International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 14 (3), pp. 001-008, March, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Molecular characterization and phylogenetic analysis of neuraminidase gene of avian Influenza H9N2 viruses isolated from commercial broiler chicken in Iran

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Accepted 18 September, 2019

Avian influenza (AI) outbreaks due to H9N2 subtype of avian influenza virus (AIV) occurred in poultry industry in Iran, throughout the past decade and caused serious economic losses as a result of decreased egg production, increased mortality and the cost of vaccination in Iranian poultry industry. Our aim was to find the phylogenic and genetic relationships among avian influenza viruses recently isolated from poultry in Iran with other Asian H9N2 lineages (1998-2007). The complete nucleotide sequences of the neuraminidase (NA) genes of ten H9N2 influenza virus strain isolated from diseased chickens in different farms in Iran during 1998-2007, were amplified and sequenced and phylogenetic relationships between these and previously reported N2 genes were investigated. The viruses surface glycoprotein genes was >90% similar to those of A/Quail/Hongkong/G1/97 (H9N2) lineage. The NA stalks regions in these viruses had no deletion or insertion or shortening as compared to that A/DK/HK/Y280/97 lineage (CK/Bei-like viruses) and the 2 human isolates A/HK/1073/99, A/HK/1074/99. The hemadsorbing site of NA had up to 3 amino acid substitution and is different from those of earlier Iranian viruses and ten Iranian isolates possessed seven potential glycosylation sites like the most H9N2 isolates. Phylogenetic analysis of NA gene showed that they belonged to the same A/Quail/Hong Kong/G1/97 like virus sub lineage.

Key words: Avian influenza virus, A/Quail/Hongkong/G1/97 (H9N2), neuraminidase gene, phylogenetic analysis, Iran.

INTRODUCTION

Influenza A viruses are enveloped, single-negative-stranded and segmented ribonucleic acid (RNA) viruses belonging to the Orthomyxoviridae family; they are highly infectious respiratory pathogens in their respective natural hosts. These viruses are classified into three types A, B and C, on the basis of their internal nucleoprotein and matrix protein antigens. Both antigens are regarded as common to all strain of same type. Influenza viruses are further categorized into subtypes, according to their surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins (Swayne and Halverson, 2003). Avian influenza viruses (AIVs) belong to type A and 16 H subtypes (H1-H16) and 9 N subtypes (N1-N9) have been reported (Fouchier et al., 2005). Based on the pathogenicity of AIVs to domestic poultry.These viruses are subclassified into 4 pathotype groups of highly pathogenic avian influenza (HPAI) viruses, causing rapid mortality in poultry which often

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approaches 100% and non-highly pathogenic avian influenza (nHPAI) viruses including mildly pathogenic (MP), low pathogenic (LP) and non pathogenic (NP). NHPAI, causing unapparent diseases with mild respiratory signs, egg production losses and sometimes with slightly elevated mortality (Capua and Alexander, 2006).

To date, all HPAI isolates have been of H5 and H7 subtypes, although viruses of these subtypes do not necessarily cause HPAI (Swayne et al., 2003; Zanella et al., 2001; Capua and Alexander, 2006). These viruses are listed as A group diseases by the Office International des Epizooties (OIE) (OIE, 2005).

Avian influenza (AI) disease due to H9N2 subtype in poultry during later part of the 1990s has been noticeably increased worldwide. The H9N2 subtype outbreaks occurred in domestic ducks, chickens and turkeys in Germany during 1995 and 1998, in chickens in Italy in 1994 and 1996, in pheasants in Ireland in 1997,ostriches in South Africa in 1995, turkeys in the USA in 1995 and 1996 and in chickens in Korea in 1996 (Bano et al., 2003; Capua and Alexander, 2004; Naeem et al., 1999). More recently, H9N2 viruses have been reported in Middle Eastern countries and have been responsible for widespread and serious disease problems in commercial chickens in Iran, Pakistan, Saudi Arabia and United Arab Emirates (Aamir et al., 2007; Alexander, 2007; Banks et al., 2000; Capua and Alexander, 2004; Naeem et al., 1999; Nagarajan et al., 2009; Nili and Asasi, 2002; Vasfi Marandi and Bozorgmehri Fard, 2002). Avian influenza due to H9N2 subtype was occurred in densely populated area of Tehran province of Iran and caused serious economic losses in poultry industry in 1998. Since these highly contagious viruses were spreading in poultry flocks of other provinces of country, a vaccination strategy by using inactivated H9N2 vaccine was adopted to control AI disease in poultry industry (Vasfi and Bozorgmehri, 2002).

