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RESEARCH ARTICLE

# Isolation and Sequence Analysis of Reassortant Low Pathogenic Avian Influenza Virus H4N6 from Duck and Chicken in Live Bird Markets from Pakistan

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**ABSTRACT**

The Live bird Markets (LBM) can serve as a paramount source of AIV infections. During routine Avian Influenza surveillance in Pakistan, low-pathogenic avian influenza virus subtype H4N6 was isolated first time from Khaki Campbell duck (*Anas platyrhynchos*) during 2010 and from broiler chicken during 2011 in the live bird markets (LBMs) from the port city of Karachi in Sindh province. Whole genome sequencing revealed introduction of a new reassortant Eurasian avian virus strain. Phylogenetically HA, NA, M, NP and PB2 genes clustered mostly with Russian strains of influenza viruses and PA gene with AI isolates from Netherlands, whereas NS and PB1 genes clustered with a Pakistani isolate of H3N1. Sequence analysis revealed a LP amino acid motif (PEKASR), avian-like receptors, conservation of amino acids at the receptor binding sites and the amino acids known to be associated with sensitivities to antiviral drugs, loss of glycosylation site in NA gene and attainment of unique PDZ domain motif (ESEI) at the C terminal of NS gene. The seroconversion against H4N6 subtype was observed mostly in the bird populations of Sindh and Khyber Pakhtoon Khawa provinces. The isolation divulged the role of LBM where mingling of different bird species provides an excellent environment for dissemination and potential reassortment of AIV. Moreover, various point mutations in these H4N6 isolates and close relationship with Pakistani H3N1 and other Eurasian strains also reflect prevailing diversity among AIVs circulating in the local LBMs.



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## INTRODUCTION

Influenza A viruses originate from non-pathogenic viruses that are circulating in migratory water birds of order Anseriformes and Charadriformes, especially in ducks, and their nesting lake water (Webster *et al.*, 1992; Uchida *et al.*, 2008). Therefore role of duck species is crucial to maintain the genetic pool of these low pathogenic avian influenza viruses (LPAIs) by asymptomatic infections (Munster *et al.*, 2006). Avian influenza virus (AIV) subtype H4 shows a high prevalence in duck populations (Qingqing *et al*., 2013). HA of H4 subtype is distantly related, by genetic and antigenic means, to that of the H5 subtype (Gilbert *et al*., 2006).

Pakistan homes a wide variety of migratory birds flocking up from Siberia and Russia during winters. The largest commune among the visitor-birds is the waterfowl group. The common migratory waterfowls that regularly immigrate to Pakistan in large numbers include Mallard, Pintail, Green-winged Teal, Shoveler, Gadwall, Wigeon and Pochard. The uncommon ducks that visit the region occasionally and usually in small numbers are Shelduck, Ruddy Shelduck, Garganey, Scaup and Tufted (Shirazi, 2005).

The newly evolved AIVs have been continuously generated by reassortment events in duck species with the potential of expanding the host range to mammalians. Continued monitoring of poultry population, in particular quails and ducks, in LBM and backyard poultry is needed to better understand the influenza ecology and interspecies transmission, as a component of pandemic preparedness (Lee *et al.*, 2004). LBMs are considered a major source

of influenza A virus dissemination and its potential reassortment (Webster, 2004; Cardona *et al.*, 2009).

During the routine surveillance of AIVs in domestic bird species, LPAI subtype H4N6 was isolated for the first time from southern part of the country. The LP AI subtype was isolated first from Khaki Campbell duck (*Anas platyrhynchos*) in LBM of port city of Karachi during December, 2010 and second time in 2011 from broiler chickens. Both the isolates were sequenced and their phylogenetic analysis was performed followed by serological investigation to determine its circulation level in the surrounding poultry population.

