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# Iranian H9N2 Avian Influenza Virus is closely related to Pakistani Parakeet Influenza Virus isolates

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## ABSTRACT

Sequence analysis and phylogenetic study of hemagglutinin (HA) gene of H9N2 subtype of avian influenza virus isolates (outbreaks of 1998-2002) in Tehran province (Iran) were studied. PCR products of a 430-bp fragment of 16 isolates were sequenced and then were aligned with the reported sequences in Genbank. Nucleotide sequence comparisons of HA gene from Iranian isolates showed 97-99% identity within the group, and 98% homology with the two isolates [A/Parakeet/Narita/92A/98 (H9N2)] and [A/Parakeet/Chiba/1/97 (H9N2)] from Pakistani parakeets imported to Japan. On the basis of phylogenetic evidence and even sequence comparison of Neuraminidase (N) gene of [A/Parakeet/Chiba/1/97 (H9N2)] and [A/Chicken/Iran/IT/99 (H9N2)](Li,2002) it is proposed that the emergence of H9N2 avian influenza infection in Iran originated in Pakistan. Due to the high percentage of H9N2 homology isolates of Iran with other isolates, namley A/quail/HongKong/G1, in Genbank and based on published reports for high similarity with infecting human H5N1 isolates, it seems that the potential of Iranian avian influenza isolates to infect human should be considered.

***Keywords***: Avian influenza (AI), Hemagglutinin (HA), Neuraminidase (N), H9N2 subtype, Phylogeny

## INTRODUCTION

Influenza is caused by a zoonotic virus that occurs in lower animals and birds as well as in humans. Influenza viruses belong to the *Orthomyxoviridae* family of RNA viruses and are divided into four genera: Influenza A, B, and C virus, and Thogovirus. Avian influenza (AI) is a highly contagious disease caused by type A influenza viruses, which have segmented negative-strand genomes [1, 2]. These viruses, on the basis of their two surface glycoproteins, are divided into two subtypes: hemagglutinin (HA) and neura-minidase (NA) [3]. Serological analysis has identified 15 HA (H1-15) and 9 NA (N1-9) subtypes among the influenza A viruses [1, 4]. HA is the major antigen for neutralizing antibodies and is involved in the binding of virus particles to receptors on host cells [5]. All 15 HA subtypes of influenza virus are found in aquatic birds, which serve as the primordial reservoir of influenza A viruses [4]. AI viruses, based on their ability to cause disease, are divided into two distinct groups: the highly pathogenic (HP) and non-highly or low pathogenic (nHP or LP) avian viruses. The HP AI viruses that may result in mortality as high as 100%, have been restricted to H5 and H7 subtypes, although, not all viruses of these subtypes are HP [6]. In recent years, outbreaks in poultry due to viruses of H9 subtypes, specially H9N2, have been widespread [7]. During the second half of the 1990s, outbreaks due to H9N2 subtypes have been reported in Germany, Italy, Ireland, South Africa, USA, Korea, China, the Middle East, Saudi Arabia and Pakistan [4, 8]. Since 1998, H9N2 AI outbreaks have been one of the major problems in Iranian poultry industry [9, 10]. In the present study, a part of the HA gene of H9N2 AI virus, with a highly conserved region corresponding to that which was used in the studies of Banks *et al*. [8] was sequenced. The isolates were from Hashtgerd and Shahriar regions in Tehran province (Iran). To determine divergency and the possible origin of AI viruses isolated in Iran, the resultant sequences were compared with other H9N2 AI virus sequences in Genbank.

## MATERIALS AND METHODS

***Viruses.*** In this study, the 16 H9N2 virus samples were prepared from the Poultry Diseases Section of Razi Vaccine and Serum Research Institute, Karaj (Iran). The identification of viruse subtype was determined by a standard hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests using polyclonal chicken antisera. These viruses were isolated from influenza outbreaks during 1998-2002 in Tehran (Hashtgerd district) [TH], and Tehran (Shahriar district) [TS] where the first influenza outbreaks were reported. Nine samples of Hashtgerd district were: TH9/02, TH1/01, TH2/01, TH3/00, TH8/00, TH4/99, TH5/99, TH6/98, and TH7/98. Seven samples from Shahriar district were: TS7/01, TS6/00, TS8/00, TS5/99, TS9/99, TS4/98, and TS10/98. The samples were grown in SPF embryonated chicken eggs as described by Peiris *et al.* [11]. Nucleotide sequences reported in this paper are available in the European Molecular Biology Laboratory (EMBL), Genbank and DNA Data Bank of Japan (DDBJ) database under the following accession numbers: TH1 to TH5: AY198313 to AY198317, TH6: AY264870, TH7: AY198318, TH8: AY264872, TH9 (TN1): AY264871, TS4 to TS7: AY264873 to AY26486, TS8 to TS10: AY198319 to AY198321. For comparison, the tree was constructed by Megalign Software of the DNASTAR package (Windows 3.03, 1993-1995).

