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Phylogenetic Study on Nonstructural (NS) Gene of H9N2 Isolated from Broilers in Iran During 1998-2007

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Abstract: Since 1998, H9N2 AI outbreaks have been one of the major problems in Iranian poultry industry. The association of high mortality and case report of H5N1 and H9N2 influenza virus in wild birds in recent years raised the specter of a possible new genetic modified AI virus. In this study, we do phylogenetic analysis on Full- length Nonstructural (NS) genes of seven H9N2 Isolates from Broilers in Iran, Tehran province during 1998-2007. Phylogenetic analysis clearly shows that Iranian H9N2 isolates gene pools, corresponding to just NS allele A. Comparison of nucleotide sequences of isolated viruses revealed a substantial number of silent mutations, which results in high degree of homology in amino acid sequences. In addition, the cluster of Iranian H9N2 isolates could be subdivided into two subgroups, which matched their times of isolation especially around 2006 time line. The high degree of similarity between the NS genes of the Iranian H9N2 isolates supports the hypothesis that these genes originated from a single predecessor. Present result provides useful molecular epidemiological data to understand the dynamics of H9N2 evolution during 9 years in Iran and support earlier phylogenetic observations.

Key words: Influenza, H9N2, nonstructural genes, H5N1 virus

INTRODUCTION

Avian Influenza (AI) has emerged as a disease with significant potential to disrupt commercial poultry production often resulting in extensive losses (Alexander, 2000). Avian influenza virus H9N2 and H5N1 subtypes are considered the most probable precursors of a pandemic influenza virus (Banet-Noach *et al*., 2007). The genome of avian influenza viruses consist of eight separate segments of single-stranded negative sense RNA, which code 10 viral proteins. Two non-structural proteins (NS1 and NS2) are coded by segment 8, which is the smallest viral RNA segment (890 nucleotides). These two proteins share ten amino terminal amino acids (aa) and the NS2 gene is spliced together with a downstream sequence (Lamb and Krug, 2007). The NEP coding sequence partially overlaps the NS1 protein at the amino terminal end and the carboxy terminal end is a result of alternative splicing of its mRNA. The NEP protein, previously known as NS2, was thought to be a non-structural protein but it is present in small amounts in the virions in association with the ribonucleoprotein (RNP) through interaction with the M1 protein.

The NS gene segment phylogenetically separates into two groups, referred to as groups or alleles A and B (Treanor *et al*., 1989). For non-structural gene subtype A viruses, five subdivisions were distinguished in the nucleotide phylogenetic tree including a human-swine group, American avian-equine group, Eurasian avian-swine group, a unique gull isolate group and a group with a single member, A/Equine/Prague/56. At the nucleotide sequence level the B subtype was subdivided into two groups, including the American avian group and Eurasian avian-equine group (Suarez and Perdue, 1998). Allele A accounts for viruses from human, equine, swine and avian species; whereas allele B comprises one equine and many avian, influenza isolates (Ludwig *et al*., 1991; Treanor *et al*., 1989; Wang *et al*., 2005). Nucleotide sequence similarities within alleles A and B have been found to be 86.5-99.4 and 89.4-99.6%, respectively and at most only 72.3% between the alleles (Kawaoka *et al*., 1998). The avian and human NS genes of the A group were derived from a common ancestor existing at about 1912.

The influenza virus NS1 protein has been shown to be a multifunctional immune modulator and a virulence

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factor for this virus. Among its multiple functions are the inhibition of the type I interferon (IFN) system in infected cells, the binding and sequestration of dsRNA, the interference with the host mRNA processing, the facilitation of preferential viral mRNA translation and the inhibition of Dendritic Cell (DC) activation. The combination of all these functions makes the NS1 protein a very potent inhibitor of immunity and allows influenza virus to efficiently escape the immune surveillance and to establish infection in the host (Fernandez-Sesma, 2007).

H9N2 have been isolated from outbreaks in poultry in several countries such as Germany, Italy, Ireland, Saudi Arabia, Israel, Pakistan, China, Hong Kong, South Africa and the United States (Alexander, 2003; Banet-Noach *et al*., 2007). Since 1998, H9N2 AI outbreaks have been one of the major problems in Iranian poultry industry (Nili and Asasi, 2003). Mortality rate of H9N2 influenza virus outbreak in broilers chicken farms during 1998-2001 in Iran was 20-60% (Nili and Asasi, 2002). Banks *et al*. (2000) have reported low-virulence H9N2 subtypes isolated from different countries, inclusive of two AI isolates from Iran. Till this time, just one NS sequence from H9N2 Iranian isolates submitted in gene bank by (Accession number of AAQ04999) by Li *et al*. (2004).

