Research Article

Characterization and Phylogenetic Analysis of Avian Influenza Virus Subtype H9N2 in Pakistan

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Abstract | H9N2 avian influenza viruses are currently endemic in Pakistan and outbreaks are continuously being reported with high morbidity, drop in egg production and reduced weight gain. Besides economic losses to the poultry industry, these viruses pose a significant threat to public health. Owing to significant involvements of neuraminidase (NA) gene in virus pathobiology by mediating virus entry and release, the present study was conducted to determine the phylogenetic relationship of currently prevailing H9N2 isolates in Pakistan. A sum of ten H9N2 AIVs were isolated from 400 suspected samples and were confirmed through serological and molecular assays. The sequence analysis of NA genes shown 99% homology with the H9N2 AIV recently isolated from Pakistan and their phylogenetic analysis revealed that all the isolates belonged to the G- lineage. Amino acid analysis of NA stalk regions of these isolates illustrated no insertion, deletion and substitutions compared to A/DK/HK/Y280/97 lineage and human isolates (A/HK/1073/99 and A/HK/1074/99). Several amino acid substitutions at various regions of the NA protein were determined with unknown functions and consequences. These findings warrant future studies for analysis of whole genome sequencing of prevailing H9N2 viruses in Pakistan which may further contribute towards the better understanding of the genetic nature and evolutionary behavior of these viruses in the country.

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Introduction

Avian influenza is a viral infection of birds which leads to economic losses to the poultry industry throughout world. The distribution of the infections in commercial poultry and migratory wild shore birds has been widely reported, and aquatic birds particularly ducks and waterfowls serve as a reservoir for the virus (Stallknecht and Shane, 1988). The avian influenza viruses (AIV) can transmit to highly susceptible species such as turkeys and chickens from these natural hosts (Davison et al., 1999).

AIVs are enveloped viruses that belong to the family *Orthomyxoviridae* and have segmented, negative sense, single stranded ribonucleic acid which encodes at least 11 different viral proteins. AIVs are highly infectious pathogens of respiratory system in their particular natural hosts. Based on their matrix (M) and internal nucleoprotein (NP) antigens, the influenza

viruses have been classified into influenza A, B and C viruses. The M and NP proteins are considered common to all the strains of same type. On the basis of their surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins, the AIV are further divided into subtypes and clades (Vatandour et al., 2011).

Currently, eighteen and ten antigenically distinctive subtypes of HA and NA glycoproteins of influenza A viruses have been recognized (Tong et al., 2013). Based on their virulence and pathogenicity, the AIV produce two different disease forms in poultry birds i.e. the low pathogenic avian influenza (LPAI) and the highly pathogenic avian influenza (HPAI) (Capua and Alexander, 2004). Primarily the viruses of H5 and H7 subtypes cause the highly pathogenic avian influenza while the other low pathogenic sub-types of virus also result in significant economic losses to poultry industry. The H9N2 subtype avian influenza viruses of low pathogenic form are especially notable due to their extensive spread in domestic poultry stretching from the Far East to the Middle East (Iqbal et al., 2013). Besides causing significant economic losses to the commercial poultry industry, the H9N2 AIV also pose severe risks to the public health (Munir et al., 2013).

These H9N2 AIV have been reported occasionally among human beings. In 1999, the H9N2 AIV caused infection in Chinese children, however, the viruses were unable to establish widespread infections in humans (Butt et al., 2011). Cases of human beings infected with H9N2 viruses have also been recorded in Hong Kong, although any death was not reported yet (Riedel, 2006). As the H9N2 AIV carry the potential to cause infection in humans, it is important to monitor the genetic variation and the ability to switch over the hosts. The H9N2 AIV has achieved a great importance because its infection has touched the panzootic proportions during the recent years (Cameron et al., 2000).

Neuraminidase (NA) is an important glycoprotein that is present on the surface of the H9N2 avian influenza virus which owns the enzymatic activity necessary for viral infection and elution. The neuraminidase protein was documented by Alfred Gottschalk and his colleagues and they recognized NA as an exosialidase or neuraminidase which causes the cleavage of α-ketosidic linkages amongst the sialic acid and the neighboring sugar residues (Stiver, 2004). Polypeptide chain of influenza virus neuraminidase is comprised of 470 amino acids and is coded by the 6th RNA segment. The crystalline structure of neuraminidase displays that it is tetramer comprising of four similar subunits composed of six 4-stranded antiparallel beta (β) sheets. It is composed of box like square head attached to a long slender stalk and has a knob at its end. The neuraminidase is inserted in lipid bilayer of the viral membrane (Laver and Valentine, 1969).