## MATERIALS AND METHODS

During routine AIV surveillance 46 clinical specimens (cloacal and tracheal swabs) were collected from live bird market in Karachi by surveillance unit. The samples were received at the National Reference Lab for Poultry Diseases (NRLPD) located in Islamabad. The samples were processed and subjected to virological evaluation through in-ovo inoculation according to the standard protocols (Senne, 1998; OIE, 2012). Then the HA and HI test were performed following standard protocol (OIE, 2015), for subtype identification of the samples showing hemagglutination activity. Experimental Infections were performed by carrying out the standard methods of Intravenous pathogenicity testing (Alexander, 1996). Seroconversion against AIV-H4N6 in bird's population of LBMs was determined using the standard HI protocols (OIE, 2015). In this regard a total of 2746 serum samples were collected from different bird species in LBMs of various geographical regions of Pakistan. These geographical areas included the four provinces of Pakistan, Sindh, Balochistan, Khyber Pakhtoon Khawa (KPK) and Punjab along with Islamabad Capital Territory (ICT) and Azad Jammu and Kashmir (AJK) state. Briefly, 508 serum samples from Sindh Province, 470 from Punjab Province, 532 from Khyber Pakhtoon Khawa (KPK) province, 404 from Baluchistan province, 412 from Azad Kashmir and 424 serum samples were collected from Islamabad capital territory.

**Sequencing and Phylogenetic analysis:** Detailed phylogenetic analysis and molecular characterization of 8 gene viral segments of H4N6 virus was performed to find out the evolutionary status of the virus and the possible routes of transmission into domestic ducks and broiler. In this regard viral RNA was extracted using QIAamp viral RNA mini kit according to manufacturer's instructions (QIAGEN). One step RT PCR was performed using Superscript $^{Tm}$ One step RT-PCR with Platinum Taq kit (Invitrogen). The purified PCR products were directly used for cycle sequencing reactions using BigDye$^{R}$ Terminator v3.1 kit, ABI. The products of the sequencing reactions were cleaned using PERFORMA $^{R}$ Ultra 96-Well kit (Edge BioSystems) and sequenced in a 4 capillary ABI PRISM 3130 Genetic Analyzer (ABI). The consensus sequence of the nucleotide sequences were generated using SeqScape software version 2.6 (ABI). Representative sequences of the H4N6 viruses were selected from the Genbank based on sequence identity. Sequences obtained for this study were submitted to Genbank (Table 1). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura *et al*., 2007) Multiple nucleotide and amino acid sequence alignments for all the eight genes were performed using Clustal W (Higgins *et al.*, 1996). Phylogenetic trees were generated by using neighbor-joining analysis in the Mega program (version 4.0).

## RESULTS

The sero-surveillance study was carried out to determine the seroconversion against isolated H4N6 AIVs. The serological data revealed a highest sero-prevalence of 24.80% among the birds population in LBM of Sindh province followed by 16.16% in KPK-province, 8.72% in Punjab province, 4.36% in AJK-province and 6.60% in Islamabad territory. However, no seroconversion was recorded in Balochistan province (Figure 1. The IVPI index value was found to be 0.20.

**Phylogenetic analysis:** The HA gene of Pakistani H4N6 was supposed to be Asian in origin by showing maximum sequence homology of 97.5% with three Russian isolates [A/common pochard /Aktau/1455/2006(H4N6), A/Coot/Aktau/1454/2006(H4 N6) and A/tufft/duck/Aktau /1465 /2006 (H4N6)]. While the NA gene showed maximum sequence identity of 97.4% with A/Duck/Vietnam/OIE-2454/2009 (H4N6). Both the surface proteins showed 84.8-97.3% and 90.0-96.0% sequence homologies with other Asian and European AI strains respectively (Figs 2 and 3, Table 2; Table Sup. 1).

Noticeably, the NS and PB1 genes were possibly Asian in origin by showing highest sequence homology of 99.0-99.6% with Pakistani H3N1 [A/D poultry/Pakistan /NARC /16945 /10]. Within the cluster of Asian and European AI isolates both these genes also showed 90.03-98.8% and 92.09-98.2% sequence homology respectively. Moreover phylogenetic comparison with the other earlier reported Pakistani AIVs showed a range of 90.3-96.9% (Figs 4 and 5, Tables 2; Table Sup. 1).