***Primer design.*** To identify AI viruses by PCR, two sets of primers, based on conserved sequences of the HA gene, were designed. The conserved sequences were selected by comparison and alignment of HA sequences retrieved from Genbank. The HA-specific primers were HAF1 (forward): 5'-GCATACATCATCCACCCAC-3' and HAR1 (reverse): 5'-TTGATCTAGCAGGCAC GTTC-3' .

***Nested PCR.*** To confirm the reliability of amplified fragments of HAF1 and HAR1, the following primers were also designed: HAF2 5'-AACAAGCGTGACAACAGAAG-3' (forward) and HAR2 5'-CCGACTGCCAGTTTGAGAC-3' (re-verse).

***RNA extraction.*** Viral RNA was extracted from infected allantoic fluid using an RNX reagent (Cinnagen, Iran). Briefly, allantoic fluid (1 ml) was centrifuged at 85,000 ×g at 4ºC for 2 h. The pellete was resuspended in 200 µl of PBS, then in 400 µl of RNX reagent, mixed well, and left in room temperature for 5 min. Extraction was done with 0.2 ml chloroform/isoamylalcohol (24:1). The RNA, in the aqueous solution, was precipitated by adding an equal volume of isopropanol. The mixture was then centrifuged at 10,000 ×g for 20 min. The pellete was washed by 75% ethanol and dissolved in 20 µl of RNase-free water.

***Reverse transcription polymerase chain reaction (RT-PCR).*** RT followed by PCR was performed using specific primers for the 430-bp fragment of HA gene (nucleotides 595 to 1024). RT-PCR was carried out in 50 µl reaction mixture containing 10 µl of 5× reaction buffer, 4 µl of mixed dNTPs (2.5 mM each), 1 µl of AMV enzyme (Titan one tube RT-PCR system kit, Roche Diagnostic, Germany), 1µl of each primer (10 ρmol each), 4 µl of RNA template, 2.5 µl DTT, 3 µl 25mM $MgCl_2$, and 23.5 µl of $H_2O$. PCR program was 42ºC for 30 min, 94ºC for 3 min, 30 cycles of 94ºC for 30 s, 52ºC for 30 s, 72ºC for 40 s, followed by 72ºC for 5 min.

***DNA sequencing.*** The PCR products were purified using PCR product purification kit (Roche Diagnostic, Germany). PCR products of 16 isolates were applied to low melting point agarose (LMP) and the distinct bands were purified from gel for direct sequencing (MWG Co., Germany). Purified fragments were sequenced from both directions. The sequence was compiled using Megalign Package.

**RESULTS AND DISCUSSION**

PCR was able to amplify the desirable fragment of HA gene of H9N2 influenza virus. Nuclotide sequence comparisons of Iranian isolates showed a range of minimum homology (91.1%) between TH9/02 and TH7/98 to a maximum homology (100%) between TH1/01- TH2/01. The data in this study underline the complexity of the ecology of AI. There are some expectable divergences within isolates of a district either in Hashtgerd or Shahriar districts.

However, there is 96.6% homology between two isolates of TH8/00 and TS5/99 from these areas which could be due to the common veterinary services in adjacent regions and other means of infection transmission such as human. Sequence comparisons of Iranian isolates with other H9N2 sequences in Genbank were consistent with other phylogenetic data [8]. Interestingly, as shown in Table 2, the maximum similarity percentage in this study was in concurrence with homology between TH5/99, one of the isolates in this study, and A/parakeet/Narita/92A/98 isolate from Japan (97.8%). Furthermore, the first reported sequences from Iran in Genbank: IRAN/16/98 and IR/11T/99, show 98.6% and 98.9% identity with Parakeet/ Narita/98 isolate, respectively. The TH5/99 isolate also showed 97% similarity with A/parakeet /chiba/97 (data not shown). The other 15 remaining isolates in this study showed 91.7% to 97.4% homology with A/parakeet/Narita/92A/98.As an explanation, the A/parakeet/Narita/ 92A/98 isolate was imported from Pakistan to Japan in 1998. Also, A/parakeet/chiba/97 virus was isolated from a parakeet in a petshop in Japan with the same origin [12]. These two isolates were genetically closely related to each other (>99% as determined by nucleotide analysis of eight RNA segments) indicating that both belong to the same lineage [12].