Human cases of H9N2 viruses were recorded in Hong Kong although, no death was reported (Peiris et al.,1999; Riedel, 2006). Crossing the species barrier to mammals highlights the pandemic potential of H9N2 virus with the emergence of non-human H9N2 isolates with avian characteristics, it is important to study the H9N2 isolates from avian hosts in addition to those obtained from humans for pandemic influenza understanding and readiness (Wenjun et al., 2009).

Two major surface glycoproteins, HA and NA, provides distinct activities important for virus replication. NA is an important functional surface glycoprotein of influenza virus.The mutations in the HB site were previously seen in viruses that bind to α-2,6-linked receptors (Rashid et al., 2009). HA binds to terminal sialic acid groups on cell surface glycoconjugates for cell surface attachment. NA possesses an enzymatic activity that removes sialic acid from sialoglycoconjugates. This activity enables release of virions from infected cells and removes sialic acid from newly synthesized HA and NA molecules to prevent self-aggregation of virions.The influenza NA plays a critical role in spread of influenza A virus (Olivier et al., 2008).

In the present study, NA genes and proteins isolated from ten Commercial broiler chicken in Iran between 1998-2007 were characterized.This isolates resulted in rapid mortality due to tracheitis and respiratory congestion. Their genomes, HB sites of NA gene of isolates were delineated and phylogenetic relationship to the other Asian H9N2 lineages were established.

MATERIALS AND METHODS

Sampling and virus isolation

Ten H9N2 influenza virus strains used in this study were isolated from diseased or dead chickens among which there was 10-35% mortality between 1998-2007 from various part of the country. In Razi Vaccine and Serum Research Institute, initial isolation of the virus was performed in l0-day-old SPF embryonated chicken eggs (ECE) following the standard protocol (OIE, 2005).

Briefly, triturated tissue samples treated with 2 antibiotic and antimycotic solution (pecillin 10000 unit mL^{-1}, streptomycin 10000 unit mL^{-1} and nystatin 20000 unit mL^{-1}) for 30 min at 37°C and clarified by centrifugation at 1500 RPM for 10 min were inoculated through allantoic routes. The allantoic fluid was harvested after incubation at 37°C for 72-96 h and clarified by centrifugation at 1500 g for 15 min at 4°C. The HA test negative samples were given two more passages and tested again before being declared negative for AIV isolation. Subtype identification of the viruses were determined in standard hemagglu-tination inhibition and PCR tests using specific antisera to the reference strains of influenza viruses (Shortridge et al., 1997). Allantoic fluids were harvested from ECE-passaged virus and used as a stock in Razi Institute for sequence analysis. The 10 virus isolates used in this study were named as follows:

(A/Ck/Ir/661/98,A/Ck/Ir/772/99,A/Ck/Ir/584/00,A/Ck/Ir/450/01,A/Ck/Ir/466/02,
A/Ck/Ir/284/02,A/Ck/Ir/92/03,A/Ck/Ir/133/04,A/Ck/Ir/68/06,
A/Ck/Ir/sh-2/07)

RNA extraction and RT-PCR

The viral RNA was extracted from allantoic fluid by means of the high pure viral Nucleotid Acid Kit (Roche Germany). Purified genomic RNA was used to generate deoxyribonucleic acid (DNA) clones by [real time polymerase chain reaction (RT-PCR)] according to the standard procedure as previously described (Guan et al., 2000). The RT-PCR was performed using spesific primers for 980 (nucleotides 1 to 980) and 560 (nucleotides 920 to 1470) bp fragment of NA. Primers used for NA amplication were:

Forward primer(980 bp):(5'-GCAAAAGCAGGAGTGAAATGA-3')
Reverse primer(980 bp):(5'AGTCCTGAGCACAAATAATGC-3')
Forward primer(560 bp):(5'-TTAGATGTGTTTGCAGGGCAC-3')
Reverse primer(560 bp):(5'-GGTTCTAAAATTGCGAAAGCC-3')

Gene sequence and phylogenetic analysis

The PCR products were purified using high pure product

Table 1. Accession No. for the Nucleotide sequences obtained in this study.

