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Research Article

GENETIC EVOLUTION OF LOW PATHOGENICITY H9N2 AVIAN INFLUENZA VIRUSES IN IRAN: ACQUISITION OF NEW MUTATIONS IN POLYMERASE ACIDIC (PA) GENES

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ABSTRACT
The present study aimed to analyze and evolution of H9N2 influenza viruses in Iran from 2008 to 2009.. To determine the genetic relationship of Iranian viruses, the PA genes from ten isolates of H9N2 viruses isolated from commercial chickens in Iran during 2008–2009 were amplified and sequenced. The Iranian isolates did not show insertions or deletions within polymerase acidic (PA) gene with compared to the prototype, A/turkey/winconsin/66, but rather numerous point mutations were registered. The PA gene of Iranian viruses showed great genetic diversity and shared a high level of similarity with PA genes from H7 subtype rather than with established H9N2 Eurasian lineages. Our findings demonstrated striking extensive reassortment in the H9N2 viruses in Iran which has led to produce of novel genotypes. The emergence of these novel genotypes of H9N2 viruses and the sustained prevalence of these viruses in poultry warrant further surveillance of H9N2 viruses by completing genomic analysis.

Keywords: *Genetic Analysis; Avian Influenza Virus; PA Gene; Iran*

Abbreviations: *PA: Polymerase Acidic, HA: Hemagglutinin, NA: Neuraminidase, NP: Nucleoprotein, bp: base pair, Dk: Duck, Ck:chicken*

INTRODUCTION
Influenza viruses are the members of the family Orthomyxoviridae with a genome of single-stranded negative-sense RNA composed of 8 gene segments encoding at least 10 proteins (Lee and Saif, 2009). These viruses are classified into three major types A, B, C based on the antigenic differences in their nucleoprotein and matrix protein. The type A virus is pleomorphic and spherical (approximately 120 nm in diameter) and can be further classified into subtypes according to the antigenicity of two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (Plese and Bouvier, 2008). To date, 16 HA subtypes and 9 NA subtypes of influenza A virus have been identified (Fouchier *et al.,* 2005). The eight segments of the influenza virus RNA genome are packaged into ribonucleoprotein particles (RNPs) containing the nucleoprotein (NP) and the trimeric RNA-dependent RNA polymerase complex, which comprises subunits PA, PB1 and PB2. The polymerase operates in two distinct modes: in the first, it transcribes virally encoded genes, using a cap-snatching mechanism to prime transcription and ensure proper 5' capping of viral messenger RNA; in the second, it replicates full-length viral RNA to produce first positive-strand complementary RNA and then progeny viral RNA. The role that the PA protein plays within the polymerase is less clear. The classical genetic data indicated that it is important for viral RNA replication but a number of site-directed mutations generated over the years relate the PA protein with cRNA synthesis, cap snatching, and cap binding and vRNA promoter recognition. The only biochemical activity reported for PA protein relates to protein degradation (Plese and Bouvier, 2008). The influenza A viral heterotrimeric polymerase complex (PA, PB1, PB2) is known to be involved in many aspects of viral replication and to interact with host factors, there by having a role in host specificity (Fouchier *et al.,* 2005).
In this study, we characterized polymerase complex (PA) genes and proteins of 10 Iranian isolates which have been isolated from Commercial broiler chicken in the Iran between 2008 and 2009. These isolates resulted in rapid mortality due to tracheitis and respiratory congestion. We delineated the polymerase

complex (PA) gene of these field isolates and we also established their phylogenetic relationship to the other Asian H9N2 viruses.

