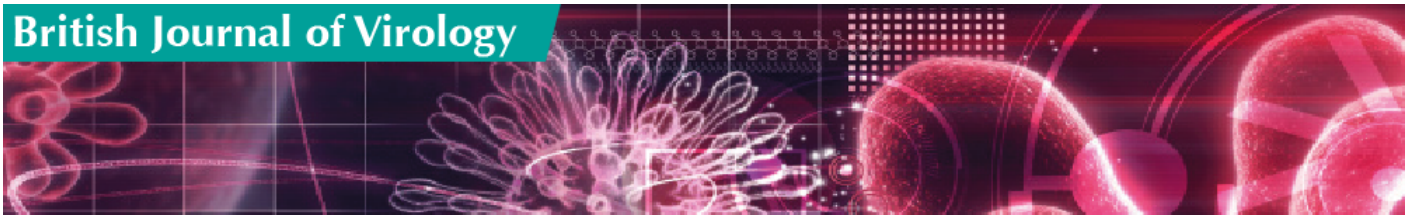

Management of clinical cases of peste des petits ruminants (PPR) disease in goats.

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Detection and phylogenetic analysis of *Peste des Petits Ruminants Virus* isolated from outbreaks in Punjab, Pakistan

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Keywords: Peste des Petits ruminants, sheep, goats, Pakistan, DNA sequencing

Running title: Characterization of PPR from Pakistan

Summary

Peste des Petits Ruminants (PPR) is an important viral disease of small ruminants and is endemic in Pakistan. In the following study, two outbreaks of PPRV in goats have been subjected to laboratory investigations. The PPRV genome was detected using both conventional and real-time PCR. Genetic characterization of the local PPRV field isolates was conducted by sequencing 322 bp of the fusion (F) and 255 bp of the nucleoprotein (N) genes. The phylogenetic tree, based on the F gene, clustered isolates from both outbreaks into lineage 4 along with other Asian isolates and more specifically into subclusters 1 along with isolates from Middle East. Conversely, analysis of N gene revealed a different pattern in branching where Pakistani isolates clustered with Chinese, Tajikistani and Iranian isolates which probably represent the true geographical pattern of circulation of the virus. This is the first report presenting the phylogenetic tree based on N gene and performing a parallel comparison of the trees of F and N gene together from Pakistani isolates, despite the fact that PPRV is one of the most devastating diseases for small ruminants in the country. The results of this study shed light on the PPR virus population in Pakistan and emphasize the importance of using molecular methods to understand the epidemiology. Such understanding is essential in any efforts to control the number and impact of outbreaks that are occurring in endemic countries such as Pakistan especially in the current scenario when OIE and FAO are eager to control and subsequently eradicate PPR from the globe, as it has been practices for Rinderpest.

Introduction

Peste des petits ruminants (PPR) is a highly contagious and often fatal morbilli-viral infection of both wild and domestic small ruminants, and is characterized by high pyrexia (up to 106 °F), anorexia, nasal and ocular discharges, erosive stomatitis, conjunctivitis, gastroenteritis and pneumonia. *Peste des Petits Ruminats virus* (PPRV), a causative agent for PPR, is a single-stranded negative sense RNA morbillivirus. Clinically, PPRV affects

goats severely but sheep undergo a mild form of the disease while cattle may experience sub-clinical infection (Anderson and McKay, 1994). PPR causes serious economic losses in Asia, the Middle East and Sub-Saharan Africa.

Based on specific regions of the nucleocapsid (N) and fusion (F) gene, PPRV isolates are grouped into four distinct lineages while only one serotype is reported (Shaila et al., 1996). Lineages II and I are found exclusively in West Africa while lineage III has been isolated in eastern Africa (Ethiopia), Arabia (Oman, Yemen) and unexpectedly once from southern India. The fourth lineage (IV) is believed to be confined completely in the Middle East, Arabia, south Asia and China (Shaila et al., 1996; Wang et al., 2009).

Since the first confirmation of PPRV in Pakistan in 1994, a huge number of outbreaks have occurred in the country; however only a limited number of these outbreaks are documented due to a lack of awareness. After successful eradication of Rinderpest from Pakistan in 2007, PPRV gained attention and it's economic importance and transboundary nature was recognised. Recently, a number of studies, including this one, have been conducted to ascertain the magnitude of the PPR problem, characterize the virus population and to define the trends and causes of its occurrence in Pakistan (Hussain et al., 1998; Ahmad et al., 2005; Khan et al., 2008; Munir et al., 2008; Abubakar et al., 2008; Abubakar et al., 2009; Munir et al., 2009; Abubakar et al., 2010). Previous studies have almost always been based on either laboratory confirmation by detecting antibodies/antigen using ELISA or based on clinico-epidemiological observations. However, given the increase in circulation of PPR virus among susceptible populations and for future eradication concern of the FAO and OIE (Normile, 2010), it is of paramount importance to delve into the molecular and genetic characterization of the field PPR viruses. The objective of this communication was to detect PPRV in suspected outbreaks in the region by employing conventional and real-time PCR and then to use sequencing to provide phylogenetic analysis of nucleoprotein and fusion protein gene segments to get an insight genetic picture of PPRV.

Materials and Methods

Clinical history and sample collection

A total of 12 samples including nasal swabs and blood were collected from two outbreaks in Pakistan. A representative sample from each outbreak was subsequently taken for sequencing. The detailed information of samples is presented in Table 1. Lyophilized freeze-dried inactivated PPR vaccine (Nigeria 75/1) obtained from CIRAD, France was used as the reference virus during the course of study. Biological material was stored on QIAcard FTA Indicator Four Spots (Qiagen), which preserve nucleic acids and inactivate the samples. The samples were shipped at ambient temperature from Pakistan to the Swedish University of Agricultural Sciences, Uppsala, Sweden for processing.

Screening of the samples using real-time PCR

The RNA was extracted from Qiacard FTA Indicator (Qiagen) impregnated with clinical samples as recommended by manufacturer (preparation of isolated RNA from FTA® Cards, Rev 1 10/17/07, Whatman) with the following modifications. Using a 2.0mm diameter Harris micro punch (Whatman), one punch for each sample was removed according to manufacturers protocol (BD09, Whatman) and placed in separate 1.5 ml microfuge tubes. 200µl Tris-EDTA buffer (10mM Tris-HCl, pH 8.0, 0.1mM EDTA) was used instead of RNA processing buffer and incubated for 15 minute (flicking tubes 3 times over the course of incubation) on ice, not at room temperature.

PCR amplification and detection was performed in Rotor-Gene 6000 real-time analyzer (Qiagen) using AgPath ID one-step RT-PCR kit (Applied Biosystem) as recommended. The primers in the N region named NPPRf TAQ: GAG TCT AGT CAA AAC CCT CGT GAG and NPPRr TAQ: TCT CCC TCC TCC TGG TCC TC and probe sequence NPPRp TAQ: FAM-5'-CGG CTG AGG CAC TCT TCA GGC TGC-3'-BHQ1 were used. The reporter dye (FAM) signal was measured at the annealing step of each

cycle and the threshold cycle (C_t) for each sample was calculated. The samples having C_t value <35 of samples were considered positive.

