Amino acid numbering. In research work that did by Camreon *et al* on comparison of Human H9N2 case and Pakistani H9N2 Isolated they declare four unique substitutions in Pakistani isolates (Q52, V371, A373, and K430) (17) but as been shown, Iranian H9N2 isolates are more unique substitution with human H9N2 isolate on NP gene. In another study on Amino acid residues of the H9N2 viruses that correlate with mouse replication phenotype, researchers found M 239 and Q 398 in H9N2 viruses that replicate in mice (18). In this study all Iranian isolates have the similar substitution. These viruses all possessed the typical avian residue at each of these locations excepting positions residue 34 in the NP protein, which was G and not A, the mouse preferred counterpart, or the more typical avian residue Aspartic acid that previously reported by Iqbal et al on Pakistani H9N2 isolates (16). It means that Iranian AI H9N2 isolates are more similar to mammalian origin H9N2 Isolates.

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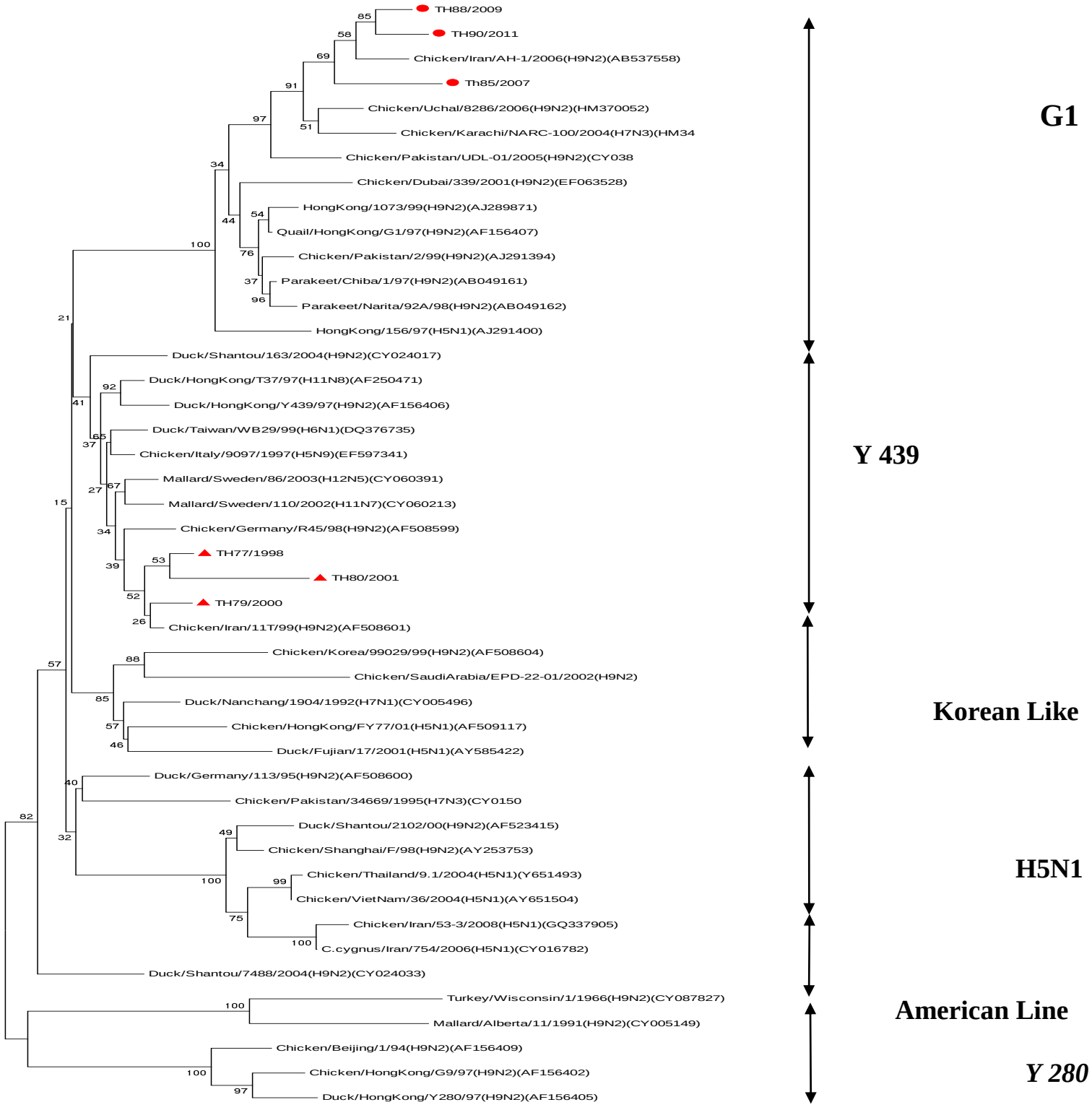

Fig. 1. Phylogenetic relationships of NP genes of H9N2 avian influenza viruses isolated from Iran between 1998 and 2011. The Phylogenetic tree was generated using Neighboring Joining model with MEGA (version 5.1). Numbers below branches indicate bootstrap value percentages from 1000 replicates, bootstrap 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 red Circle (Group 2) and Triangle (Group 2). The sequences obtained from GenBank.