MATERIALS AND METHODS

Sampling and Virus Isolation

Samples collected from 30 farms were received between April 2008 and February 2009, from various parts of the country. Sample collection was performed according to the standard method for clinical poultry specimens (Swayne *et al.,* 1998). Lung and trachea specimens were submitted during the period 2008 to 2009 and were stored at −70 °C until used. They were collected in a 2× phosphate buffer solution (PBS, pH 7.4) containing antibiotics and antifungals (Penicillin10000 unit/ml, Streptomycin 10000 unit/ml and Nystatin 20000unit/ml). Initial viral isolation was performed in 10-day-old SPF (Specific Pathogen Free) embryonated chicken eggs (ECEs). Eggs were candled daily, and embryos dying within 24-h post inoculation (PI) were discarded. Allantoic fluids were collected from the eggs, and the presence of viruses was determined by haemagglutination. Subtype identification of the viruses was determined by standard haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests using polyclonal chicken antisera as described previously (Alexander and Spackman, 1981). 10 virus isolates obtained in this study were named as follows:
(A/chicken/Iran/RZ28/2008,A/chicken/Iran/RZ36/2008,A/chicken/Iran/RZ37/2008,A/chicken/Iran/RZ42/2009,A/chicken/Iran/RZ53/2008,A/chicken/Iran/RZ69/2008,A/chicken/Iran/RZ70/2008,A/chicken/Iran/RZ71/2009,Achicken/Iran/RZ75/2009, A/chicken/Iran/RZ77/2009)

RT-PCR and sequence analysis:

The viral RNA was extracted directly from the allantoic fluid by means of the High pure viral Nucleic Acid Kit (Roche Germany).Purified genomic RNA was used to generate cDNA clones by (RT-PCR) according to the standard procedure (Lee *et al.,* 2001).

Primers used for PA amplication were:

Forward primer (1668 bp):

5'- GCAAAAGCAGGAGTGAAAATG-3'

Reverse primer (1668 bp):

5'- AGTCCTGAGCACAAATAACTGG-3'

The PCR products were purified by using High pure product purification kit (Roche Germany).PCR products were applied to low melting point agarose (LMP) and the distinct bands were purified from gel for sequencing (MWG co, Germany)

Nucleotide and deduced amino acid sequences of the PA gene were edited with the Editseq (DNASTER Software package Version 5.2) Nucleotide and deduced amino acid sequences were aligned by ClustalW, Version 1.4.

Nucleotide sequences of the PA gene were used for phylogenetic tree construction. The phylogenetic analysis was performed with the MegAlign program 2.8.

RESULTS AND DISCUSSION

Results

In this study 1668 base pair of the PA genes were sequenced and amino acid sequences 556 of the PA genes 10 isolates were deduced from the nucleotide sequence.

The Iranian isolates did not exhibit insertions or deletions within (PA) gene as compare with their prototype A/turkey/winconsin/66 but rather numerous point mutations were registered.

These viruses differed from other viruses by 8 amino acid substitutions at the following positions: 8 (I to M) ،220 (N to C) ، 276 (H to R) 289 (N to S) ، 385 (R to I) ، 391 (H to k) ، 410 (N to L).

Phylogenetic analysis of the PA gene showed that all the PA genes of the Iranian H9N2 viruse fell in two groups, unknown avian and Dk1(duck) (Figure1). All of the H9N2 viruses isolated in 2008-2009 except A/chicken/Iran/RZ53/2008 belonged to the unknown avian sublineage which grouped with the 2004

Pakistani H7N3 viruses. A/chicken/Iran/RZ53/2008 clustered with Dk2 sublineage, which is most closely related to Dk/ST/163/04, an isolate possibly from migratory duck.

Base on sequence comparison and our previous studies we identified at least two different genotypes, designated F and B, among these 10 viruses (Figure 2)

The PA polymerase genes of the recent H9N2 viruses originated from 2 sublineages. The PA gene of A/chicken/Iran/RZ53/2008 (genotype G) belonged to the Dk2 sublineage. However, most PA genes of the H9N2 viruses isolated in 2008-2009(genotypes F) were similar to those of the H7N3 viruses isolated in the Pakistan.

A number of residues in the polymerase proteins (PB1, PB2 and PA) are known to play a key role in the host range of avian influenza viruses to increase virulence or replication in the mammalian host. One of the Iranian isolates (A/chicken/Iran/RZ71/2009) carry amino acid substitution N 615 K in the PA gene, a mutation that correlates with the adaptation of H9N2 avian influenza virus to mice and humans.