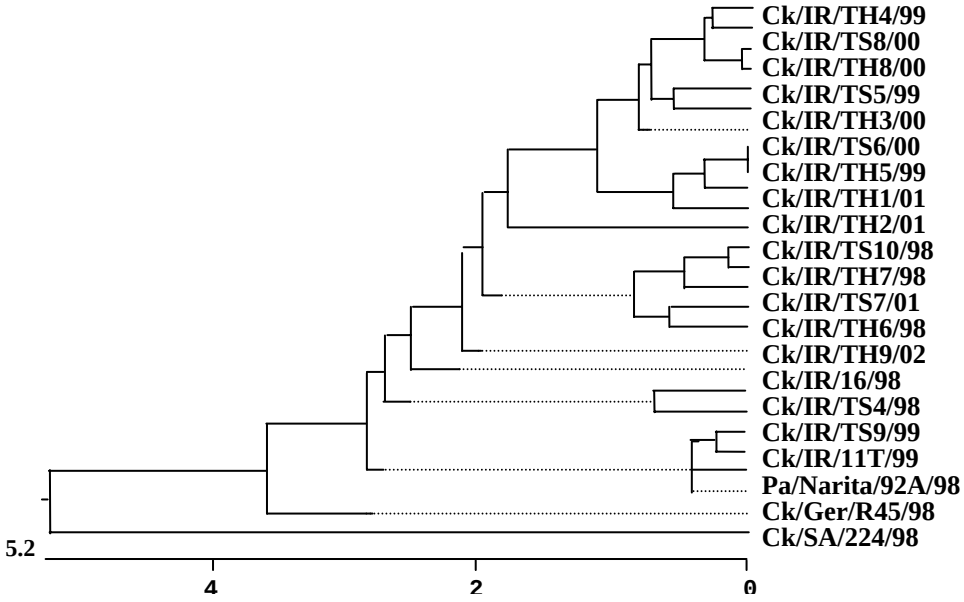


**Fig. 1.** Phylogenetic relationship amongst the HA genes of H9N2 AI viruses in Iranian isolates, and the other isolates in Genbank having high nucleotide sequence homology. The phylogram is constructed from 27 H9N2 viruses using 430 nucleotide sequences of the HA gene. The Iranian isolates show fairly close relationship to each other. The length of the horizontal lines are proportional to the genetic differences (%). Vertical lines are for spacing branches. Ck, chicken; IR, Iran; TH, Tehran-Hashtgerd; TS, Tehran-Shahriar; Pa, parakeet; Ger, Germany; SA, Saudi Arabia; Pk, Pakistan; Qa, quail; HK, Hong Kong.

As an explanation, the A/parakeet/Narita/ 92A/98 isolate was imported from Pakistan to Japan in 1998. Also, A/parakeet/chiba/97 virus was isolated from a parakeet in a petshop in Japan with the same origin [12]. These two isolates were genetically closely related to each other (>99% as determined by nucleotide analysis of eight RNA segments) indicating that both belong to the same lineage [12]. Since the viruses were identified one year apart, their lineage must have been established in Pakistan for at least one year. These isolates also showed a high similarity with other chicken isolates from Pakistan (Ck/Pk/4/99 and Ck/Pk/5/99), Germany (Ck/Ger/R45/98), Saudi Arabia (Ck/SA/224/98) in Genbank (Fig. 1), and less similarity to isolates from Malaysia (A/pekin-duck/Malaysia/F20/1/98), and Singapore (A/pekin duck/Singapore/F91/5/ 9/97) (data of the last two isolates are not shown). The first reported outbreak of H9N2 AI in Pakistan was in 1999 [13]. The isolations of H9N2 AI from Pakistani parakeets in Japan were in 1997 and 1998 [12]. The lack of H9N2 AI report in chickens in Pakistan before 1998 [14] was due to low mortality, concurrent infections, and insufficient attention to detect H9N2 subtype. Also, the first outbreak of H9N2 AI in Iran was in 1998 [9, 12]. Regarding these data, we postulate that the H9N2 infection had been circulating in some bird species and even chicken farms in Pakistan before the first observation was documented. The highest homology between Iran and Pakistan isolates showed that the emergence of H9N2 infection in Iran was probably originated from Pakistan.

On the other hand, preliminary comparisons of partial HA gene sequences indicated that some H9N2 viruses, recently isolated in Pakistan, Saudi Arabia, Germany and Iran were indeed closely related to their HA to the human H9N2 isolates [15]. Influenza surveillance studies of live poultry markets in Hong Kong have demonstrated that the most common influenza viruses isolated from these areas are H5N1 subtype [18]. Following the outbreak of "Bird flu" due to H5N1 in Hong Kong in 1997, and the isolation of H9N2 subtype viruses from patients both in China and Hong Kong, raised the specter of a possible influenza pandemic [16]. Analysis of complete genome of a Pakistani isolate (A/chicken/Pakistan/2/99) shows that it is genetically closely related in its all eight genes (97-99% homology) to the human H9N2 isolates. Sequence analysis of HA gene of the H9N2 parakeet viruses (A/Parakeet/Chiba/1/97, and A/Parakeet/Narita/92A/98) showed >97% identity with those of the H9N2 viruses isolated from humans in 1999, and G1 (A/quail/HongKong/G1) in 1997 [12]. Furthermore, the six out of eigth genes encoding internal components of the same virus are similar to the corresponding genes of the human H5N1 viruses that caused 6 (out of 18) fatal cases of human infection in China and Hong Kong [15, 17, 18]. Existing 96-98% homology among the Iranian and Pakistani isolates indicates that the Iranian isolates could have the same potential in infecting humans. Based on these data, the potential of Iranian isolates, as a serious threat to the human health, should be considered. Taking these results together, and due to reassortment specification of AI viruses, it is imperative to characterize and compare the complete genome of avian and human isolates in order to monitor any transferring threat of AI virus to humans.

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