In 2006, H5N1 report in swans in northern of Iran firstly but to this time we don't have official report from commercial flocks in Iran (Wave). Although, Fereidouni *et al*. (2005) report detection of other subtypes of avian influenza virus from migratory birds in Iran. In this study, we present phylogenetic study on seven viruses isolated from Broilers in the Tehran province in Iran during 1998 to 2007.

MATERIALS AND METHODS

(Viruses) sample collection: Sample collection was performed according to the standard method from suspected clinical broiler specimens in Tehran Province. Specimens (lung and intestine) were stored at -70°C until use. Samples were collected in a 2X phosphate buffer solution (PBS, pH 7.4) containing antibiotics (10.000 IU mL^{-1} penicillin, 1 mg mL^{-1} streptomycin sulphate) and anti antifungal (20 IU mL^{-1} Nystatin) (SIGMA, St. Louis, MO, USA). Other virus samples (5 isolates) were available in central lab of department of clinical science in faculty of veterinary medicine, University Of Tehran. All of these viruses used were isolated in embryonated eggs from swab specimens obtained from the tracheas and intestine of affected chickens in Tehran Province (Kendal *et al*., 1982; Peiris *et al*., 1999).

Virus isolation: Ten-day-old SPF embryonated chicken eggs were inoculated and incubated at 37 °C for 48 h. Eggs were candled daily and embryos dying within 24 h post inoculation were discarded. Allantoic fluids were collected from the eggs and the presence of viruses was determined by hemagglutination assay. The identification of viruses subtype was determined by a standard Hemagglutination Inhibition (HI) and Neuraminidase Inhibition (NI) tests using polyclonal chicken antisera. The pathogenicity of the isolates was determined by means of the intravenous pathogenicity index test (IVPI). The allantoic fluids containing virus were harvested and stored at -70°C until use. All of HA negative allantoic fluids were inoculated for the second passage (Peiris *et al*., 1999).

RNA extraction: Viral RNA was extracted from infected allantoic fluid using RNX ™-Plus reagent according to the manufacturer's instruction (Cinnagen, Iran) that based on by phenol-chloroform extraction and ethanol precipitation (Sambrook and Russell, 2001). Briefly, in an RNAase free 1.5 mL tube, 800 μL of RNX ™-Plus solution was added to 200 μL allantoic fluids. After shaking, 200 μL of chloroform was added and the mixture was centrifuged at 14,000 RPM (Eppendorf,) at 4°C for 15 min. Equal volume of Isopropanol was added to the upper phase in a new tube. The mixture was centrifuged at 12,000 RPM at 4°C for 15 min. The supernatant was discarded and 500 μL of 75% ethanol was added to the pellet. After centrifugation at 7,500 RPM for 10 min at 4°C, the supernatant was discarded and the pellet was dried at room temperature for few minutes. Finally, the pellet was diluted in 20 μL DEPC treated water. To help dissolving, the tube was incubated at 55-60°C in water bath for 10 min and stored at -70°C for RT-PCR reaction .All of chemical reagents that used were supplied from Merck Company.

RT and PCR reaction: Reverse transcription was done by using oligonucleotide influenza universal primer, uni12, with "Revert Aid" first strand cDNA synthesis Kit (Fermentas, Canada) (Hoffmann *et al*., 2001). Amplification of the NS gene was carried out by PCR as described by using specific primers. Primers 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 25 μL Cinnagen PCR master mix (Cinnagen, Iran). The amplification protocol was: one step of denaturation at 94°C for 3 min, 35 cycles of 94°C /45, 55°C/45 and 72°C/60 Sec and final extension at 72°C for 10 min.

Sequencing and phylogenetic analysis: The PCR products were separated by electrophoresis using 1% agarose gel and insert to AT vector. PCR products were purified with the QIA quick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and send for Sequencing and subsequently sequenced in both directions by a CEQTM 2000 Dye Terminator Cycle Sequencing kit (Beckman Coulter, Fullerton, CA) using an automated CEQTM 2000XL Micro capillary DNA Analysis Sequencing Apparatus (Beckman Coulter).