Viruse strain	Accession No.
A/Ck/Ir/661/98	HM165464
A/Ck/Ir/772/99	HM165465
A/Ck/Ir/584/00	HM165466
A/Ck/Ir/450/01	HM165467
A/Ck/Ir/284/02	HM165468
A/Ck/Ir/466/02	HM165469
A/Ck/Ir/92/03	HM165470
A/Ck/Ir/133/04	HM165471
A/Ck/Ir/68/06	HM165472
A/Ck/Ir/sh-2/07	HM165473

purification kit (Roche Germany). The PCR products were applied to low melting point (LMP) agarose and the distinct bands were purified from gel for sequencing (MWG Co., Germany).Nucleotide and deduced amino acid sequences of the NA genes were edited with the Editseq (DNASTAR Lasergene Software package Version 7.1.0) then, Nucleotide and deduced amino acid sequences were aligned by ClustalW Method. phylogenetic tree was performed with the MegAlign program version 7.1.0 and established their relationship to the Asian H9N2 lineages for example China, Korea, Hong Kong, Pakistan, Saudi Arabi, Dubai and Turkey.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study are available from GenBank under accession No.: HM165464- HM165473 (Table 1).

RESULTS

Analysis of the NA sequence of the H9N2 viruses

In this study, 1410 base pairs of the NA genes (ORF region) were sequenced and amino acid sequences (469) of the NA of the 10 isolates were deduced from the nucleotide sequence. The amino acid sequence of the isolates showed high identity (90.7-99.7%) to each other. Amino acids of HB_site in the NA stalk are three location which include, first position 366-373, second position 399-404 and third position 431-433. HB_site at position 366-373 are IKKDSRAG except for A/chicken/Iran/133/2004, A/chicken/Iran/450/2001 and A/chicken/Iran/68/2006 isolates which are IKQDSRAG and A/chicken/Iran/sh-2/2007 isolate which is IKKDSREG. The other situation being contained single type DSDNLS at position 399-404 and one type PQE at position 431-433 (Table 2).

The NA stalk regions in these viruses had no deletion or insertion or shortening as compared to that other G1 sublineage (Table 2).

Analysis of NA protein sequences showed that these H9N2 viruses have seven glycosylation sites with the N-X-T/S motif in which X may be any amino acid except proline. Seven of these sites were located in the NA protein [positions 61, 69 (70), 86, 146, 200, 234 and 402] (Table 3). These regions between the N2 NA proteins was compared and demonstrated that H9N2 virus isolates that has been obtained in this study are like the most H9N2 viruses (Table 3).

Phylogenetic analysis

In this study phylogenetic analysis of the N2 gene indicate that H9N2 influenza viruses prevalent in chicken population in Iran belonged to the same A/Qail/Hong Kong/G 1/97-like virus sublineage (Figure 1). These findings further confirm the Asian origins of the Iranian viruses. In turn, the Iranian isolates (this isolates and the other isolates) can be divided in to three subgroups: The first of these includes viruses isolated from 1999 to 2004. The Iranian strains isolated during 2004-2006 fall in to the second subgroup. The strains isolated after 2006 and until the end of the study(A/chicken/Iran/sh-2/2007) fall into the third subgroup (Figure 1). Thus these three subgroup are roaming in the country since 1998.

However the samples for several years fell within distinct clusters. It seems that Iran is being faced with two phenomena: accumulation of mutations and reassortment in the G1-like sublineage.