The influenza virus neuraminidase plays vital functions which help entry and release of the viruses from the infection site. It is responsible in initiating the cleavage of a (2-3) – (2-6) - ketosidic linkage that is present between adjacent sugar residue and a terminal sialic acid. In the respiratory tract, the breakage of this bond has several imperative functions which help in the spread of the progeny virions. The cleavage of sialic acid by NA in the mucous of the respiratory tract prevents inactivation of the virus and stimulates the penetration of the virus into the respiratory tract epithelial cells (Colman, 1994). During the previous avian influenza outbreaks the prime emphasis was given on diagnosis and control of the infection by strategic vaccination and adopting the strict biosecurity measures. The information about the biological and genetic characteristics of H9N2 AIV remained largely unexplored. Owing to the remarkable role of the NA gene in pathogenicity, entry and elution of the virus, it is important to study the characteristics of the NA gene of avian influenza virus subtype H9N2 isolated from poultry in Pakistan.

Materials and Methods

Sampling and virus isolation

A sum of 200 samples were collected from live as well as diseased birds from broiler flocks located at different cities in Pakistan. Initial screening and virus isolation was performed in 10 days old embryonated chicken eggs (ECEs) at Influenza Laboratory, Department of Microbiology, University of Veterinary and Animal Sciences Lahore Pakistan using the standard protocol described by (OIE, 2005). Briefly the samples of trachea and lungs were chopped and treated with antifungal and anti-mycotic solutions and incubated at 37^{0}C for 30 minutes. After incubation the samples were centrifuged at 4000xg for clarification and supernatant was collected. The collected supernatant was inoculated in embryonated eggs via the allantoic sac route and incubated at 37^{o}C. The

allantoic fluid was harvested 48-72 hours post-inoculation and centrifuged at 4000xg at 4°C for clarification. Haemagglutination assay was performed for the screening of AIV positive samples. Subtype identification of AIV was performed using the specific antisera (GD Diagnostics Netherlands) against H9N2 according to previously described protocol (Gough et al., 1977). The molecular confirmation was performed by RT-PCR and specific primer against H9 and H2 genes (Sarwar et al., 2013). A total of 10 samples were confirmed through PCR and out of these isolates three samples were selected for further analysis. These samples were named as: A/Chicken/Pakistan/UVAS-426/2015(H9N2), A/Chicken/Pakistan/UVAS-511/2015(H9N2) and A/Chicken/Pakistan/UVAS-953/2016(H9N2).

Viral RNA extraction and RT-PCR

Viral RNA of the selected positive samples was extracted using FavorPrep Viral Nucleic Acid Extraction Kit (Favrogen, Cat # FAVNK001-2) according to protocol provided by the manufacturer. Both quality and quantity of extracted viral RNA was analyzed by nanodrop 2000 (Thermo scientific company, USA). The cDNA synthesis was performed by Thermo Scientific RevertAid First Strand cDNA kit (Cat: K1822). Near full length amplification of N2 gene was performed using self-designed primers listed below: (N2-945bp-F): 5'-TAGCACTTGGCTCTGCTTCT-3' (N2-945bp-R): 5'-CAACAAGTCCTGAGCACACA-3' (N2-460bp-F): 5'-CTCCAATAGACCCGTACTAT-3' and (N2-460bp-R): 5'-CCTGAAGTCCCACAAAATAC-3'. RT-PCR was performed in thermal cycler using 25µl reaction mixture containing 2.5 µl $MgCl_2$ (50mM), 2.5 µl dNTPs (2.5mM), 2 µl *Taq* Buffer (10X), 1 µl of each forward and reverse primer (10 pmol), 9 µl of nuclease free water and 5 µl of cDNA. The amplification of reaction mixture was carried out according to following program; initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55.4°C (945 bp) and 53.7°C (460 bp) for 30 seconds and extension at 72°C for 60 seconds up to 35 cycles, followed by a final extension at 72°C for 10 min. The N2 gene amplification was confirmed by running the PCR product on 1.2% agarose gel. Electrophoresis was performed at 110 Volts for 30 minutes. The amplified PCR product was further purified using Gene Jet Gel Extraction Kit (cat K0691) according to instructions provided by the manufacturer.