The Matrix gene mostly clustered with Asian AI strains by showing highest sequence homology of 99.2% with Russian AI strains [A/gargany/Altai/1216/2007 (H3N6) and A/mallard/Altai/1208/2007(H3N6)]. The NP gene formed cluster with the AI isolates of Asian lineage by showing highest sequence homology of 99.0% with A/mute swan/Aktau/1460/2006(H5N1). The PB2 gene phylogenetically clustered with Russian AI isolates. Noticeably within the group of five Russian H3 AI strains, the highest sequence homology range of 98.6 and 98.5% was observed with A/mallard/Altai/ 1208/2007(H3N6) and A/gargany/Altai/1216/2007 (H3N6). The PA gene was found to be in close nucleotide sequence relation of 98.2% with A/Mallard/ Netharlands/17/2007(H11N8). Moreover these four genes (M, NP, PB2 and PA) also showed sequence similarities of 87.0-99.0% with Asian AI isolates and 89.9-96.8% with European strains. However, sequence identities with the previously reported Pakistani AI strains (H5N1, H7N3 and H9N2) were 85.0-95.2% (Figs Sup1, Sup2, Sup3 and Sup4, Tables 2 Sup. 1).

**Table 1:** Pakistani LPAI H4N6 isolates with Gene Accession Numbers

| Sr. No | Genes | A/Khak I Campbell Duck/ Pakistan / NARC 23963/ 10(H4N6) | A/Chicken/Pakistán/ NARC-28842 / 2011 (H4N6) |
|---|---|---|---|
| 1 | HA | JN714468 | KF113545 |
| 2 | NA | JN714469 | KF113546 |
| 3 | M | JN714471 | KF113547 |
| 4 | NS | JN714470 | KF113548 |
| 5 | NP | JN714472 | KF113549 |
| 6 | PA | JN714473 | KF113550 |
| 7 | PB1 | JN714474 | KF113551 |
| 8 | PB2 | JN714475 | KF113552 |

**Sequence analysis:** The amino acids at the HA cleavage site of Pakistani H4N6 were PEKASR. The sequence analysis (H4 numbering used) revealed conservation of amino acids at receptor binding sites (110Y, 165W, 167V, 196H, 203E, 207L and 208Y), at the left edge of receptor binding pocket (237R, 238G, 239Q, 240S, 214G, 242R) and at the right edge of receptor biding pocket (146G, 147K, 148S, 149G, and 150A). The Pakistani H4N6 strain was characterized by full length NA gene. The amino acids residues His275 in NA gene, and Leu26, Val27, Ala30, Ser31 and Gly34 in Matrix gene were conserved. The Pakistani H4N6 virus strain also retained Glu627 in PB2 gene and Alanine149 in NS genes. Surprisingly, at the C-terminal of NS1 gene of Pakistani H4N6 virus isolated from duck, Valine was mutated to Isoleucine at 230 amino acid position (Tables 3 and 4).

The HA gene of Pakistani H4 isolates possessed five Asparagine linked glycosylation sites (four in HA1 protein and one in HA2 protein) at 18, 34,178, 310 and 497 amino acid positions. On the other hand, NA gene of Pakistani H4N6 isolates retained 9 N-linked glycosylation sites at amino acid positions 28, 32, 51, 54, 67, 70, 86, 146 and 201 while one glycosylation site at amino acid position 62 was missing due to the mutation of Asparagine to Serine.