DISCUSSION

The H9N2 AIVs have been circulating in the domestic poultry in Iran for 12 years and were still isolated from chickens, even from some vaccinated flocks. Although, the H9N2 viruses are described as low pathogenic avian influenza (LPAI) viruses, which may cause high morbidity and mortality (Aamir et al., 2007; Kim et al., 2006; Nili and Asasi, 2002; Guo et al., 2000). Guo et al. (2000) have reported that Ck/Beijing/1/94 is lethal for chickens in the field and also under experimental condition. Because of the significant role of NA gene, in this present study the NA gene of ten H9N2 influenza virus strains isolated from poultry farms suspected to Avian Influenza in Iran on 1998-2007 were sequenced. The main function of influenza virus NA is responsible for the release and spread of new virus from the cell by its action on the neuraminic acid in the receptors (Kim et al., 2006)

The hemadsorbing (HB) site is located on the NA surface away from the enzymatic site. Amino acid at the HB site of NA of influenza viruses is conserved among aquatic bird viruses and equine viruses. The NA of ten isolates carried substitutions (in spite of earlier Iranian isolates) in the HB site similar to those of other avian H9N2 viruses from Asia and human H9N2 viruses (Table 2). This conservation of NA hemadsorption activity among many isolates of avian viruses representing most subtypes of NA suggests that it is maintained for a

Table 2. Amino acid substitutions in the hemadsorbing sites and deletion in stalk.

Virus	Subtype	Deletion in stalk	HB-Site		
			366-373	399-404	431-433
CK/IR/661/98	H9N2	-	IKKDSRAG	DSDNLS	PQE
CK/IR/772/99	H9N2	-	IKKDSRAG	DSDNLS	PQE
CK/IR/584/00	H9N2	-	IKKDSRAG	DSDNLS	PQE
CK/IR/450/01	H9N2	-	IKQDSRAG	DSDNLS	PQE
CK/IR/466/02	H9N2	-	IKKDSRAG	DSDNLS	PQE
CK/IR/92/03	H9N2	-	IKKDSRAG	DSDNLS	PQE
CK/IR/133/04	H9N2	-	IKQDSRAG	DSDNLS	PQE
CK/IR/284/02	H9N2	-	IKKDSRAG	DSDNLS	PQE
CK/IR/68/06	H9N2	-	IKQDSRAG	DSDNLS	PQE
CK/IR/SH-2/07	H9N2	-	IKKDSREG	DSDNLS	PQE
CK/IR/RZ42/08	H9N2	-	IRKDSREG	DSDNWS	PQE
CK/IR/RZ53/08	H9N2	-	IKQDSRAG	DSDNLS	PQE
CK/IR/ZMT-10/98	H9N2	-	IKKDSRAG	DSDNLS	PQE
QA/HK/G1/97	H9N2	38-39	IKKDSRSG	DSDIRS	PQE
HK/1074/99	H9N2	38-39	IKKDSRSG	DSDNWS	PQE
DK/HK/Y280/97	H9N2	63-65	IKEDSRSG	DSDNWS	PQE
CK/IR/L252/03	H9N2	-	IKQDSRAG	DSDNLS	PQE
CK/IR/B263/04	H9N2	-	IKKDSRAG	DSDNLS	PQE
CK/DUB/338/01	H9N2	-	IKKDLRAG	DSDNWS	PQE
TUR/WISC/1/66	H9N2	-	ISKDSRAG	DSDNWS	PQE
CK/BJ/1/95	H9N2	63-65	IKEDSRSG	DSDNWS	-
CK/IS/1465/03	H9N2	-	IKKDSRAG	DSDNLS	PQ-
CK/GER/113/95	H9N2	57-81	ISKDSRSG	DSDNWS	PQE
CK/KOR/MS96/96	H9N2	-	INKDSRSG	DSDNWS	PQE
CK/IR/NGV-1/06	H9N2	-	IKKDSRAG	DSDNWS	PQE
CK/SD/1/02	H9N2	63-65	IKEDSRSG	DSENWS	-
CK/PK/4/99	H9N2	-	IKKDSRAG	DSNWS	PQE

Table 3. Sequence of amino acids in glycosilation sites between the N2 neuraminidase proteins.

Virus	Amino acid site (s)						
	61	69, 70	86	146	200	234	402
CK/IR/661/98	N	N	N	N	N	N	N
CK/IR/772/99	N	N	N	N	N	N	N
CK/IR/584/00	N	N	N	N	N	N	N
CK/IR/450/01	N	N	N	N	N	N	N
CK/IR/466/02	N	N	N	N	N	N	N
CK/IR/92/03	N	N	N	N	N	N	N
CK/IR/133/04	N	N	N	N	N	N	N
CK/IR/284/02	N	N	N	N	N	N	N
CK/IR/68/06	N	N	N	N	N	N	N
CK/IR/SH-2/07	N	N	N	N	N	N	N
CK/IR/RZ42/08	N	N	N	N	N	N	N
CK/IR/RZ53/08	N	N	N	N	N	N	N
CK/IR/ZMT-10/98	N	N	N	N	N	N	N
QA/HK/G1/97	N	N	N	N	N	N	I
HK/1074/99	N	N	N	N	N	N	N
DK/HK/Y280/97	N	N	N	N	N	N	N

Table 3. Contd.