Gene sequencing and bioinformatics analysis

The purified amplicons were sequenced by Dye-terminator Sanger's sequencing method. The sequences were retrieved using Bioedit program and peaks of sequences were carefully observed. Sequences were edited using Genious R7 7.0.2 and analyzed using BLAST (Basic Local Alignment Search Tool). Nucleotide alignment and translated amino acid sequence was obtained using the Bioedit program. With the help of MEGA 6.0 software package phylogenetic relationship of these viruses was determined with other H9N2 AIV lineages of Iran, UAE, Turkey, India, China and Korea.

Genbank accession numbers

The nucleotides sequences obtained in this study are available in NCBI Genbank under the following accession numbers: KY028776, KY02877 and KY028778.

Results and Discussion

In this study, 1300 base pairs of the NA genes (partial cds) were sequenced and amino acid sequences of the NA gene of these three isolates were deduced from the nucleotide sequence. The amino acid sequence of the isolates showed high identity (more than 99%) to each other. Amino acids of haemadsorbing site (HB site) in the N2 stalk include, 366-373, 399-404 and third 431-433 motifs. The HB site at position 366-373 was predicted to be IEKDSRTG for all three isolates. Rest of isolates contained single type DSGNRS at position 399-404 and PQE at position 431-433 (Table 1). The NA stalk regions in these viruses had no deletion or insertion or shortening as compared to G1 sublineage. Analysis of NA protein sequences showed that these H9N2 viruses have eight potential glycosylation sites with the N-X-T/S motif in which X may be any amino acid except proline. Eight conserved potential glycosylation sites were located at 44NSSE48, 61NITE65, 69NGTI73, 46NGTT150, 202NATA206, 411NNST415, 411NNST415 and 412NSTX416 and 437NGST441.

The phylogenetic analysis of the N2 gene of these isolates indicates that H9N2 influenza viruses prevalent in chicken population in Pakistan 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 Pakistani viruses.