## DISCUSSION

The National Reference Lab for poultry disease (NRLPD) in Pakistan confirmed the first isolation of LPAI H4N6 from Khaki Campbell Duck (*Anas platyrhynchos*) during 2010 and second isolation was confirmed from chicken during 2011 in LBM of Sindh province. One of the probable reasons for high percentage of sero-conversion against AIV H4N6 in Sindh (24.80%) and KPK provinces (16.16%) is the coastal belt in Sindh Province while the attractive birding sites along with water bodies (Lakes, Rivers) in the hilly areas of KPK province which provide opportunities for frequent movements of migratory waterfowls, wild migratory birds and their intermingling with the local bird species.

**Table 2:** Highest nucleotide sequence homology of whole genome of Pakistani H4N6 isolates compared to nucleotide sequences available in Genbank database

| Genes | Viruses with the highest percentage of nucleotide identity | Accession Numbers | Percentage nucleotide identity |
|---|---|---|---|
| PB2 | A/mallard/Altai/1208/2007(H3N6) | CY049783 | 98.6% |
| | A/gargany/Altai /1216 /2007(H3N6) | CY049767 | |
| PB1 | A/Chicken/Pakistan/NARC-16945/2010(H3N1) | HQ165994 | 99.0% |
| PA | A/Mallard/Netharlands/17/2007(H11N8) | CY043885 | 98.2% |
| HA | A/Common Pochard /Aktau /1455/2006(H4N6) | FJ434370 | 97.5% |
| | A/Coot/Aktau/1454/2006(H4N6) | FJ434369 | |
| NP | A/Mute Swan/Aktau/1460/2006(H5N1) | FJ434377 | 99.0% |
| NA | A/Duck/Vietnam/OIE-2454/2009(H4N6) | AB545604 | 97.4% |
| M | A/gargany/Altai/1216/2007(H3N6) A/mallard/Altai/1208/2007(H3N6) | CY049783 CY049767 | 99.2% |
| NS | A/Chicken/Pakistan/NARC-16945/2010(H3N1) | HQ166000 | 99.6% |

**Table 3:** Genetic analysis of the amino acids at the HA cleavage site and receptor binding site of the HA gene of Pakistani H4N6 and other closely related AIV strains

| Virus Nomenclature | HA Cleavage site motif | Amino Acid Residues at HA Receptor binding site (H4 Amino acid Position) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 338-343 | 110 | 165 | 167 | 196 | 203 | 207 | 208 |
| A/Khaki Campbell Duck/Pak/NARC- 23963/ 10(H4N6) | PEKASR | Y | W | V | H | E | L | Y |
| A/Chicken/Pakistan/NARC-28842/2011(H4N6) | PEKASR | Y | W | V | H | E | L | Y |
| A/Coot/Aktau/1454/2006 (H4N6) | PEKASR | Y | W | V | H | E | L | Y |
| A/Avian/Japan/8K10185/2008(H4N6) | PEKASR | Y | W | V | H | E | L | Y |
| A/Duck/Vietnam/OIE-2454/2009(H4N6) | PEKASR | Y | W | V | H | E | L | Y |
| A/Mallards/Netherlands/4/2006(H4N6) | PEKASR | Y | W | V | H | E | L | Y |
| A/Mallard/Sweden/62/2003(H4N6) | PEKASR | Y | W | V | H | E | L | Y |
| A/Muscovyduck/Thailand/CULM1973/09(H4N6) | PEKASR | Y | W | V | H | E | L | Y |

**Table 4:** Known molecular signatures found in various gene products of Pakistani H4N6 and reference AIV strains

| Virus Nomenclature | NA | | M2 | | | | | NS1 | | PB2 |
|---|---|---|---|---|---|---|---|---|---|---|
| | AA 275 | 11 AA deletion 53-63 | AA 26 | AA 27 | AA 30 | AA 31 | AA 34 | AA 149 | PDZ Binding Domain | AA 627 |
| A/Khaki Campbell Duck/Pak/NARC-23963/10(H4N6) | H | NO | L | V | A | S | G | A | ESEI | E |
| A/Chicken/Pak/NARC-28842 /11(H4N6) | H | NO | L | V | A | S | G | A | ESEV | E |
| A/Coot/Aktau/1454/2006 (H4N6) | H | NO | L | V | A | S | G | A | ESEV | E |
| A/Avían/Japan/8K10185/2008(H4N6) | H | NO | L | V | A | S | G | A | ESEV | E |
| A/Duck/Vietnam/OIE-2454/2009(H4N6) | H | NO | L | V | A | S | G | A | ESEV | E |
| A/Mallards/Netherlands/4/2006(H4N6) | H | NO | L | V | A | S | G | A | ESEV | E |

(Amino acids associated with antiviral drug sensitivity, cellular signaling pathways, interferon resistance and virulence).