CK/IR/L252/03	N	N	N	N	N	N	N
CK/IR/B263/04	N	N	N	N	N	N	N
CK/DUB/338/01	N	N	N	N	N	N	N
TUR/WISC/1/66	N	N	N	N	N	N	N
CK/BJ/1/95	S	NS	N	N	N	N	N
CK/IS/1465/03	-	-	-	-	-	-	N
CK/GER/113/95	-	-	N	N	N	D	N
CK/KOR/MS96/96	N	NN	N	N	N	N	N
CK/IR/NGV-1/06	N	NG	N	N	N	N	N
CK/SD/1/02	S	NS	N	N	N	N	N
CK/PK/4/99	N	NS	N	N	N	N	N

specific role in virus replication (Fereidouni et al., 2009). It is believed that the HB site in the NA of Asian H9N2 viruses to be under positive selection pressure for mutations, which results in compatible combinations of HA and NA (Matrosovich et al., 2001). However, avian viruses co-circulating in mammals such as pigs and humans accumulate various substitutions in the HB site, thus decreasing their HB capacity (Baum and Paulson, 1991; Lee et al., 2009). Together, human virus-like receptor specificity and similar substitutions in the HB region of the NA in these recent isolates suggest the possible role of poultry species such as chickens in the zoonotic transmission of influenza viruses from aquatic birds to humans (Gambarian et al., 2002; Moosakhani et al., 2010). This study showed that it is possible the replacement were observed in HB site in the NA genes of Middle East influenza H9N2 viruses, as a result of the virus circulation in the region since the advent of H9N2 viruses in 1997. Despite all these changes in viruses of Middle East, including the A/chicken/Iran/sh-2/2007, they still have a relationship to the G1 sublineage.

The sequence and length of the stalk region is known to vary among and within NA subtypes. Shortening of the NA stalk by deletion of the amino acid is characteristic of the highly pathogenic H5 and H7 influenza viruses (Matrosovich et al., 1999). However it is not known whether deletion in the NA of H9N2 virus is correlated with pathogenicity in chickens. The NA stalk regions in these ten viruses, similar to the Iranian sequence available in GenBank had no deletions in the stalk (Table 2).

Over all, the NA genes H9N2 viruses classified into a single group within a G1-like sublineage,which the H9N2 influenza viruses isolated from humans in Hong Kong also had deletion in positions 38 and 39, however, No deletion was observed in H9N2 influenza viruses isolated from Pakistan and United Arab Emirates. One can say that although each sublineage might be formed in two separate groups, but these groups have common ancestor. The whole NAs of the isolates H9N2 influenza Viruses prevalent in Chickens in China during 1995-2002, had 3 amino acid deletions at positions 63-65 in the stalk, which confirmed that these isolates were closely related (Liu et al., 2003). No deletion was observed in the N2 of Korean, human and swine, and Ck/HK/G9/97 group viruses .

Pattern of glycosylation sites (ASP aminoacid at positions 61, 69 or 70, 86, 146, 200, 234 and 402) between all types of N2 NA proteins viruses were similar (Guan et al.,2000). Viruses isolated in this study and other Iranian H9N2 viruses which are available in GenBank , show similar patterns of glycosylation sites. It is note worthy that glycosylation site at position146 among N2 NA proteins viruses was protected (Guo et al., 2000).