Discussion

H9N2 viruses circulated widely in the Middle East (Iran, United Arab Emirates Israel) and were associated with serious disease in poultry (Aamir *et al.,* 2007; Alexander, 2007; Mosleh *et al.,* 2009; Perk *et al.,* 2008). In this study, we have reported the genetic analysis Of PA gene of H9N2 avian influenza viruses and found that Iranian viruses had undergone genetic reassortment . The molecular basis of host-range restriction and of adaptation of influenza A viruses to a new host species has not yet been defined. Previous studies suggested that mutation of the polymerase complex is required for adaptation to a new host and may enhance replication and transcription of the adapted virus in a mammalian host (Gabriel *et al.,* 2005; Wu *et al.,* 2009). Earlier work focused on the role of the HA and M1 proteins in host-range restriction and adaptation (Govorkova *et al.,* 2000; Wan and Perez, 2007). But numerous studies now indicate that the virulence of influenza viruses is likely to be a multigenic trait (Gabriel *et al.,* 2005; Chen *et al.,* 2007). Amino acid N615K of PA is a known determinant of pathogenicity and host specificity. Gabriel *et al.,* (2005) have indicated that the N 615 K substitution may be crucial for adaptation to mammalian hosts (Gabriel *et al.,* 2005). We surmise that the substitution PA N615K observed here in Iranian viruses may also lead to increased pathogenicity.

Instead of Lys at position 615 within the PA protein, Arg was found in human H1N1, H5N1, and H9N2 isolates including A/HK/483/97, A/HK/485/97, and A/HK/1073/99. The avian strains A/Teal/HK/W312/97 (H6N1) and A/Quail/HK/G1/97 (H9N2), proposed donors of internal genes of H5N1 viruses, were found as well, emphasizing the relevance of PA 615Arg for host change (Gabriel *et al.,* 2005). Previous studies have shown that the Eurasian lineage consists of at least three sublineages represented by their prototype strains: A/chicken/ Korea/38349-p96323/96 (Korean-like), A/duck/Hong Kong/ Y280/97 (Y280-like), and A/quail/Hong Kong/G1/97 (G1- like) (Guan *et al.,* 2000; Matrosovich *et al.,* 2001). As Xu *et al.,* (2007) reported, our result also showed that PA gene of H9N2 viruses formed different sublineages including G1-like , Ck/ Beijing –like (Y280-like), three duck lineages (Dk1, Dk2,Dk3) and unknown avian (Xu *et al.,* 2007).

Our previous studies indicated that Iranian surface glycoprotein genes (HA and NA) and one internal genes (NP) are similar to G1-like virus represented by Qa/HK/G1/97 (Soltanialvar *et al.,* 2011; Soltanialvar *et al.,* 2010) whereas the PA genes of the Iranian H9N2 viruses, formed a distinct group compared to G1-, Korean- and Y280-like sublineage.

Comparison of the extent of PA gene sequence homologies of the Iranian isolates showed more similarity with a H7N3 chicken isolate from Pakistan (A/Chicken/ Karachi /NARC-100/2004 (92.5–95.5%) compared to Qa/HK/G1/97 (85.3–86.6%), Dk/HK/Y280/97 (84.7–86.9%) and Ck/Korea/323/96 (88.2–89.9%) (Table 1). Based on the genetic similarities and phylogenetic analysis, our findings suggest that the Iranian viruses had undergone genetic reassortment with other influenza subtypes including H7 viruses.

Like the Iranian isolates, reassortment between H9N2 and the highly pathogenic avian influenza virus H7N3 subtype was reported in Pakistan (Abbas *et al.,* 2010). It is also noted that the viruses from Dubai

and Pakistan shared an outgroup relationship with the Iranian viruses in the PA gene tree suggesting that these viruses are derived from the same gene pool.