Initially, Nucleotide and deduced amino acid sequences were edited with the MEGA4 Package, version 5 (Tamura *et al*., 2007). Nucleotide and deduced amino acid sequences were aligned using Free CLC Bio workbench (Larkin *et al*., 2007).The sequences obtained in the present study and other sequences of NS genes of influenza viruses from the Gene Bank database were used for phylogenetic analysis. Nucleotide acids sequences of the NS gene were used to study and construct the respective phylogenetic trees. Phylogenetic analysis based on Nucleotide sequence was performed by MEGA4 software. Phylogenetic trees were constructed by the neighbor-joining method (Zhang and Sun, 2008). Bootstrap re-sampling and reconstruction was performed 1,000 times to confirm the reliability of phylogenetic trees (Dopazo, 1994).

Table 1: Iranian Isolates characterized in this study

Accession No.	Virus strain	IVIP
FJ205646	A/Chicken/Iran/TH77/1998(H9N2)	0
FJ205647	A/Chicken/Iran/TH78/1999(H9N2)	0
FJ205648	A/Chicken/Iran/TH80/2001(H9N2)	0
FJ205649	A/Chicken/Iran/TH82/2003/(H9N2)	0
FJ205650	A/Chicken/Iran/TH83/2004(H9N2)	0
FJ205651	A/Chicken/Iran/TH85/2006(H9N2)	0
FJ205652	A/Chicken/Iran/TH86/2007(H9N2)	0

IVPI: Intravenous pathogenicity index

Nucleotide sequence accession numbers: The nucleotide sequences for all H9N2 influenza viruses used in this study are available in Gen Bank under accession numbers FJ205646 through FJ205652. The accession numbers are provided in Table 1.

RESULTS

All of Iranian isolates contains 230 acid amine as the same number as most of H9N2 strains isolated elsewhere except two recently H9N2 isolates TH85 and TH186 (217 aa) (Table 2).

Compared with the HA genes of the H9N2 viruses isolated Iran, only a few phylogenetic analyses of the NS genes have been reported. Phylogenetic tree among Iranian isolates and among other world, isolates are presented in Fig. 1 and 2, respectively.

Table 2: Percentage homology comparison of deduced amino acid sequence of NSI genes among seven Iranian H9N2 influenza virus isolates from broilers during 1998-2007. Percentage Homology was generated by CLUSTAL W software. A/CK/IR was not shown in the name of all Iranian H9N2 isolates in this table

	TH77 (1998)	TH78 (1999)	TH80 (2001)	TH82 (2003)	TH83 (2004)	TH85 (2006)	TH86 (2007)
TH77 (1998)		94	90	95	94	88	87
TH78 (1999)	94		93	93	93	87	87
TH80 (2001)	90	93		88	88	85	84
TH82 (2003)	95	93	88		96	86	87
TH83 (2004)	94	93	88	96		90	90
TH85 (2006)	88	87	85	86	90		88
TH86 (2007)	87	87	84	87	90	88	

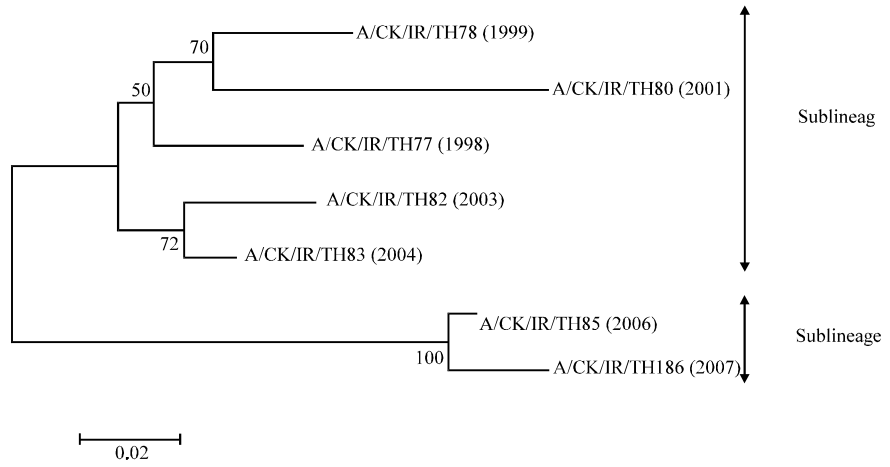

Fig. 1: Phylogenetic relationship of NS genes among seven Iranian H9N2 influenza virus isolates from broilers during 1998-2007. The Nucleotide coding region tree was generated by neighbor-joining analysis, using MEGA 4. Numbers below key nodes indicate the percentage of bootstrap values of 10000 replicates