PCR amplification, TOPO cloning and sequencing of F and N genes

One sample from each outbreak (Multan and Faisalabad) was selected for sequence analysis. A fragment of 380 bp was amplified from the most variable region at the 3′ end of the N gene using primers; PPR-N-F (5′- TCTCGGAAATCGCCTCACAGACTG-3′) and PPR-N-R (5′-CCTCCTCCTGGTCCTCCAGAATCT-3′). The F gene was amplified using PPRV-F-F 5'-ATCACAGTGTTAAAGCCTGTAGAGG-3', PPRV-F-R 5-'GAGACTGAGTTTGTGACCTACAAGC-3' primers. In both PCRs, 3µl of RNA was used in a 25µl reaction using One-Step RT-PCR kit (Qiagen) as recommended. The amplified PCR products were gel extracted and cloned in pCR3.0 using TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. At least 3 recombinant plasmids were processed with Miniprep (Promega) for DNA extraction and processed for sequencing using ABI PRISM BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Sequences were analyzed with an automated nucleic acid analyzer (ABI PRISM 3100, Applied Biosystems, Foster City, CA). Each DNA fragment was sequenced at least twice in both directions.

Sequence and phylogenetic analysis

Sequence assembly and editing were performed using the SEQMAN programme of DNASTAR Lasergene 8 (version 8.0.2 13). Construction of phylogenetic trees was performed with the Neighbour-Joining method using Kimura two-parameter model in Mega5 version 5. BLAST program was used to search GenBank for homologous gene sequences in NCBI database. To look for variation among the sequences under comparison, a phylogenetic network was drawn for the nucleotide (F gene) or amino acid (N gene). The data was processed by star contraction algorithm (Forster et al., 2001) and then by Median-Joining (MJ) network algorithm (Bandelt et al., 1999) (www.fluxus-engineering.com). The sequences of the genes analyzed here have been

submitted to the GenBank. The accession numbers for F gene are JN009671 and JN009672 and for N gene JN009673 and JN009674.

Results

The Qiacard FTA Indicator (Qiagen) appeared to have been an appropriate sampling system for this study. The results from the clinical material revealed that all the samples tested from each outbreak were positive when tested with real-time PCR. However, conventional PCR showed two and three positive from Multan and Faisalabad districts, respectively (Table 1).

The analysis of 322 bp F gene sequences revealed that the homology between the two isolates in the present study ranged between 99-100% whereas the similarity of F gene with previously characterized Pakistani isolates range from 95% to 98%. Similarly, the sequence homology of N gene between isolate of each outbreak range between 99-100%. Since there is no N gene sequence available from Pakistani origin, no similarity can be calculated for previously characterized Pakistani PPRV isolates.

A phylogenetic tree for each gene (F and N) was constructed based on the sequences retrieved from GenBank, representing all the lineages. The F gene tree depicted that Pakistani isolates under study clustered together with isolates of Middle East (Fig. 1). Further, lineage IV in F gene tree can be divided into subclusters (SC): SC1, SC2 and SC3. Pakistani isolates were grouped in either SC2 (Pakistan/SAH/Pak/07 and Pakistan/FSD/PK/07) or in SC1 (Pakistan/94 and the isolates under study). The N gene based tree shows that all the four lineages are clearly separated from each other, however in this tree, the Pakistani isolates clustered together with isolates from Tajikistan, Iran and China (Fig. 2).

The network analysis based on F gene sequences showed that all the four lineages are well separated from each other (Fig. 3), each represented by different colour. A central

node was formed by 26 isolates of lineage IV, which is mainly constituted by Indian isolates. Another isolate (IN00 AF464879) made a second node, outside the central node. The previously characterized Pakistani isolate differs from the central node by 3 mutations, with the new isolates differing by further 5 and 7 mutations. Isolates from Saudi Arabia and Kuwait were also clustered in this minor taxon. The network analysis for the N protein showed that the isolates from the present study were separated from a central node made by Indian isolates in lineage IV with a difference of 3 and 6 mutations (Fig. 4). Lineage 1 showed a close relationship with lineage IV, however all the lineages are clustered in separate branches.

Discussion

There have been several outbreaks of PPR in Pakistan throughout the year. Several studies have confirmed the diagnosis of PPR based on immunoglobulin detection from clinical samples (Hussain et al., 1998; Ahmad et al., 2005; Khan et al., 2008; Munir et al., 2008; Abubakar et al., 2008; Abubakar et al., 2009; Munir et al., 2009; Abubakar et al., 2010). In order to understand the epidemiology of these PPRV outbreaks, it is essential to characterize these viruses to get better insight into the virus population. For the conservation of biological samples, especially where cold chain is unavailable, filter papers with inactivation properties containing dried blood or clinical samples appears to be a practical sampling system. It has been practiced for many RNA and DNA viruses (Michaud et al., 2007). The fact that all the samples in this study were detected by real-time PCR indicates that it is a convenient, inexpensive and appropriate source of sample collection and transportation for PPRV from goats, which have clinical signs.

PPRV has enormous economic importance, which is due to its highly infectious nature (Abubakar et al., 2011) and serious clinical implications. The high morbidity (100%) and mortality (90%) in PPRV susceptible populations is mainly contributed by secondary infections, which cause symptoms such as pneumonia or gastroenteritis. The majority of animals in the outbreaks were either malnurished or had heavy parasitism. It is plausible that malnutrition/parasitism are factors for increasing the clinical relevance of

PPRV infections. A compromised immune status caused by parasites and/or malnutrition may make animals more susceptible to catching and have serious symptoms result from PPRV.

To assess the genetic similarity and divergence between the isolates of these two outbreaks and their relationship with previously characterized isolates submitted to GenBank, the partial F and N gene sequences were phylogenetically analyzed. An overall topology of the trees indicated that PPRVs from different geographical regions vary to a larger extent in their N gene than F gene sequences. Previously characterized Pakistani PPRV isolates were grouped in either subcluster 1 or 2 in the lineage IV (based on F gene) where as the isolates in current study were grouped into subcluster 1 which constitute the majority of PPRV isolates sequenced so far (Shaila et al., 1996; Kerur et al., 2008). Because the N sequences of Pakistani PPRV isolates are not available (or not sequenced before) it is hard to predict the homology and rate of variations over the time. It appears from the tree constructed on N gene that Pakistani isolates are closely related to Chinese, Tajikistani and Iranian isolates. It is logical that PPRV could have come from these countries, as they are geographically proximate to Pakistan. On the other hand, the F gene based tree showed that Pakistani isolates are close to isolates from Middle East with whom Pakistan has immense trade. Recent evidence indicates that N gene appears to provide the better epidemiological picture of PPRV and is now in favour over F gene classification (Shaila et al., 1996). However, given the fact that PPRV has tendency to mutate, phylogenetic analysis based on both F and N genes should provide a more informative characterization. This is demonstrated clearly in results of this study, which has the new isolates clustering differently for the two genes.

Due to the high number of silent mutations in the 3rd codon, the amount of amino acidic variations in the F-gene MJ network was too low to get a good geographical resolution (data not shown). The network based on the nucleotide variation gives a better resolution (Fig. 3). The F gene based network analysis indicated that branching of PK10_MULTAN and PK10_FAISALABAD matched the branching pattern shown by

Neighbour-joining method for the corresponding sequences. Further, the new isolates clustered with Indian and Iranian isolates in a different taxon than previously characterized Pakistani PPRV isolates. The isolates in the present study along with another Pakistani isolate characterized in 1994 branched in a separate taxon with in lineage IV. This indicates either a number of introductions into Pakistan or, a high mutation rate, or both. In any case, further studies at larger scale are warranted. Network analysis of for the N protein was in concordance with the branching pattern shown by the consensus tree.