Phylogenetic analysis of the NA genes of the influenza viruses revealed that there was three distinct lineages: North American avian, human and swine, and Eurasian avian lineages. The Eurasian avian lineage consists of three groups. Group I includes the Korean strains(CK/Kor/MS96/96). Group II represented by Qa/HK/G1/97 consists of human H9N2(A/HK/1074/99) and Pakistan H9N2 viruses, Group III consists of only Hong Kong H9N2 viruses isolated in 1997 (DK/HK/Y280/97). According to the phylogenetic analysis, all the Iranian isolates belonged to a single lineage which is closer to viruses from the so-called G1 lineage, a finding that may indicate a common origin of all Iranian isolates form a comprised single progenitor and confirm the Asian origins of these. In turn, this group divides into three main subgroups: The first of these includes viruses isolated from 1999 to 2004 . The Iranian strains isolated during 2004-2006 fall in to the second subgroup. The strains isolated after 2006 and until the end of the study(A/chicken/Iran/sh-2/2007)fall into the third subgroup (Figure 1). No Ck/Bei-like viruses and Korean like viruses was found in chicken population since the outbreak of H9N2 influenza in Iran in 1997. This evidence indicates that (HA genes) and (NA gene) of H9N2 influenza virus circulating in Iran during the past years were well conserved and the earliest Iranian isolates or neighboring countries may be considered to