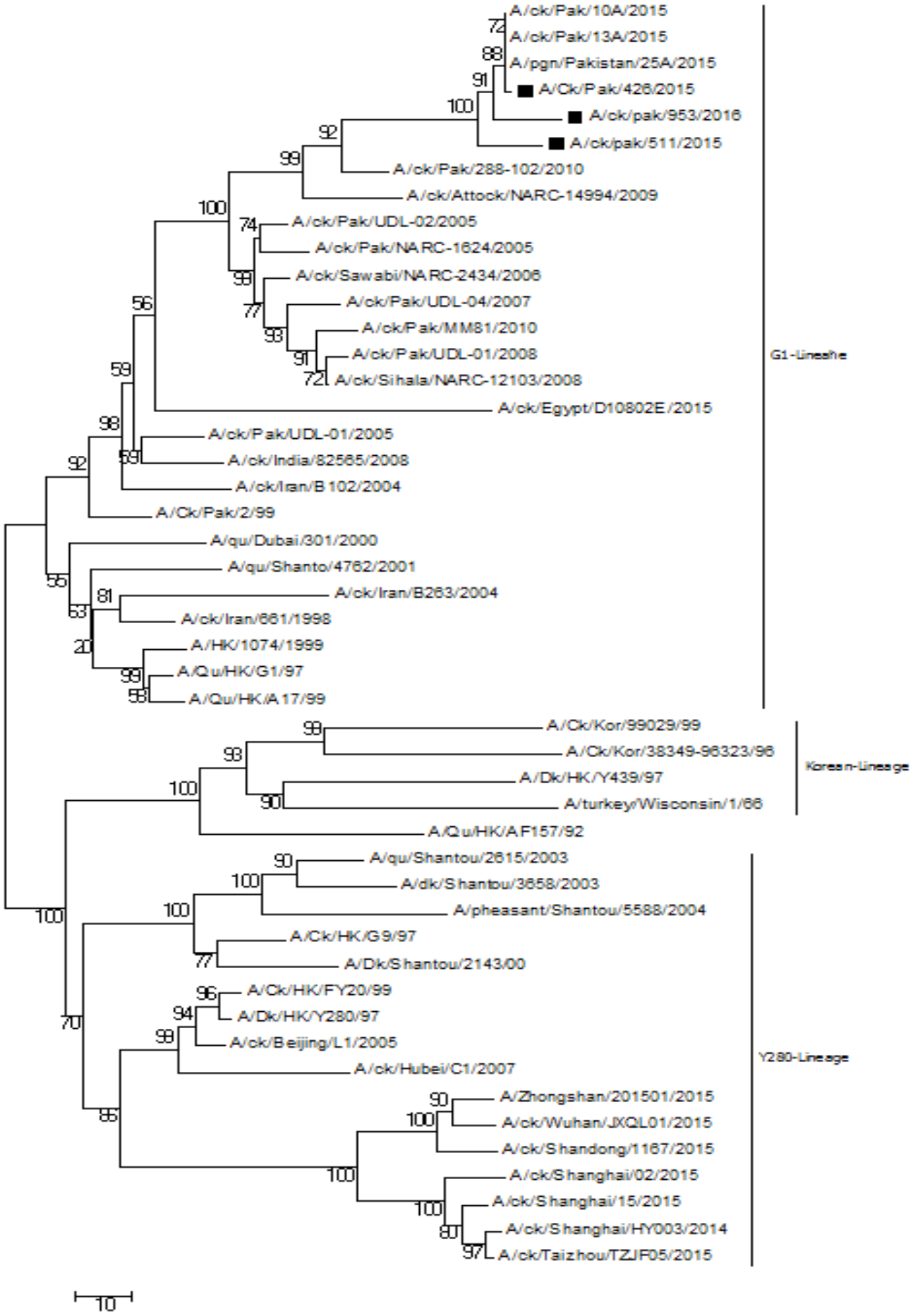

Figure 1: *Phylogenetic relationships of Neuraminidase genes from H9N2 viruses isolated in Pakistan during 2015 and 2016 with other representative AIV of H9N2 subtype.*

Table 1: *The amino acid sequence of the isolates*

Virus Subtype	Deletion in stalk	HB-Site_		
		366-373	399-404	431-433
A/CK/PK/426/2015	------	IEKDSRTG	DSGNRS	PQE
A/CK/PK/511/2015	------	IEKDSRTG	DSGNRS	PQE
A/CK/PK/953/2015	------	IEKDSRTG	DSGNRS	PQE
QA/HK/G1/97 H9N2	38-39	IKKDSRSG	DSDIRS	PQE
A/CK/PK/13A/2015	------	IEKDSRTG	DSGNRS	PQE
HK/1074/99 H9N2	38-39	IKKDSRSG	DSDNWS	PQE
A/CK/PK/10A/2015	------	IEKDSRTG	DSGNRS	PQE
DK/HK/Y280/97 H9N2	63-65	IKEDSRSG	DSDNWS	PQE
CK/IR/L252/03 H9N2	------	IKQDSRAG	DSDNLS	PQE
A/CK/PK/MM81/2010	------	IKKDSRAG	DSDNRS	PQE
A/CK/PK/UDL/2008	------	IKKDSRAG	DSDNRS	PQE
A/CK/PK/UDL-02/2005	------	IKKDSRAG	DSDNRS	PQE
A/CK/PK/UDL-04/2007	------	IKKDSRTG	DSDNRS	PQE
A/CK/PK/288-102/2010	------	IEKDSRAG	DSDNRS	PQE
A/CK/PK/NARC-1617/2005	------	IKKDSRAG	DSDNWS	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	PQCK
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

The H9N2 AIVs have been classified into three major lineages i.e. Eurasian lineage, human and swine and the North American lineage. The Eurasian lineage has been further subdivided to three different sub lineages represented by prototypes A/Quail/ Hong Kong/ G1/97 (G1), A/Duck/Hong Kong/Y280/97 (Y280) and A/ Chicken/Korea/38349-p96323/96 (Korean) (Liu et al., 2003). Avian influenza H9N2 is prevalent in poultry industry in Pakistan since last 2 decades. Although it is low pathogenic yet it is the leading cause of the respiratory infections resulting in great economic losses in terms of reduced egg production, weight loss and high morbidity (Aamir et al., 2007). The H9N2 avian influenza virus A/Ck/Beijing/1/94 is lethal for the chickens both in the field as well as under experimental conditions (Guo et al., 2000). Because of the significant roles of NA gene in pathogenicity and release of the virus, the present study was conducted to characterize and to determine the phylogenetic analysis of N2 genes of recently isolated viruses from poultry in Pakistan with the previously isolated H9N2 viruses.