To conclude, based on clinical signs and the detection of PPRV using real-time and conventional PCR, the disease outbreaks in the current study were caused by PPRV. The phylogenetic analysis of the F and N genes showed that Pakistani PPRV isolates clustered into lineage IV, as expected. In order to address the problem, real-time or RT-PCR and sequencing should be added to regular serological surveillance to allow characterization of PPRV in the region. The OIE and FAO should recommend this test to enable investigations into the cause and control of outbreaks. Given the economic importance of PPRV due to high case fatality, morbidity and mortality and due to high production losses, it is extremely important that such monitoring take place. To further reduce the economic impact of PPRV on small ruminant industry in Pakistan, an awareness campaign to the livestock owners should be considered.

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Table. 1: Description of the samples used in the study.

Outbreak districts	Clinical picture	Samples collected	Parasitism	Malnutrition	Real-time PCR	Conventional PCR
Multan	Beetal Breed, Diarrhoea, Weakness, Oral necrosis, Fever (104 °F)	4	Yes	Yes	Positive (all)	Positive=2, Negative=2
Faisalabad	Desi Breed, Sever diarrhoea, Weakness, Shivering, Lesions around oral cavity, Fever (103 °F)	8	No	Yes	Positive (all)	Positive=3, Negative=5

Fig. 1. Majority rule consensus tree of PPR viruses based on the variable region of the F gene (322 bp) constructed using the neighbour-joining method in the Kimura-two-parameter model in Mega5 version 5. Numbers indicate the bootstrap values (1000 replicates) and only the values above 50% are shown in the figure. Horizontal distances are proportional to sequence distances. Figure indicates the clear division of the four lineages of PPRV. The samples used in this study are marked with round black spot (●).

Fig. 2. Majority rule consensus tree of PPR viruses based on the variable region of the N gene (255 bp) constructed using the neighbour-joining method in the Kimura-two-parameter model in Mega5 version 5. Numbers indicate the bootstrap values (1000 replicates) and only the values above 50% are shown in the figure. Horizontal distances are proportional to sequence distances. Figure indicates the clear division of the four lineages of PPRV. The samples used in this study are marked with round black spot

(●).

Fig. 3. Phylogenetic network analysis based on the F gene of the field isolates with corresponding sequences published in the GenBank. Numbers along the branches represent the nucleotide changes separating the nodes. Based this network analysis, the isolates from the present study clustered in the single taxon with 7 mutations (PK10_MULTAN) or 5 mutations (PK10_FAISALABAD) compared to previously characterized Pakistani isolate (PK94_FR667646). Samples used in this study are marked in circle.

Fig. 4. Phylogenetic network analysis based on the N protein of the field isolates with corresponding sequences published in the GenBank. Numbers along the branches represent the amino acid changes separating the nodes. Samples used in this study are marked in circle.

Molecular and phylogenetic analysis of hemagglutinin (HA) and neuraminidase (NA) genes of H9N2 avian influenza viruses isolated from vaccinated broiler chickens in Iran in 2011

Abstract

H9N2 avian influenza outbreaks have been one of the major problems of poultry industry in Iran. A vaccination program has been implemented to control disease since 1988, but some outbreaks continued to occur in several broiler flocks. In this study four strains which were isolated in 2011 from vaccinated broiler flocks with a history of respiratory illness were sequenced. Sequenca analysis HA and NA genes indicated that all strains isolated in this study shared nucleotide homologies of 91.3% to 92.4% and 90.4% to 91.1% with the vaccine strain for HA and NA genes, respectively. Extra potential glycosylation sites could be observed in HA and NA genes of current isolates versus vaccine strain. Molecular analysis of all four isolated strains showed KSSR motif of cleavage site in the HA genes, while it is RSSR for typical strains in Iran. Phylogenetic analysis of HA and NA genes showed that all strains isolated in this study fell into the same subgroup and they belong to A/Quail/HongKong/G1/97 H9N2 lineage but in comparison with vaccine strain they had some deviation in the decade and fell into different subgroup. Other changes included loss, addition and variation in predicted epitope in HA and NA genes of field strains. These results suggest that the commercial vaccine genetically is not enough coordinated with current viruses in region. Therefore,

constant evaluation of important genes induced immunity and vaccine efficiency in protection against these strains would be essential.

Key words: Phylogenetic analysis-Hemagglutinin-Neuraminidase-Influenza virus-Glycosylation-Epitope-Iran-Vaccine

Introduction

Avian influenza is caused by type A influenza virus and is categorized based on the activity of two surface glycoprotein; Hemagglutinin and neuraminidase(Webster et al., 1992; Guo et al., 2000). There are 16 HA and 9 NA subtypes responsible for causing infection. HA plays an essential role in the early stages of infection and is responsible for the virus binding to its receptor, sialic acid, which is present on the host cell surface and promotes fusion of viral and endosomal membranes and eventually facilitates viral entry into the host cell (Webster et al., 1992). The NA surface glycoprotein of influenza viruses prevents virus aggregation by cleaving the α-ketosodic linkage between sialic acid and adjacent sugar residue. This results in destruction of receptors recognized by HA and facilitates the to and fro movement of the virus from the site of infection (Gubareva et al., 2000; Guo et al., 2000). Avian influenza virus (AIV) H9N2 which is known to infect poultry populations throughout the world, is derived from the Eurasian and the North American influenza virus genes(Fouchier et al., 2005). In chickens 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 (Banks et al., 2000; Nili and Asasi. 2002; Vasfi Marandi and Bozorgmehri-Fard. 2002; Capua and Alexander. 2006; Nagarajan et al., 2009; Bozorgi et al., 2012)

The first report of AIV (H9N2) in poultry flocks in Iran was in 1998. Since 1998 H9N2 avian influenza outbreaks have been one of the major problems in Iranian poultry industry(Karimi et al., 2004). These highly contagious viruses were spreading in poultry flocks of other provinces of country, thus a vaccination strategy by using inactivated H9N2 vaccine based on A/Ck/Iran/av1221/98 was adopted to control AI disease in poultry industry (Vasfi Marandi and Bozorgmehri-Fard. 2002). In spite of using of this vaccine, some outbreaks continued to occur in several broiler flocks with great economic losses. All H9N2 subtypes isolated from vaccinated or unvaccinated chickens belonged to non HPAI till now(Vasfi Marandi and Bozorgmehri-Fard. 2002; Soltanialvar et al., 2010)

Present study was conducted to carry out phylogenetic analysis and molecular characterization of new strains isolated in Iran and establish the phylogenetic relationships between Iranian Isolates and neighboring or Eurasian sublineages since 1998 until 2011; also to identify and predict epitopes for antigenic changes and

variations in glycosylation sites, and to compare circulating viruses with vaccine strain yearly.

Material and Methods

Sampling and virus isolation: samples were collected from 25 farms in various parts of country since February until December 2011. Tissue samples usually included trachea and lung. All samples were collected from flocks with the history of acute respiratory illness and vaccination against H9N2 influenza virus. Virus isolation was carried out in 9-11 days old SPF embryonated eggs respecting the standard protocol (OIE,2005). Virus subtypes were determined by a standard Hemagglutination Inhibition (HI) and Neuraminidase Inhibition (NI) test (Shortridge et al,1997). The four isolated viruses in this study were named as follow: (A/chicken/Iran/186/2011,A/chicken/Iran/187/2011,A/chicken/Iran/189/2011,A/chicken/Iran/191/2011). The A/Ck/Iran/av1221/98 H9N2 virus which is the selected strain for producing vaccine in iran since 1998 was used for molecular and phylogenetic analysis.