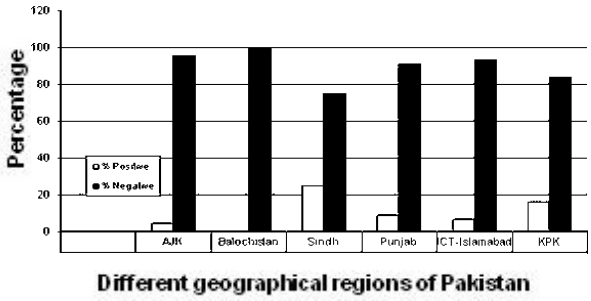


**Fig. 1:** Seroconversion observed against AIV-H4N6 in birds population of live bird markets in various geographical regions of Pakistan.

Phylogenetic analyses revealed the introduction of new avian reassortant Eurasian strain into Pakistan. The data indicated that HA, NA, Matrix, NP, and PB2 genes were of Asian origin and possibly derived from Russian AI strains while PA gene was European in origin and possibly derived from non-H4 AI isolates from Netherlands. This fact revealed the possible role of migratory wild birds for introduction of this new reassortant H4 strain from Europe and Russia (Siberia) into Pakistan through international migratory route 4 (Indus flyway). Analogous to this study, different subtypes of AIVs including H4N6 strains isolated from three species of dabbling ducks (Mallard, Wigeon and Garagny) in Ukraine were phylogenetically related to the AIV strains from Siberia and Europe (Kulak *et al*., 2010). Conspicuously the PB1 and NS genes were also Asian in origin and possible progenitor might be the Pakistani H3N1 strain (Siddique *et al*., 2012). The H3N1 strain (A/Dpoultry/Pakistan/NARC/16945/10) strain was isolated from northern part of the country during March, 2010 while, Pakistani H4N6 strains (A/Khaki Campbell duck/Pakistan-Khi/NARC-23963/10) and A/ck/Pakistan/ NARC-28842/ 2011 were isolated from southern part of the country during December, 2010 and December, 2011 respectively. These observations suggested that these LP strain are consistently circulating in the wild and domestic bird population of Pakistan and sporadically detected. The close sequence homology of genes between these two H4 isolates also revealed that the mingling of bird species in LBM provides an excellent environment for transmission of AIVs among different bird species. In LBMs large number of birds from various sources is kept in wire stacked cages which contains dumbly packed and mingled bird species. Likewise, isolation of H4N6 and H4N9 AIV strains have also earlier been reported from live birds markets in Thailand (Wisedchanwet *et al*., 2011).

The presence of multiple amino acids such as Lysine (K) and Arginine (R) at the HA cleavage site may turn LPAI into HPAI (Horimoto *et al*., 1995). The amino acids at the HA cleavage site of Pakistani H4N6 were PEKASR indicated the low pathogenic characteristics further confirmed by IVPI (0.1-0.2). The receptor binding sites Gln239 and Gly241 of Pakistani H4N6 were similar to all H4N6 viruses found in Eurasian, Australian and African lineages. Thus it was evident, that Pakistani H4N6 strain would preferably recognize a-2, 3-gal linkages receptors prevalent in avian species.