Phylogenetic analysis of the Iranian PA gene revealed at least two different genotypes. Our identification of novel genotypes of H9N2 viruses in 2008-2009 was markedly similar to those of a recent study conducted by Igbal *et al.*, (2009) in the Pakistan (Iqbal *et al.*, 2009). This finding suggests a high degree of diversity among the

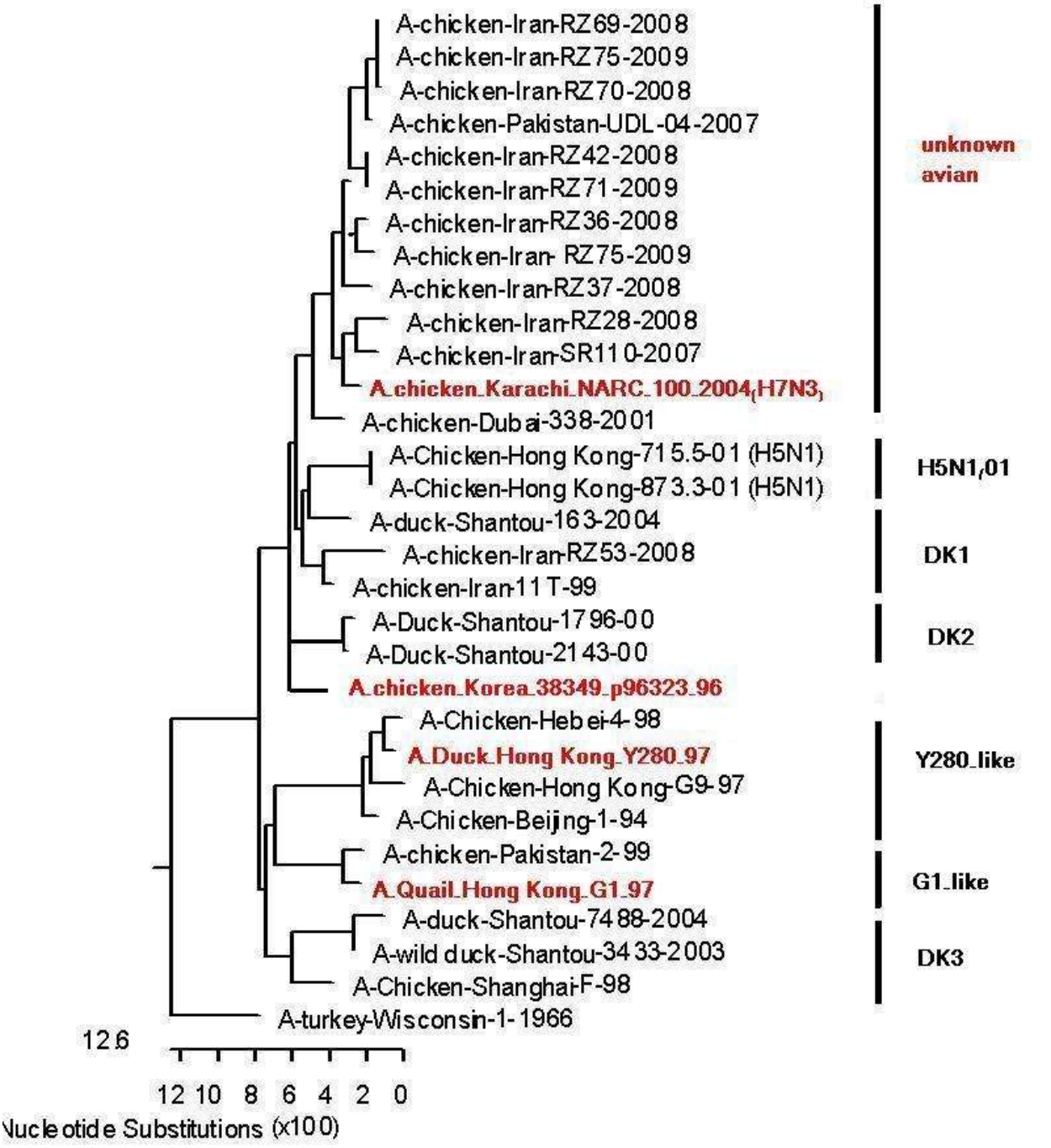

Figure 1: Phylogenetic analysis of the PA gene showed that all the PA genes of the Iranian H9N2 viruse fell in two groups, unknown avian and Dk1