RT-PCR and sequence analysis: The field viruses RNA were extracted directly from the allantoic fluid by means of High Pure viral Nucleic Acid Kit (Roche Germany).The RNA of vaccine strain was extract from a commercial vaccine in Iran.
Purified genomic RNA was used to generate cDNA clones by RT-PCR through the standard procedure which was using specific primers

Primers used for HA amplification were:

Forward primer (1720 bp): 5'-AGCAAAAGCAGGGG-3'

Reverse primer (1720 bp): 5'-AGTAGAAACAAGGGTGTT-3'

Primers used for NA amplification were:

Forward primer (1470 bp): 5'-GCAAAAGCAGGAGTGAAATGA-3'

Reverse primer (1470 bP): 5'-GGTTCTAAAATTGCGAAAGCC-3'

The PCR products were purified using High Pure product purification kit (Roche Germany). These products were applied to low Melting Point (LMP) agarose and distinct bands were purified from gel for sequencing (MWG,Germany).

Nucleotide and deduced amino acid sequences of HA and NA genes from new isolated strains were edited by BioEdit version 7. Nucleotid and amino acid sequences were aligned by ClustalW, In order to compare H9N2 viruses isolated in this study with circulating virus since 1998 to 2011, a computerized search of all available sequences of avian influenza Virus subtype H9N2 reported in poultry flocks in neighboring countries of Iran and the Eurasian G1 sublineage reference was conducted. The search was performed with National Centre for Biotechnology Information (NCBI) Flu Database(Bao et al., 2008) and sequences of NA and HA were retrieved and downloaded (data not shown).The phylogenetic analysis was performed with the Mega 5 program(Tamura et al., 2011)

Determination of glycosylation site

To determine glycosylation sites of viral strain, an online server application, ScanProsite (de Castro et al., 2006) was used to compare and identify N-glycosylation sites.

Epitope analysis of HA and NA for antigenic variations

Epitope prediction was performed by CTL (Cytotoxic T Lymphocyte) epitope prediction method (Bhasin and Raghava. 2004). The amino acid sequences of HA and NA proteins from selected strains were used in this step and the predicted antigenic sites from each sequence were compared so that composition similarities and differences were determined.

Phylogenetic analysis of HA and NA nucleotides.

Sequencing avian influenza H9N2 viruses isolated from Iran since 1998 until 2011 was done by MEGA 5 (Tamura et al., 2011).. The selected nucleotide sequences based on local alignment and homology searches using BLAST were aligned by using CLUSTALW. Phylogenetic trees were constructed by using neighbor-joining tree with maximum composite likelihood. Internal branching probabilities were determined by bootstrap analysis of 1000 replicates and were indicated by percentage value on each branch.

Results

Disease signs in the field: The symptoms included anorexia, reduced feed consumption, gasping and conjunctivitis with facial edema. The major post mortem findings were tracheitis, respiratory congestion. Mortality was up to 10%, resulting in considerable economic losses.

Analysis of HA and NA sequences of the H9N2 viruses

The sequenced part of the HA gene contains 1720 nucleotide coding 566 amino acid residues. This protein region includes a complete receptor-binding pocket, cleavage site, glycosylation site and various antigenic epitopes. In order to analyse them we compared the HA amino acid sequences (deduced from the nucleotide sequences) of new strains isolated in this study with those of other Iranian isolates between 1998 and 2011, vaccine strain and the Eurasian G1 sublineage reference strain (A/Quail/HongKong/G1/97).

Nucleotide sequence analysis of the new HA genes showed homologies of 92/2-99/6%and shared a homology of (90.8-92%) with H9N2 isolates of A/QA/HK/G1/97 lineage.

In this study, 1410 base pairs of the NA genes (ORF region) were sequenced and amino acid sequences (469) of the 4 isolates were deduced from the nucleotide sequence. The nucleotid sequence of the isolates were highly identical (95.4-99.6%) and shared a homology of (88.3 - 89%) with H9N2 isolates of A/QA/HK/G1/97 lineage.

The deduced amino acid sequence revealed that the HA cleavage site motif was

KSSR/GLF.

The receptor binding site motif

Analysis of the HA receptor binding pocket in particular showed that, except for A/Ck/Ir/584/20, A/Ck/Ir/TH81/02, A/Ck/Ir/TH81/02andA/Ck/Ir/119/05 isolates, other H9N2 isolates from Iran contained Leucine (L) at position 234(Table 1)

N-Glycosylation site of HA and NA

Potential glycosylation sites with the N-X-T/S motif (in which X may be any amino acid except proline) were predicted by ScanProsite for HA and NA viral proteins isolated from Iran shown in Tables 3 and 4 respectively. A total of seven predicted glycosylation sites (PGS) at position (29-32; 105-108; 141-144; 298-301; 305-308; 492-495; 551-554) were obtained for each protein sequence of HA isolated since 1998 until 2011. With the Exception of vaccine strain in 1998 which did not have glycosylation site at position(551-554).

More amino acid substitutions and variations were also observed for NA protein sequences. Nine glycosylation sites for the dominant viral strains from 1998 to 2006 were similar to each other but viral strains isolated in 2011 revealed five new modifications at N-glycosylation site NSSK, NGTI, NRSG, NSSE, NVTE at positions 44-47, 69-72, 402-405, 44-77, 61-64 in A/Ck/Ir/N101/2011, A/CK/Ir/191/2011, A/Ck/Ir/187/2011, A/Ck/Ir/186/2011, A/Ck/Ir/198/2011 Respectively and one new glycosylation site NSSY at position 313-316 in A/Ck/Ir/189/2011.

The hemadsorbing (HB) site

Amino acids of HB site in the NA stalk are at three locations, the first position is 366-373, the second one is 399-404 and the third one is 431-433. HB sites at position 366-373 were IKKDSRAG except for A/ck/Iran/b111/2004 and A/ck/Iran/68/2006 isolates which was IKQDSRAG and A/ck/Iran/SH2/2007 isolate which was IKKDSREG. A/ck/Iran/RZ36/2008 and A/ck/Iran/RZ71/2009 were IRKDSRAG. HB site at position 399-404 was DSDNLS except for A/ck/Iran/RZ36/2008 and A/ck/Iran/RZ71/2009 which was DSDNWS and all the strains isolated in this study which was DSDNRS. The other situation contained single type PQE at position 431-433 (Table 2).

Phylogenetic analysis

Evolutionary relationship of HA and NA nucleotides sequence were determined by comparing these Iranian isolates and selected H9N2 viruses isolated in several other countries with the established Eurasian H9N2 lineages: namely, the G1-lineage, the Y280-lineage and the Korean-lineage represented by prototype viruses A/Quail/Hong Kong/G1/97, A/Duck/Hong Kong/Y280/97 and A/Chicken/Korea/38349-p96323/96 respectively, and with viruses identified in the Far East as emerging H9N2 lineages(Xu et al., 2007a).The strain of current vaccine was also included in this analysis. Phylogenetic analysis of the HA gene indicates that all Iranian isolates belong to the A/Qail/HongKong/G1/97-like virus sublineage, (Figure 1). The strains which were

isolated in this study had close relationship with other Iranian strains which were dominant during 2009-2011 suggesting that they are from common source. Moreover the strong relationship was observed with H9N2 viruses from Pakistan, Saudi Arabia, Egypt and Israel respectively. In turn, the Iranian isolates (this isolates and the others) can be divided in to three subgroups: The first of these are viruses isolated since 1998 to 2005. The Iranian strains isolated during 2004-2008 fall in to the second subgroup. Those strains isolated during 2009 to 2011 and new strains in this study fall into the third subgroup (Figure1). Therefore these three subgroups are present in the country since 1998. None of the sequences clustered within the Y280 and Korean-like lineages.