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AI H9N2 in Iran in 2006, has been showed the NP gene was located in G1 clade with similarity rate of 86.4% to first reported H9N2 11T isolate in Genbank (19).

As shown in phylogenetic tree, Iranian AI H9N2 Isolates divided in two distinct clusters according to their isolation year. Group 1 is located in Y-439 clade and Group 2 is located in G1 Clade.

In the group 1, there is just isolate 11T, which were be sequenced and we were shown primary isolates of AI H9N2 from Iran after outbreak 98 (TH77/1998) are located with 11T in same clade. Iranian H9N2 Isolates including TH77, TH79 and TH80 have high similarity and classify in Clade G1. These results are agreed with another works from Iran, Pakistan and India (19-21).

Several studies have been carried out on NP gene of H9N2 isolate in different countries. In study on H9N2 Isolates from United Arab Emirates 2000 to 2003, their NP gene was also similar to those of the Middle East lineage of H9N2 viruses, and ultimately to those of the G1 lineage (22).

Li *et al* through work on Evolution of H9N2 viruses from domestic poultry in Mainland China, showed that the NP genes share very high homology and form a large sub lineage rooted to CK/BJ /1/9 4, and NP genes of CK/ HLJ/ 48/01, CK/SH /10/01, and CK/GD/56/01 share a 97–99% homology with CK/SH /F/98 and form a distinct sub lineage (18).

In Kim *et al*. study on NP gene of 2004 Korean H9N2 isolates, they conclude NP gene of those isolates were clustered with those of Korean H9N2 and of H5N1 viruses isolated in China and Hong Kong In Kim *et al* study on NP gene of 2004 Korean H9N2 isolates, they conclude NP gene of those isolates were clustered with those of Korean H9N2 and of H5N1 viruses isolated in China and Hong Kong (23).

Tosh et al. regarding the NP gene tree, reported that all the Indian H9N2 viruses formed a well-supported cluster (100% bootstrap value) in the G1-like sub lineage and also showed a nucleotide homology of 96.7–97.5% with Qa/HK/G1/97 reference isolate (21) .

Iqbal *et al* showed the NP gene of H9N2 viruses have close identity with Wih those of isolated in Pakistan in 1999 and clustered in the A/Quail/Hong Kong/G1/97 virus lineage. But NP gene of H7N3 viruses isolated from Pakistan was located in deferent cluster (16). NP genes from the H7N3 Pakistani isolates were closely related with nucleotide identity above 99.7% with the exception of 100/Karachi-04. The most closely related lineages to the main clade were wild bird and poultry isolates from Europe and Asia collected in the 1990's with around 95% identity. The 100/Karachi -04 isolate show 91% identity with the NP genes from other H7N3 viruses and the most closely related lineage was the 1999 and 2005-2008 H9N2 viruses from Pakistan (24).

As you could find NP gene of primary isolates of Iran have high similarities with Wild bird origin NP genes. 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 (25-27). But this is the first report that one internal gene of primary isolates of H9N2 (11T, TH77) in Iran has a different origin. It is possible that H9N2 that entered to Iran and caused outbreak in Iran (1998) was emerge as re assortment isolate between Natita origin isolate and wild bird originate and similar to Pakistani H7N3 isolates. Also, it may be we have H7 strains in Iran that new virus are emerged by re assortment process between H9N2 and H7. We do Also more sequences and phylogenetic study on other internal genes of Iranian AI H9N2 that analyzed in this research. As we don't have any intensive program for surveillance of H7 subtype in Iran, It is possible that we have H7 in Iran and veterinary organization should be increase their molecular surveillance of for H7 subtype in different region of Iran. On the other hand fereiroduni *et al* detected H7 subtype in migratory birds in Iran (28), but the NP gene of these isolate don't deposited in gene bank for include them for epidemiological studies. This suggests that the primary lineage of H9N2 in Iran are the result of a single initial introduction, probably from wild birds and

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have circulated long enough to evolve into separate clades from other lineages of AIV.
But After 2005, Iranian clade based on NP gene has been changed and all of them are categorized in G1 Clade that Jeidari *et al* pointed to this (19).
For Changing of the clade of Iranian H9N2 Isolates based on NP gene during 11 years several hypotheses to be proposed: The first is the origin of second dominant group is originated from Pakistan H9N2 isolated. As studies on other genes of H9N2 isolates identified that they are located in G1 clade. It is possible the H9N2 are circulated between Pakistan and Iran. The second hypothesis is NP Reassortment possibility of H9N2 with NP from G1- like Isolates.
Also the third hypothesis is the result of vaccine pressure on inducing mutation within Iranian Isolates. Since 1998 in Iran, killed h9n2 vaccine has been used in poultry farms.
These results indicate that Iranian AI H9N2 isolates of avian influenza virus show more amino acid substitutions Compare to those found in Human H9N2 isolates. These results may represent the tendency of avian H9N2 to adaptation in mammalian hosts. These findings provide additional information regarding the genetic heterogeneity of H9N2 avian influenza viruses circulating in Iranian poultry industry. Therefore, it seems continuous and intensive avian H9N2 isolates supervision should be done worldwide. Authors suggest common project on molecular epidemiology of H9N2 are designed between regional counties Iran, Pakistan and India.

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