The Pakistani H4N6 strain was characterized by full length NA gene, as observed in most of the closely related Eurasian AI strains. Pakistani H4N6 viruses revealed sensitivity to antiviral viral drugs Oseltamivir, Zanamivir and Amantadine by possessing His275 in NA gene, and Leu26, Val27, Ala30, Ser31 and Gly34 in Matrix gene similar to previous study (Wan *et al*., 2008). The Pakistani H4N6 viruses also retained Glu627 in PB2 gene and Alanine149 in NS genes which are known to be associated with host, range and anti-viral cytokines responses, respectively (Subbarao *et al.*, 1993). The NS1 C-terminal region might be involved in binding to PDZ domains on proteins involved in host cellular signaling pathways. Majority of AIV strains possessed an ESEV PDZ domain motif. Interestingly, the C-terminal of NS1 gene of Pakistani H4N6 isolated from duck was characterized by unique ESEI PDZ domain motif. These observations indicated that the newly isolated viruses are undergoing genetic drift by establishing and maintaining their genetic pool in various domestic fowl species.

Glycosylation plays crucial role in the generation of new influenza viruses and significantly affect the receptor-binding properties of the influenza virus HA protein. The HA gene of Pakistani H4 isolates possessed five N- linked glycosylation sites. On the other hand, NA gene of Pakistani H4N6 isolates retained 9 N-linked glycosylation sites while one glycosylation site at amino acid position 62 was missing due to the mutation of Asparagine to Serine. However, NA gene of closely related AIVs used for phylogenetic comparison possessed 10 glycosylation sites.

In Pakistan the largest commune among the visitor-birds is the waterfowl group, commonly known as ducks. Therefore, the AIVs surveillance and isolation in various duck species in wet lands and LBMs of Pakistan is of significance to understand the dissemination pattern of these LP AIVs in duck species.

The AIV H4 subtype circulating in LBMs can be a plausible risk to birds and human health. The shedding of viruses in feces and contamination of wire stack cages in LBM offers felicitous environment for virus dissemination among bird species kept in the cage or in the nearby cages. Consequently, an outbreak of influenza A viruses of these LP AIVs including H4 subtypes in the birds in LBM can occur and spread. In this regard isolation of H4N6 from duck and subsequently from chicken in LBM from Pakistan is noteworthy and represents the risk of infection in local birds from these reassortant AIVs. Furthermore, human infections by such avian influenza subtypes upon experimental inoculation have also been reported (Beare and Webster, 1991) in this context, therefore, the circulation of these newly reassorted influenza viruses in markets would pose a continuous threat to vendors, consumers and people who visit the live bird markets (LBMs). During winters in Pakistan live bird markets are saturated with different types of captured birds. To meet the nourishment needs and to satisfy the hobbies, a large number of bird lovers as well as devourers visit live bird markets and buy the birds of their choice, without knowing their health status. Some bird-lovers are also found busy in window-shopping in these markets. Therefore, AIVs surveillance in wet lands and LBMs of Pakistan is cardinal to understand the prevalence and dissemination pattern of these and other LP AIVs in bird species which may lead to developing some effective strategies for the control and prevention for influenza A outbreaks.