Phylogenetic analysis of N2 gene showed that all the NA genes of the Iranian H9N2 viruses fall into G1-like lineage (Fig.2). These findings further confirm the Asian origins of the Iranian viruses. In turn, this group is divided into subgroups: the first group includes viruses isolated from 1998 to 2007, the second subgroup includes strains isolated during 2008 -2011.

Epitope analysis of HA and NA for antigenic variations

Results from CTLPred epitope prediction showed that HA protein sequences isolated from Iran had some variations. Epitope GNCPKYVRV which was observed in position 312 of the vaccine strain (A/Ck/Ir/av1221/98), was absent in current strains except for Ck/Ir/191/2011 that was GTCPKYISL. Another epitope (IGLRNVPAK) at position

327-336 that was seen in Ck/Ir/189/2011 had not been found in previously reported strains in Iran. Furthermore, viruses isolated since 2004 to 2011 and viruses isolated in this study revealed SFYRNMRWL epitope at position 154-163 that was absent in vaccine strain(Table 5).

Epitopes for NA showed lots of variations in a yearly manner. More observations revealed that GANINFMSI and HRTLLMNEL epitopes at position 461-469 and 155-163, were present in 1998 and vaccine strains, respectively. But were absent in viruses isolated in this study. Instead SCYPRYPEV, SCHDGRAWL and SELGVPFHL epitopes at positions 279-287, 182-190 and 161-169 which are present in strains in 2011 were not seen in 1998 and vaccine strain. Another change was that DYNINSSYL epitope was only present in Ck/Ir/189/2011(Table 6).

Table 1 : Receptor binding site and cleavage site of H9N2 viruses

Viruses	98 (106)	153 (161)	155 (163)	183 (191)	190 (198)	194 (202)	195 (203)	226 (234)	cleavage site 335-339
A/Ck/HK/G1/97	G	W	T	H	E	L	Y	L	RSSR
A/Ir/av1221/98	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/TH77/98	-	-	-	-	A	-	-	-	RSSR

A/Ck/Ir/661/98	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/584/20	-	-	-	-	A	-	-	Q	RSSR -
A/Ck/Ir/TH81/02	-	-	-	-	A	-	-	Q	RSSR
A/Ck/Ir/320/03	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/B76/04	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/152/04	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/B102/05	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/119/05	-	-	-	-	A	-	-	Q	RSSR -
A/Ck/Ir/20/06	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/SH2/07	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/R36/08	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/SS2/08	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/EBVG/10	-	-	-	-	T	-	-	-	KSSR
A/Ck/Ir/ 186/11	-	-	-	-	A	-	-	-	KSSR
A/Ck/Ir/ 187/11	-	-	-	-	A	-	-	-	KSSR
A/Ck/Ir/ 189/11	-	-	-	-	A	-	-	-	KSSR
A/Ck/Ir/191/11	-	-	-	-	A	-	-	-	KSSR

Table 2 :Amino acid substitution in the hemadsorbing sites and deletion in stalk

		Site HB		
Viruses	Deletion in stalk	366-373	399-404	431-433
A/Ck/Iran/av1221/98	-	IKKDSRAG	DSDNLS	PQE
A/ Ck/Iran/661/1998	-	IKKDSRAG	DSDNLS	PQE
A/Ck/Iran/11T/99	-	IKKDSRAG	DSDNLS	PQE
A/Ck/Iran/284/2002	-	IKKDSRAG	DSDNLS	PQE
A/Ck/Iran/B111/2004	-	IK**Q**DSRAG	DSDNLS	PQE
A/Ck/Iran/68/2006	-	IK**Q**DSRAG	DSDNLS	PQE
A/ Ck/Iran/SH2/2007	-	IKKDSR**E**G	DSDNLS	PQE
A/ Ck/Iran/RZ36/2008	-	I**R**KDSRAG	DSDN**W**S	PQE
A/Ck/Iran/RZ53/2008	-	I**R**KDSR**E**G	DSDNLS	PQE
A/Ck/Iran/RZ71/2009	-	I**R**KDSRAG	DSDN**W**S	PQE
A/Ck/Iran/N101/2011	-	IKKDSRAG	DSDN**R**S	PQE
A/Ck/Iran/186/2011	-	IKKDSRAG	DSDN**R**S	PQE
A/Ck/Iran/187/2011	-	IKKDSRAG	DSDN**R**S	PQE

A/Ck/Iran/189/2011	-	IKKDSRAG	DSDN**R**S	PQE
A/Ck/Iran/191/2011	-	IKKDSRAG	DSDN**R**S	PQE
A/QA/HK/G1/97	38-39	IKKDSRSG	DSDIRS	PQE
A/HK/1074/99	38-39	IKKDSRSG	DSDNWS	PQE
A/DK/HK/Y280/97 H9N2	63-65	IKEDSRSG	DSDNWS	PQE

Table 3.comparison of predict N-glycosylation sites (PGS)within aminoacid sequences of HA proteins isolated between 1998-2011(H9N2).

Strain	position						
	29-32	105-108	141-144	298-301	305-308	492-495	551-554
A/Ir/av1221/98	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	-
A/Ck/Ir/661/1998	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	-
A/Ck/Ir/TH77/1998	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/TH81/2002	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/B76/2004	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/TH81/2002	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/B76/2004	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/TH186/2007	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/RZ53/2008	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/RZ36/2008	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/EBGV/2010	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/N101/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC

A/Ck/Ir/186/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/187/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/189/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/191/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC

Table 4. comparison of predict N-glycosylation sites (PGS)within aminoacid sequences of NA proteins isolated between 1998-2011(H9N2).

	position								
Viruses	44-47	61-64	69-72	70-73	86-89	146-149	200-203	234-237	313-316 402-405
A/Ck/Ir/av1221/98	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Ir/661/1998	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Ir/ZMT/101/98	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/11T/99	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/284/2002	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/B111/2004	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/68/2006	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/RZ53/2008	-	N**R**TE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/RZ36/2008	-	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	- N**W**SG

A/Ck/Iran/RZ71/2009	-	NITE	N**G**T**I**	-	NWSK	NGT	NATA	NGTC	-	N**W**SG
A/Ck/Iran/N101/2011	N**S**S**K**	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	-	N**R**SG
A/Ck/Ir/191/2011	N**S**S**K**	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	-	N**R**SG
A/Ck/Ir/187/2011	N**S**S**E**	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	-	N**R**SG
A/Ck/Ir/186/2011	N**S**S**K**	N**V**TE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	-	N**R**SG
A/Ck/Ir/189/2011	N**S**S**K**	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	**NSSY**	N**R**SG