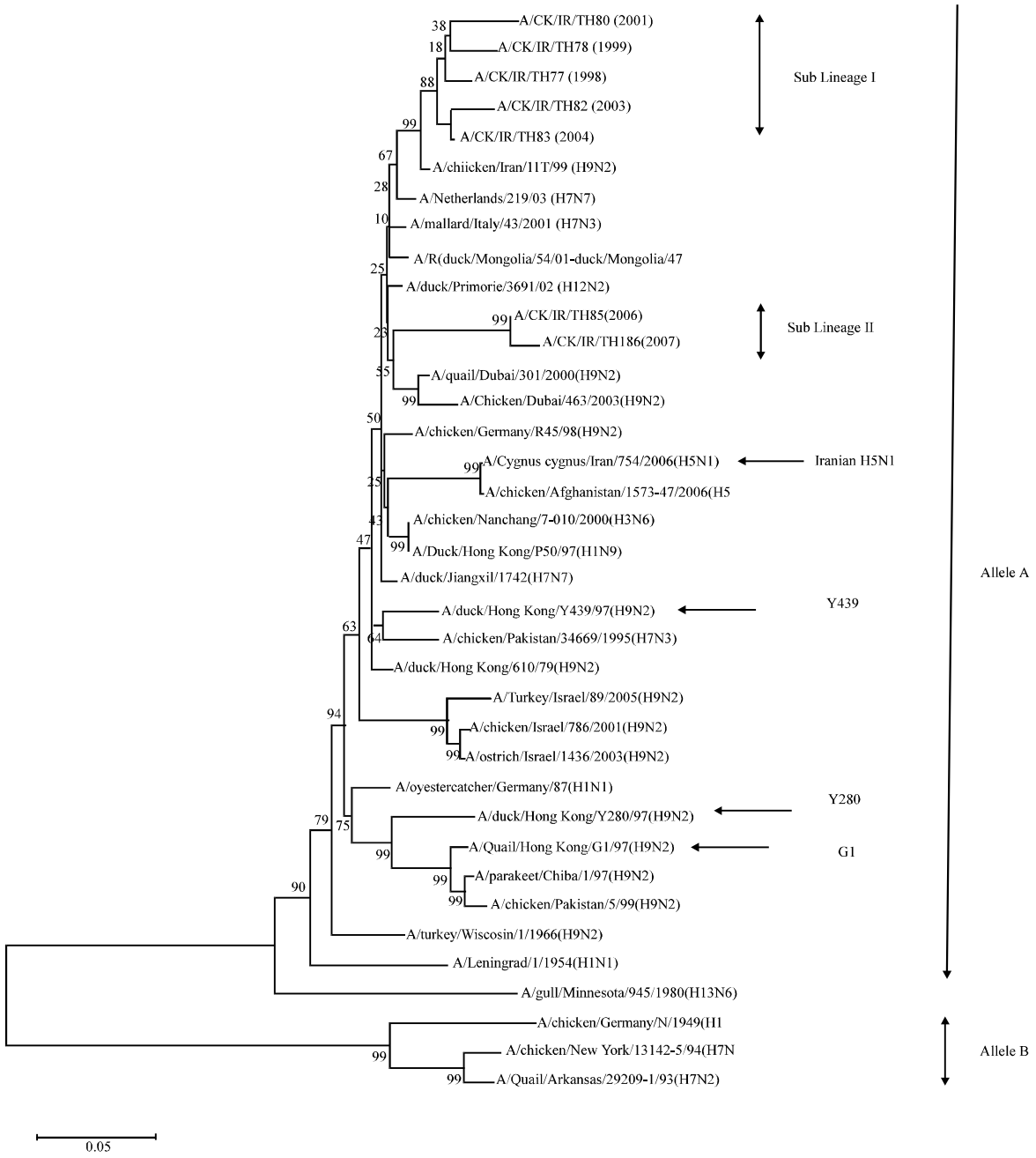

Fig. 2: A neighbor joining phylogenetic tree NS genes based on nucleotide sequence between Iranian H9N2 influenza virus isolates from broilers during 1998-2007 and other Influenza strain. Bootstrap value based on 1,000 replicates is shown at each main branch