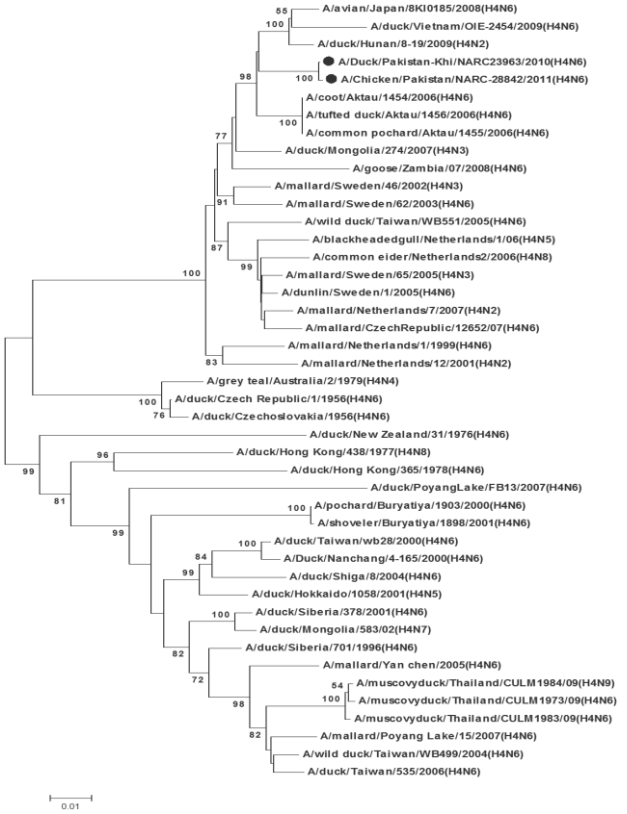


**Fig. 2:** Phylogenetic relationship of HA gene of Pakistani H4N6 viruses with other representative AIVs. The phylogenetic tree was generated using the neighbor-joining algorithm as implemented in MEGA version 4. Bootstrap analysis with 1000 replicates was performed for confirming tree topology. Scale bar indicates 0.01 nucleotide substitutions per site. The Pakistani isolates are highlighted in dark squares.

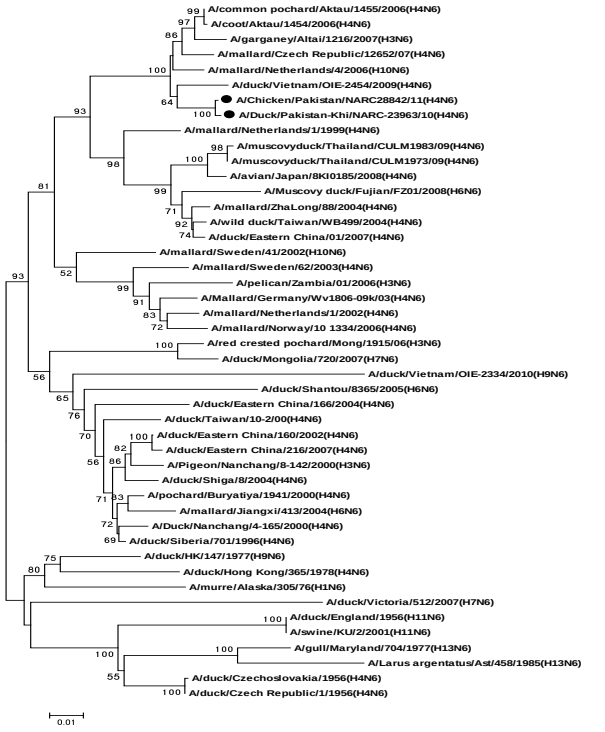


**Fig. 3:** Phylogenetic relationship of NA gene of Pakistani H4N6 viruses with other representative AIVs. The phylogenetic tree was generated using the neighbor-joining algorithm as implemented in MEGA version 4. Bootstrap analysis with 1000 replicates was performed for confirming tree topology. Scale bar indicates 0.01 nucleotide substitutions per site. The Pakistani isolates are highlighted in dark squares.