	position							
Viruses	431	108	134	154	312	324	327	493

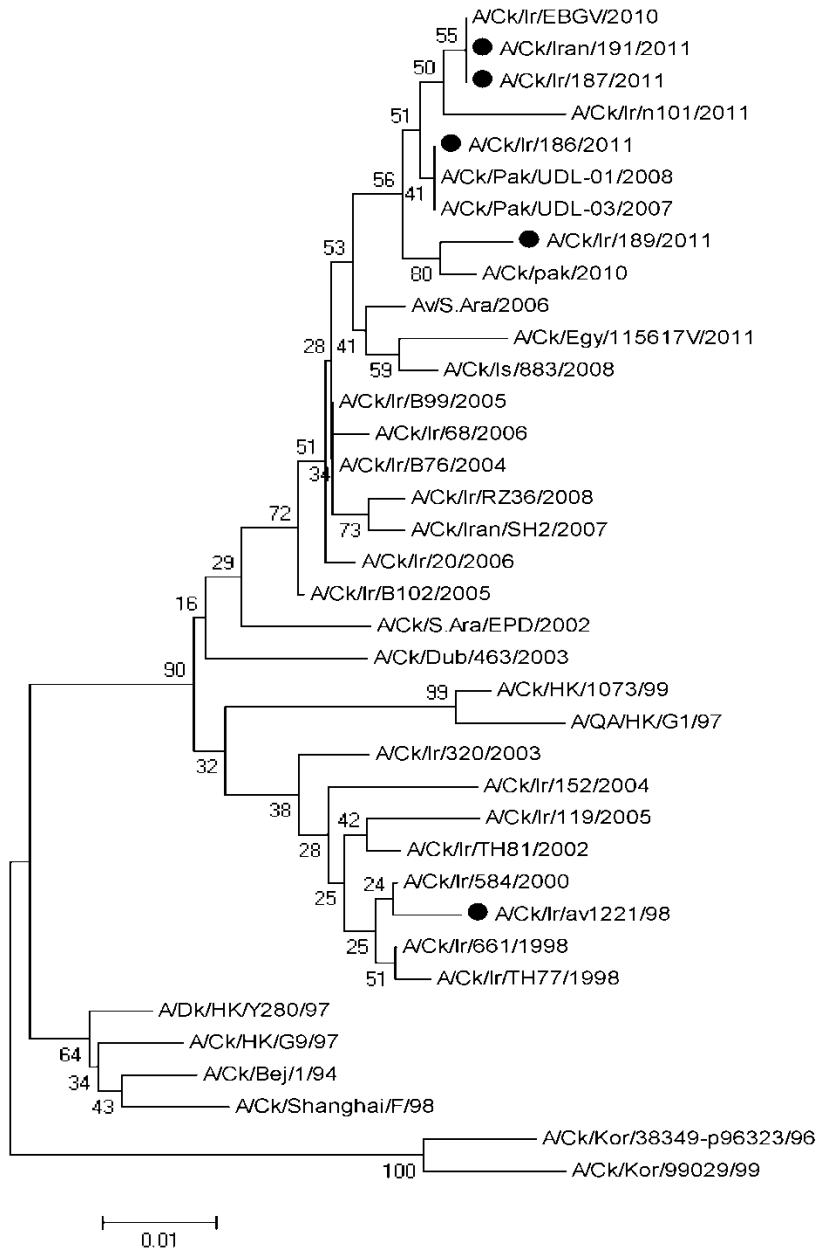
Table 5. Comparison of antigenic epitope in the amino acid sequences of HA protein (H9N2) from 1998 to 2011.

A/Ck/Ir/av1221/98	AYNAELLVL	CYPGNVENL	IFPDTIWNV	-	GNCPKYVRV	KLAVGLRNV	-	-
A/Ck/Ir/TH7798	AYNAELLVL	CYPGNVENL	IFPDTIWNV	-	GNCPKYVRV	KLAVGMRNV	-	-
A/Ck/Ir/661/98	AYNAELLVL	CYPGNVENL	IFPDTIWNV	-	GNCPKYVRV	KLAVGLRNV	-	-
A/Ck/Ir/TH81/2002	AYNAELLVL	-	IFPDTIWNV	-	GNCPKYVRV	KLAVGLRNV	-	GTYDRRKYK
A/Ck/Ir/B76/2004	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/TH186/2007	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/ Ck/Ir/SH2/2007	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/RZ53/2008	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/RZ36/2008	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/EBGV/2010	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/186/2011	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/191/2011	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	GTCPKYISL	-	-	-
A/Ck/Ir/187/2011	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/1892011	AYNAELLVL	CYPGNVENL	-	SFYRNMRWL	-	KLAVGLRNV	IGLRNVPAK	-

Table 6. Comparison of antigenic epitope in the amino acid sequences of NA protein (H9N2) from 1998 to 2011.

	position									
Viruses	**100**	**297**	**248**	**461**	**155**	**279**	**299**	**182**	**161**	**309**
A/Ck/Ir/av1221/98	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/661/98	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/ZMT/101/98	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/11T/99	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/284/2002	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/B112004	FSKDNSIRL	GSNRPVLYI	GRADTRILF	-	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/68/2006	FSKDNSIRL	GSNRPVLYI	GRADTRILF	-	HRTLLMNEL	SCYPRYPEV	-	-	-	-
A/Ck/Ir/SH2/2007	FSKDNSIRL	GSNRPVLYI	-	GANINFMSI	HRTLLMNEL	SCYPRYPEV	-	-	-	-
A/Ck/Ir/RZ36/2008	FSKDNSIRL	GSNRPVLYI	-	GANINFMSI	-	SCYPRYPEV	NRPVLYINM			
A/Ck/Ir/RZ53/2008	FSKDNSIRL	GSNRPVLYI	-	GANINFMSI	HRTLLMNE L	SCYPRYPEV	-	-	-	-
A/Ck/Ir/RZ71/2009	FSKDNSIRL	GSNRPVLYI	-	GANINFMSI	-	SCYPRYPEV	NRPVLYINM	-	-	-
A/Ck/Ir/N1012011	FSKDNSIRL	GSNRPVLYI	GRADTRILF	-	-	SCYPRYPEV	-	SCHDGRAWL	-	-
A/Ck/Ir/191/2011	FSKDNSIRL	GSNRPVLYI	-	-	-	SCYPRYPEV	-	SCHDGRAWL	SELGVPFHL	-