DISCUSSION

The length of NS is Like previously described and released by Wang *et al.* (2005) about China isolates. In first phylogenetic study among H9N2 Iranian Isolates base on Nucleotide sequences of NS1 coding region revealed that Iranian isolates divided to two sub lineages from 1998 to 2007 (Two distinct lineages of NS genes of the H9N2 influenza viruses were circulating in the chicken population in Iran). Tree show that from 2006, Iranian isolates located in new sub lineage. A phylogenetic tree was constructed on the basis of the complete nucleotide

sequence of the NS1 and NS2 coding region of Iranian studied isolates and selected viruses from Gen bank database. The NS genes of 7 isolates of H9N2 sequenced in the present study were used for phylogenetic study with NS genes of influenza virus references obtained from the Gene Bank. The results of this analysis indicate that the NS genes of all the Studied Iranian isolates during 1998-2007 were located in allele A. Present results show that the NS genes of the all Israeli isolates fall into allele A such as described in another countries by Banet-Noach (Banet-Noach *et al*., 2007; Wang *et al*., 2005).

Iranian isolates in phylogenetic tree located in two locations. Our phylogenetic analysis showed that H9N2 Iranian isolates in sub lineage I are a close relationship with viruses found in Northern Europe mostly from Netherland. But in sub lineage II , are a close relationship with viruses found in United Emirate. (Thus, phylogenetic analysis also showed that the NS gene of Recent Iranian isolates had a close relationship with those of the Emirates H9N2 avian strains). Interestingly result that H5N1 strain that isolate from swan (Cygnus Cygnus) in Iran and isolates from neighborhood country such as Afghanistan (Wave) located near recently H9N2 Iranian Isolates that including Sub lineage II.

It is probable H5N1 strains effect on changing sub lineage of recently Iranian isolates from Iran in 2006 and 2007 or export and import of poultry related between United Emirate and Iran affect divergenicity of Iranian Isolates I n recent year.

The NS genes of the Eurasian H9 viruses are divided into three different lineages, whose representative strains are A/duck/Hong Kong/Y439/97 (DK/HK/Y439/97), Qa/HK/G1/97 and DK/HK/Y280/97 (Liu *et al*., 2002). Phylogenetic tree of seven Iranian H9N2 isolates based on deduced amino acid are not shown here. In this study, genetic analysis showed that the NS gene of Iranian H9N2 isolates belonged to the Dk/HK/Y439/97-like lineage. Therefore, the molecular epidemiological surveillance of H9N2 viruses not only from domestic Chickens but also from migratory ducks should be continued and intensified. On the other hand, the Qa/HK/G1/97-like viruses have been considered to have potential public health risks as exemplified by the donation of the internal genes to the H5N1 viruses that resulted in the Hong Kong incident in 1997 in which avian influenza A viruses transmitted directly from chickens to humans. Even though the Qa/HK/G1/97-like viruses were not actually found on chicken farms, in this study, the implications of the zoonotic risks further stress the importance of the continued and sustained surveillance for the ultimate goal of the control of influenza.

The HA gene fragments of Iranian H9N2 isolates during 10 years belonged to the Qa/HK/G1/97-like lineage (unpublished data) But NS gene of Iranian H9N2 in this study belong Dk/HK/Y439/97-like Viruses.

In Amir *et al*. (2007) study emirates Isolates similar to isolates such as A/Duck/Hong Kong/P54/97(H11N9) and A/duck/Nanchang/1904/92 (H7N4) from the Influenza Sequence Database, formed a distinct clade with the viruses of G1 and Y280 lineages (88-91% similarity) All the "internal" genes of emirates Isolates, with the exception of the NS and PB2 genes, were highly similar to the genes of isolates from Iran and Pakistan that also belong to the A/Qa/HK/G1/97-like lineage (Aamir *et al*., 2007). The cluster of Iranian H9N2 isolates could be subdivided into two subgroups which matched their times of isolation especially around 2006 timeline. However, the differences between these subgroups exceed 10% on the other hand differences between isolates in sub lineage II and I are 5 and 6% respectably. The high degree of similarity between the NS genes of the Iranian H9N2 isolates supports the hypothesis that these genes originated from a single predecessor. The differences between the subgroups may be attributed to a few point mutations without genetic shifts.

CONCLUSION

Present result provides useful molecular epidemiological data to understand the dynamics of H9N2 evolution and support earlier phylogenetic observations. Furthermore, the ecology of AIV in the wild bird's population is an area in which research efforts will be concentrated in the future. Therefore, the molecular epidemiological surveillance of H9N2 viruses not only from domestic chickens but also from migratory birds should be continued and intensified and Molecular epidemiological surveillance of H5N1 and H9N2 viruses from domestic and wild birds should be continued and intensified. It lacks scientific study and information on live birds marketing in Iran and should more study on this epidemiological group.

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