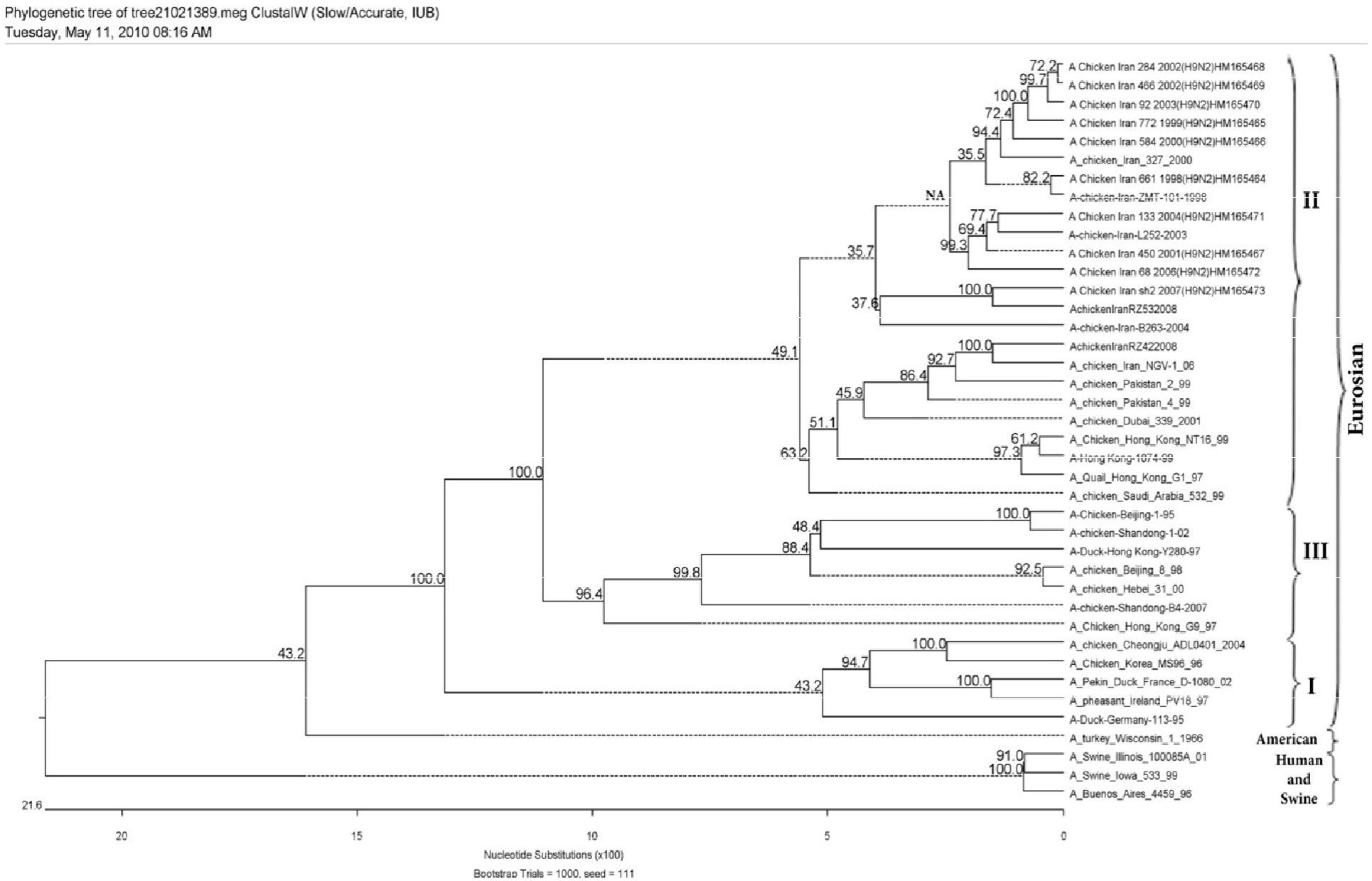

Figure 1. Phylogenetic relationships of Neuraminidase genes from H9N2 viruses isolated in Iran during 1998-2007, and other reprasantative AIVs of these subtype.

represent such a distant ancestor. Nevertheless, according to the topology of the phylogenetic tree, the Iranian group was not homogeneous. It seems that Iran is being faced with two phenomena: accumulation of mutations and reassortment in the G1-like sublineage. It is possible these differences are the result of accumulation of mutations among the viruses circulating within the poultry stock. Even though Iranian samples fell within distinct clusters and showed several reassortment events in G1 group. It seems, therefore, this new isolate (A/chicken/Iran/sh-2/2007) and together with 2008 isolate are due to form a new cluster with a variable nature(Soltanialvar et al., 2010). The wide cocirculation of distinct genetic clusters of H9N2 viruses in the Middle East may explain the NA gene changes are more predominant rather than HA gene changes. One of the factors driving the evolution of low-pathogenic influenza viruses is the immunological pressure, which increases in the case of the continuous vaccination. The postvaccinal specific immunity of the population could be conclusive as a selective factor in ensuring prevalence of a viral strain which differed from the vaccinal strain and earliest Iranian isolates; however, there are great numbers of report that indicated the group viruses in Eurasian sublineage confer an immunity to each other (Aamir et al., 2007; Xu et al., 2007).

NA genes of previous Iranian H9N2 viruses isolates (1998-1999) showed significant similarity with a G1-like sublineage and H9N2 influenza viruses isolated from humans in Hong Kong, this thought indicating that by 1999, Iranian influenza viruses had been experiencing constant changes and no new H9N2 isolates introduced. But in these new isolates similarity of H9N2 viruses has been reduced. These may suggest the introduction of new NA gene.

Overall, migratory water birds are natural reservoir of influenza A viruses and it is found that the most viruses had been introduced in Iran are H9N2 subtypes (Toroghi et al., 2006). The prevalence of LPAIV in wild birds in Iran, especially wetlands around the Caspian Sea which represent an important wintering site for migratory water birds.These birds may play an important role in the ecology and perpetuation of influenza viruses in this region (Fereidouni et al., 2010; Guo et al., 2000).

In addition, Qa/HK/G1/97-like viruses have the potential public health risk because the internal genes of the Qa/HK/ Gl/97-like viruses are similar to those of the 1997 H5Nl human and avian isolates, and human H9N2 viruses isolated in Hong Kong in 1999 also belong to Qa/HK/G 1/97 lineage (Lin et al., 2000; Cameron et al., 2000). It is, therefore, important to continue the molecular epidemiology surveillance to check whether the Qa/ HK/Gl/97-like viruses exist in chicken population in Iran.

ACKNOWLEDGEMENTS

This research was financially supported by Razi Vaccine and Serum Research Institute of Iran.

Abbreviations: AIV, Avian influenza virus; **RT-PCR,** reverse transcription polymerase chain reaction; **CK,** chicken; **DK,** duck; **HK,** Hong Kong; **Ir,** Iran; **NA,** neuraminidase; **H,** hemagglutinin; **HPAI,** highly pathogenic avian influenza; **nHPAI,** non-highly pathogenic avian influenza; **MP,** mildly pathogenic; **LP,** low pathogenic; **NP,** non pathogenic; **OIE,** Office International des Epizooties; **AI,** Avian influenza; **ECE,** embryonated chicken eggs; **DNA,** deoxyribonucleic acid; **LMP,** low melting point; **LPAI,** low pathogenic avian influenza; **HB,** hemadsorbing.

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