A/Ck/Ir/186/2011	FSKDNSIRL	GSNRPVLYI	-	-	-	SCYPRYPEV	-	SCHDGRAWL	SELGVPFHL	-	
A/Ck/Ir/189/2011	FSKDNSIRL	GSNRPVLYI	GRADTRILF	-	-	-	-	SCHDGRAWL	-	DYNINSSYL	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A/Ck/Pak/UDL-03/2007
A/Ck/Ir/189/2011
A/Ck/pak/2010
Av/S.Ara/2006
A/Ck/Egy/115617V/2011
A/Ck/Is/883/2008
A/Ck/Ir/B99/2005
A/Ck/Ir/68/2006
A/Ck/Ir/B76/2004
A/Ck/Ir/RZ36/2008
A/Ck/Iran/SH2/2007
A/Ck/Ir/20/2006
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A/Ck/S.Ara/EPD/2002
A/Ck/Dub/463/2003
A/Ck/HK/1073/99
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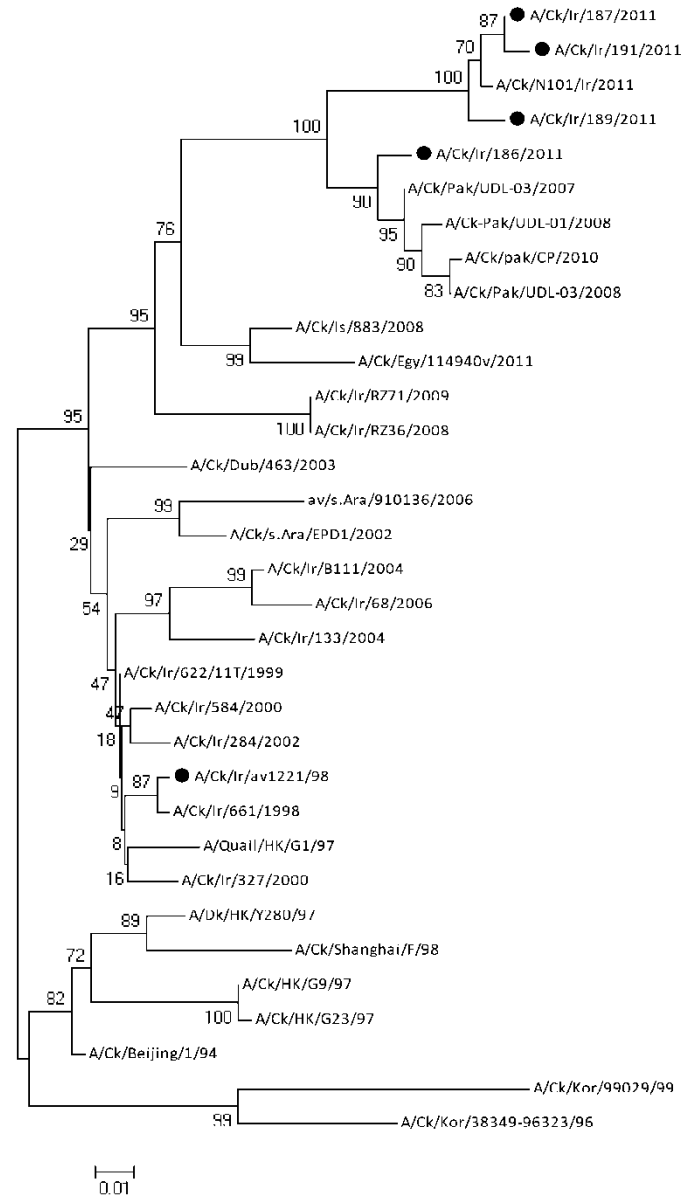
A/Ck/Ir/320/2003
A/Ck/Ir/152/2004
A/Ck/Ir/119/2005
A/Ck/Ir/TH81/2002
A/Ck/Ir/584/2000
A/Ck/Ir/av1221/98
A/Ck/Ir/661/1998
A/Ck/Ir/TH77/1998
A/Dk/HK/Y280/97
A/Ck/HK/G9/97
A/Ck/Bej/1/94
A/Ck/Shanghai/F/98
A/Ck/Kor/38349-p96323/96
A/Ck/Kor/99029/99
55
50
51
56
41
53
80
28
41
59
51
34
72
73
29
16
90
99
32
38
42
28
25
24
25
51
64
34
43
100
0.01

Figure 1:Phylogenetic relationship of HA gene from H9N2 virus isolated in Iran during 1998-2011 and other reperesentative AIV_S of this subtype. The strains presented in this study are indicated as character ●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A/Ck/Ir/584/2000
A/Ck/Ir/284/2002
A/Ck/Ir/av1221/98
A/Ck/Ir/661/1998
A/Quail/HK/G1/97
A/Ck/Ir/327/2000
A/Dk/HK/Y280/97
A/Ck/Shanghai/F/98
A/Ck/HK/G9/97
A/Ck/HK/G23/97
A/Ck/Beijing/1/94
A/Ck/Kor/99029/99
A/Ck/Kor/38349-96323/96
0.01

Figure 2:Phylogenetic relationship of NA gene from H9N2 virus isolated in Iran during 1998-2011 and other reperesentative AIV_S of this subtype.The strains presented in this study are indicated as character ●

Discution

As one of the major surface glycoproteins of Influenza A viruses, HA actually specifies several important functions. The globular tip of the HA protein contains a receptor for binding to the host cell surface (Perdue. 2008). Antibodies made by the infected host and directed at the globular tip effectively attack the HA and prevent binding and entry. However, this globular head also changes due to mutations rapidly enough to produce novel structural components that are no longer recognized by host cell antibodies. This “antigenic drift” in effect gives the virus an “escape” mechanism from antibodies to once again allow efficient binding and entry to the cell. This feature of the HA is the reason that twice yearly the World Health Organization convenes experts to evaluate antigenic and genomic changes and recommend modifications to human vaccine strains according to the changes in the HA in the seasonal influenza human strains(Perdue. 2008). Sequence and phylogenetic analysis have the potential to be effective approaches towards vaccine development(Xu et al., 2007b; Park et al., 2011).Therefore, the present study was performed to verify the extent of antigenic

variation in HA and NA genes and deduced surface proteins sequences of the H9N2 avian virus isolated in Iran.

Analysis of the HA cleavage site showed that strains isolated in 2011 had motife (KSSR/GLF) that was different from those previously reported in Iran. From 1998 to 2008, all H9N2 viruses retained a conserved amino acid pattern at the cleavage site; RSSR/GLF(Vasfi Marandi and Bozorgmehri-Fard. 2002; Karimi et al., 2004; Kianizadeh et al., 2006; Soltanialvar et al., 2010). The motif (KSSR/GLF)might indicate the LPAI nature of H9N2 strains,however the significance of this mutation on viral stability or increased pathogenicity requires further studies, as the cleavage site is considered an indicator of pathogenicity(Senne et al., 1996; Hulse et al., 2004).
It has been well documented that the receptor binding site motif of HA is critical for cellular receptor specificity and determining virus host range(Ha et al., 2001; Gambaryan et al., 2002). The presence of glutamine (Q) at position 234 (H3 numbering: 226) is the typical avian virus signature, and it has been reported that presence of this amino acid results in affinity for binding to 2,3-linked sialic acid (avian receptors), whereas in the case of leucine (L) at the same position, there is a affinity for 2,6-linked sialic acid (human receptors) and potential cause of reported human infections (Lin et al., 2000) Therefore, we could predict that the Iranian isolates in this study have affinity for binding to human like receptor which are similar to it of other strains isolated in Iran(Soltanialvar et al., 2010; Bozorgi et al., 2012)

Glycosylation plays an important role in the viral life cycle. Glycans near the antigenic epitopes interfere with antibody recognition to escape immune pressure from hosts, and those near HA0 cleavage site protect their infectivity(Skehel et al., 1984; Deshpande et al., 1987; Igarashia et al., 2008) Gain or loss of glycosylation sites near the receptor-binding domain can also affect viral receptor recognition(Das et al., 2010; Chen et al., 2011) . Deshpande demonstrated that the only difference in glycosylation between HA1 (virulent H5N2) and HA1 (avirulent H5N2) is the absence of an N-linked oligosaccharide at Asn residue 11 in HA1 (virulent H5N2). This result showed that a site-specific glycosylation can regulate the processing of HA and thus the virulence of a virus(Deshpande et al., 1987).

In this study glycosylation motifs NGTC, NISK and NGTY were located near antigenic epitopes CYPGNVENL, GTCPKYISL, GTYDRRKYK at positions 108, 312 and 493 in A/ck/Iran/187, A/ck/Iran/191 and A/ck/Iran/TH81/02, respectivly. Furthermore, glycosylation of the NA gene with motif NSSY at position 313-316 was located near DYNINSSYL epitope at position 309 in A/ck/Iran/189 strain. These variations may interfere with antibody recognition and help the virus to escape from immune responses. Computational and experimental studies of currently reported HA and NA segments from Influenza A viruses, will be useful for determining antigenic variation and its influence on virulence of these strains.