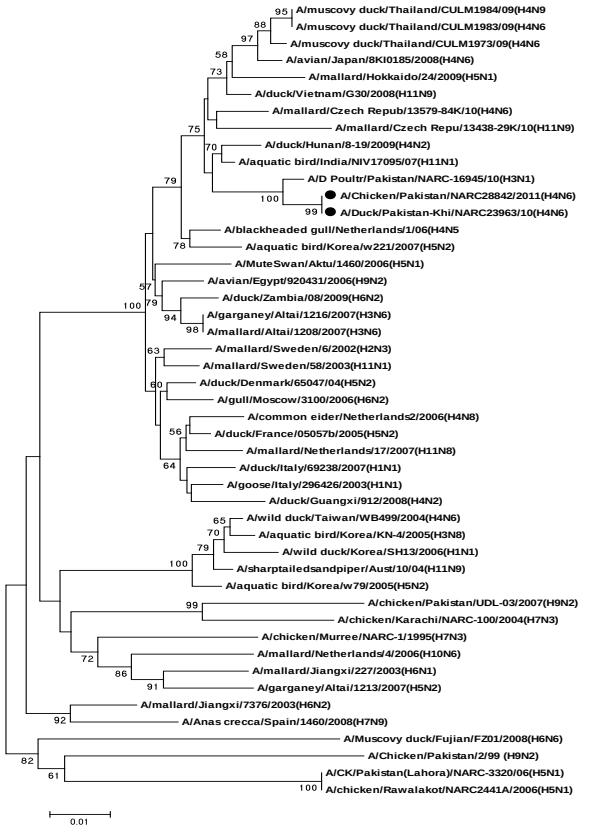


**Fig. 4:** Phylogenetic relationship of NS gene of Pakistani H4N6 viruses with other representative AIVs. The phylogenetic tree was generated using the neighbor-joining algorithm as implemented in MEGA version 4. Bootstrap analysis with 1000 replicates was performed for confirming tree topology. Scale bar indicates 0.005 nucleotide substitutions per site. The Pakistani isolates are highlighted in dark squares.

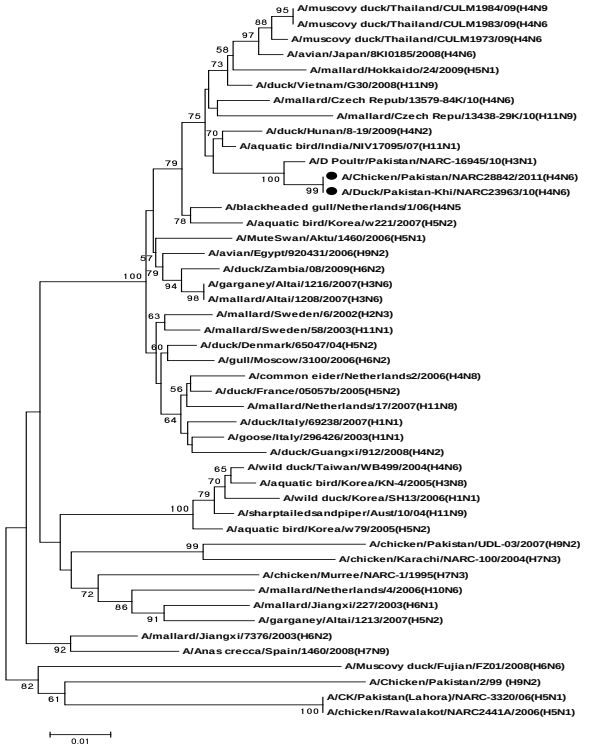


**Fig. 5:** Phylogenetic relationship of PB1 gene of Pakistani H4N6 viruses with other representative AIVs. The phylogenetic tree was generated using the neighbor-joining algorithm as implemented in MEGA version 4. Bootstrap analysis with 1000 replicates was performed for confirming tree topology. Scale bar indicates 0.01 nucleotide substitutions per site. The Pakistani isolates are highlighted in dark squares.

**Conclusions:** The isolation of AIV H4N6 from duck and chicken in live bird market of Pakistan and its close relation with previously isolated H3N1 from Pakistan as well as with other Eurasian strains from Russia and Europe reflect the status and diversity of the AI viruses circulating in local bird's population. LBM played an important role in extending genetic diversity of Influenza viruses in Pakistan. The newly evolved AIVs have been continuously generated by reassortment events in ducks in LBMs with the potential of expanding the host range to broiler chicks.

**Author's contribution:** NS, KN and ZA intended and conceived the study. NS and KN wrote the manuscript. NS and SR edited the manuscript and finalized the data. NS, SR and FR performed the molecular part. NS and IB performed Serology part. MAA and AA provided consultation and reagents. RF helped in sampling. All authors read and approved the final manuscript.

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