In this study the neuraminidase gene of three isolates was amplified and sequenced and a consensus sequence for each isolate was made and there was slight polymorphism in the analyzed sequences. The phylogenetic analysis of N2 gene revealed that all the isolates had 98-99% nucleotide similarity among the recently isolated H9N2 AIV in 2015 and had 93-95% homology with the H9N2 viruses isolated in the previous years. The isolates of this study belonged to the same G1 sub-lineage represented by A/Quail/Hong Kong/G1/97 prototype of the Eurasian lineage of H9N2 AIV as of the previously isolated viruses and clustered in the clade of newly isolate H9N2 AIV from Pakistan. The three motifs of the functional activity of N2 are the stalk length, the sialic acid binding

or HB site and the potential glycosylation sites. The amino acids for HB site are at three different positions i.e. 366-373(IKKDSRAG), 399-404 (DSDNRS) and at position 431-433(PQE). The N2 stalk length had no deletion of amino acids at position 38-39 as compared to A/Qu/HK/G1/97 and HK/1073. The shortening of the N2 stalk length by the deletion of the amino acids is characteristic feature of the highly pathogenic H5 and H7 avian influenza viruses (Matrosovich et al., 1999). However it is not known whether deletion in the N2 of H9N2 virus is correlated with pathogenicity in chickens. The amino acid sequence of the isolate in this study has been changed from the previously isolated viruses by Munir et al. (2013) and Iqbal et al. (2009). The sialic binding site or the hemadsorbing (HB) was changed to IEKDSRTG with two substitutions K367R and R371T as compared to the previous IKKDSRAG reported by Munir et al. (2013) and Iqbal et al. (2009).

The amino acid sequence at position 399-404 of HB site was changed to DSGNRS from DSDNRS as compared to Munir et al. (2013) and Iqbal et al. (2009) with one amino acid substitution. The amino acids in the HB site (431-433) were the PQE and these were same as of the (Munir et al., 2013) and (Iqbal et al., 2009). There was no unique five amino acid deletion in the N2 stalk region of these viruses as compared to A/Ck/Pak/UDL-02/05 reported by Iqbal et al. (2009) in which five amino acids at position 46-50 were absent and this five amino acid deletion was typical of human H3N2 and H2N2 viruses. The substation at position 372 was Serine (S) to Threonine (T) as compared to Iqbal et al. (2009) which was serine (S) to alanine (A). In addition substitution at position 403 from Tryptophan (W) to Arginine (R) was found in all the three isolated and was similar to (Munir et al., 2013; Iqbal et al., 2009) and this substitution was found in Qu/HK/G1/97 but not in other H9N2 viruses and the human H9N2 isolates. Similarly amino acids at positions 400S, 402N, 403R, 432Q are conserved in G1-like lineage viruses and other H9N2 viruses isolated from ducks and the migratory birds.

It has been perceived that the glycosylation sites in N2 contribute a major role in increased virulence of the influenza viruses, either because of the change in sialic acid activity due to the alteration in antigenicity. Glycosylation sites comparison between the G1-like H9N2 viruses and three isolated viruses of this study proved the presence of eight potential glycosylation sites at 44NSSE48, 61NITE65, 69NGTI73,146NGTT150, 202NATA206, 411NNST415, 411NNST415 and 412NSTX416 and 437NGST441. Among them, the 44NSSK48 which was present in A/Chicken/Pakistan/CP/2010 (Munir et al., 2013) also reported in A/Chicken/Hong Kong/G9/97 and A/Chicken/India/2048/03 whose significance is not known, was changed to 44NSSE48. However, addition or loss of potential glycosylation may contribute to increased virulence due to altered antigenicity or sialidase activity. The 86NWSK90 which was present in A/Chicken/Pakistan/CP/2010 was absent in these isolates and the have been changed to 86NWLK90.
One of the major driving factors in the evolution of low pathogenic influenza viruses is the immunological pressure which has been increased due to continuous vaccination. The post-vaccination specific immunity of the population could be a discriminating factor which ensures the prevalence of a viral strain which differed from the vaccine strain and the previous isolates of Pakistan. There are great number of reports which revealed the group viruses of the Eurasian sub-lineage give cross protection (Xu et al., 2007). Also, the Qa/HK/G1/97-like viruses have the potential risk of the public health concern because the Qa/HK/ Gl/97-like viruses have the internal genes similar to those of the H5Nl (1997) human and avian isolates, and human H9N2 viruses isolated in Hong Kong (1999) also belong to Qa/HK/G 1/97 lineage (Lin et al., 2000; Cameron et al., 2000). Therefore it is very important to continuously characterize and to study the molecular epidemiology and phylogenetic relationships of the H9N2 AIV prevalent in the country.

Authors Contribution

All the authors contributed equally for the completion of this manuscript.

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