Sequence analysis revealed that mutation of the NA hemadsorption site in H9N2 viruses resulted from a positive selective pressure to change suggesting that a loss of

the site increased virus fitness in land-based poultry (Matrosovich et al., 2001) Hemadsorption site serves to enhance the catalytic efficiency of NA and, in addition to changes in the receptor-binding specificity of the hemagglutinin, alterations of the NA are needed for the emergence of pandemic influenza viruses (Uhlendorff et al., 2009).The HB site in the NA of Asian H9N2 viruses is believed to be under positive selection pressure for mutations, which results in compatible combinations of HA and NA (Matrosovich et al., 2001).

It is remarkable that an inactive H9N2 vaccine which has extensively been used to control avian influenza is responsible for the mutation observed in HB site in the NA genes of Iranian H9N2 viruses, resulting from pressure of vaccination program on immune system. In addition, the virus circulation in the region might has also increased these modification. Despite all these changes in viruses of Iranian isolates they still have a relationship with 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 M Fau - Zhou 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 four viruses, like Iranian sequence available in GenBank had no deletions in the stalk (Table 2)

Phylogenetic analysis revealed that Iranian isolates had fallen within distinct clusters for several years. It seems as if these viruses are being faced with two phenomena: accumulation of mutations and reassortment in the G1-like sublineage(Vatandour et al., 2011). Evolution relationship showed that current strains isolated in Iran in 2011 are closely related to strains isolated in Pakistan where has common boundaries with Iran. On the other hand they are also similar to viruses isolated in Egypt in 2011 and in Israel in 2008 as well as Saudi Arabia in 2006 where do not have any common boundaries with Iran.It seems viruses isolated from Iran,Egypt,Israel and Saudi Arabia have originated from common source in spite of being geographical distances.

It was observed based on phylogenetic analysis that the vaccine strain and the 1998's strain from Iran appear in the same subgroup and shared a homology of 99.2 in both NA and HA amino acids. Viruses isolated in 2011 fall into another subgroup and compare with vaccine strain displayed amino acid homology of HA and NA ranging from 90.4% to 91.1% and 90.7% to 91.4%, respectively. Results revealed that these strains had non negligible genetic differences with the vaccine strains. The potential for differences in antigenic variations is high, especially when compared with sublineages of H9N2 viruses(Xu et al., 2007a).It has been demonstrated antigenic diversities in H9N2 viruses using monoclonal antibodies correspond with phylogenetic relationships(Xu et al., 2007b). Their findings also correspond with our results where H9N2 viruses showed sequence variations and antigenic diversities based on evolution. Three and seven differences in antigenic epitopes observed in HA and NA genes of

current isolates and vaccine strain respectivly(Table 5,6) in addition to five glycosilation motifs and one new glycosylation site at current strains(Table 4,5) might trigger antigenic diversities. Therefore, continues evaluation of important gene induced immunity and vaccine efficiency in Iran would be crucial.

Conclusion: Findings from the study support the genetic instability of influenza A (H9N2) viruses.. The genetic disparity between the current prevalent strains and vaccine strain may result to decreased protection after vaccination. Thus in the countries where a control policy is established these divergenisities should be considered. Our study would laid new foundation for genetic evaluation of H9N2 viruses compared with vaccine strain and necessitated yearly surveillance in Iran.

Abbreviations:

HA:Hemagglutinin ,NA:Neuraminidase,PGS:Potential glycosylationsite,HB:Hemadsobing site,AIV:Avian influenza virus,nHPAI:non-highly pathogenic avian influenza,LPAI:Low pathogenic avian influenza,OIE:office international des Epizootics,RT-PCR:reverse transcription polymerase chain reaction,A:influenza A, Ck:chicken,Ir:Iran,Pak:Pakistan,Egy:Egypt,Av:Avian,Ara:Arabia,Dub:Dubai,HK:HongKong,QA:Quail,Kor:Korea,DK:Duck

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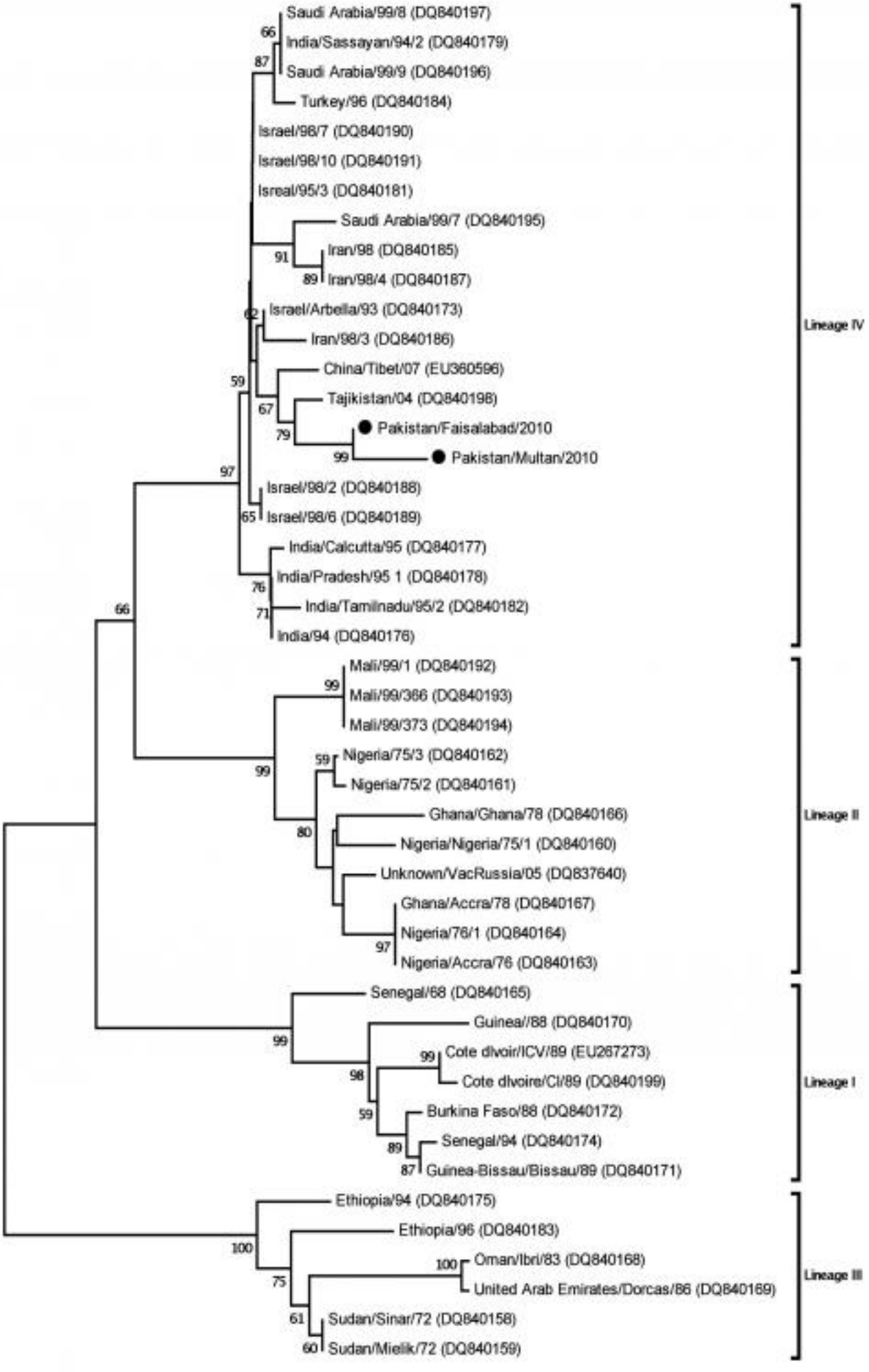
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