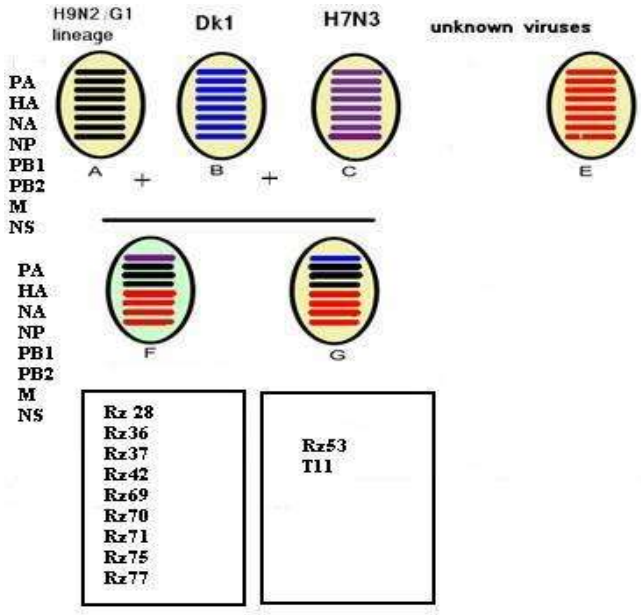

Figure 2: Phylogenetic analysis of the Iranian PA gene revealed at least two different genotypes

Table 1: Homology of PA genes of Iranian isolates with other H9N2 avian influenza viruses from GenBank

Abbreviations Qa: Quail, DK: Duck, Ck: chicken, Ir: Iran, Kch: Karachi, ST: Shantou

	Percent Homologya with				
Iranian isolates	**Qa/HK/G1/97**	**Dk/HK/ Y280/97**	**ck/Korea/323/96**	**Ck/Kch /NARC-100/04 (H7N3)**	**Dk/ST/163/04**
Ck/Ir/RZ28/08	85.3	84.7	88.2	95.3	85.1
Ck/Ir/RZ 36/08	85.4	85.7	88.4	94.4	86.6
Ck/Ir/RZ 37/08	85.3	84.8	88.3	94.2	85.7
Ck/Ir/RZ 42/08	86.4	84.7	88.9	95.1	85.5
Ck/Ir/RZ 53/08	85.4	84.8	89.2	92.4	95.4
Ck/Ir/RZ 69/08	86.0	85.7	88.5	95.1	85.3
Ck/Ir/RZ 70/08	85.3	85.1	88.2	94.4	88.5
Ck/Ir/RZ 71/09	86.6	86.3	89.9	95.1	88.2
Ck/Ir/RZ 75/09	85.7	84.7	89.2	94.7	89.9
Ck/Ir/RZ 77/09	85.5	86.9	89.1	95.1	89.2
Ck/Ir/ SR10/07	85.4	84.9	88.6	95.5	85.8
Ck/Ir /11T/99	85.3	84.7	88.2	92.5	95.3

H9N2 viruses in the region. It is also worth noting that these virus genotypes identified in this study were isolated within a 8-month period of 2008-2009; therefore, they were cocirculating rather than appearing in a stepwise fashion. This genetic heterogeneity is of some concern in view of the prevalence of H9N2 viruses in Middle East.

In subsequent years, multiple reassortant variant genotypes of H9N2 avian influenza viruses from domestic poultry in China and India have been identified and well characterized (Tosh *et al.*, 2008; Sun *et al.*, 2010; Wu *et al.*, 2008). The association of high mortality in recent years and report of H5N1 and H9N2 in wild birds in Iran (Shoushtari *et al.*, 2007) raised the probability of a new genetic modified avian influenza virus, this feature suggests the pandemic potential of the H9N2 avian influenza virus and emphasizes the need for continuous surveillance in Iran, which has been continuing since 2000.

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