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Phylogenetic and Mutational Analysis of Neuraminidase of Avian Influenza A (H9N2) Virus of B2- Sub Lineage, Isolated from Commercial Poultry Pakistan

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RS designed and conducted the experimental work. FA helped in virus isolation and manuscript preparation. SI supervised the work.

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ABSTRACT

Avian influenza A virus subtype H9N2 has been in circulation since last two decades in poultry industry of Pakistan. This virus is causing huge economic loss every year due to poor weight gain, drop in egg production and mortality of birds since first reported in 1998. Influenza viral surface protein neuraminidase enhances both virus replication and its release from host cells. In this study we isolated nineteen H9N2 viruses from infected birds of various farms from Punjab, Pakistan. Neuraminidase gene of these viruses was amplified using RT-PCR and sequenced to perform mutational analysis. Phylogenetic analysis revealed that B2 sub-lineage is endemic in the country as all isolated H9N2 strains belongs to this clade of G-1 lineage. Two isolated H9N2 strains A/Chick/Pak/303/2018 and A/Chick/Pak/436/2019 contain six glycosylation sites instead of seven as found in their ancestral strains. A comparative analysis of neuraminidase amino-acids sequences of B2 sub-lineage with Y- 280 lineage and B1 sub-lineage demonstrated that there were no human related substitutions, insertion or deletions in hem-absorbing site and loop of H9N2 isolates. Two unique substitutions, Gln39Arg and Lys47Glu have been noted in NA stalk domain of all isolates of this study which might be indirectly enhancing the virulence of virus. These findings demonstrated that new mutations are emerging in B2 sub-lineage and there is need for constant surveillance of evolving genome of H9N2 virus prevailing in the country to combat the future challenges of avian influenza out breaks in Pakistan.

INTRODUCTION

Influenza A Virus (IAV) of Orthomyxoviridae family is a single-stranded negative sense RNA virus (Wang *et al*., 2018). The virus contains lipid bilayer envelope attained from their host and nucleocapsid with inner core of matrix proteins (Lazniewski *et al*., 2018). There are eight genomic RNA segments which encode several proteins (Krejcova *et al*., 2015). Viral segment 4 and 6 encode surface glycoproteins called as hemagglutinin (HA) and neuraminidase (NA), respectively. HA and NA also function as surface antigen of IAV (Wu *et al*., 2014). Due to antigenic variation of NA and HA proteins, IAV is classified into different subtypes; 16 subtypes of HA and nine subtypes of NA have been reported (Plschka, 2012; Wang *et al*., 2018). Birds act as natural reservoir for all subtypes and have been isolated from different avian species (Alexander, 2000). Some subtypes like H1N1, H2N2 and H3N2 have been reported to cause disease in humans whereas subtypes H5N1, H7N7 and H9N2 cause diseases in birds and mammals as well (Claas *et al*., 1998). IAV H9N2 subtype has been in circulation among wild and commercial birds since its first isolation in 1966 (Li *et al*., 2012).

NA, a spike glycoprotein is embedded in lipid bilayer envelope of H9N2 virus by short hydrophobic amino acid sequences. A homotetramer of NA disintegrate the receptors sites by hydrolyzing sialic acid residues from glycoproteins to release the viral progeny from host cells (Samson *et al*., 2013; Hua-Yang *et al*., 2016). NA is about 470 amino acids long peptide which folds into four structural domains: one cytoplasmic tail, a transmembrane region, a stalk and one catalytic head having haemabsorbing site (HB site) and haemabsorbing loop (McAuley *et al*., 2019). Cytoplasmic tail is conserved among all IAV subtypes with MNPNQK sequence plays critical role in influenza virus life cycle (Mitnaul *et al*., 1996). Amino acid residues spanning from position 7 to 29 form N-terminal hydrophobic transmembrane domain and perform a significant role in the translocation of NA from

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endoplasmic reticulum to the apical membrane (McAuley *et al*., 2019). Stalk domain is formed by amino acid sequence at 38-74 positions, but this number of residues is variable among different IAV subtypes (Blok and Air, 1982). The length of stalk domain is specific to species and plays a role in viral adaptation and virulence (Park *et al*., 2017).

Amino acids 366-373, 399-404 and 431-433 form Hb site (Mosaad *et al*., 2017) which is a common feature of NAs of different subtypes of IAV. X ray crystallography has revealed that sialic acid residues can also bind at second site of IAV called as Hb site which is present adjacent to catalytic site on NA head and facilitates the binding to erythrocytes, a process called as hemabsorption (Streltsov *et al*., 2015). Amino acid residues at positions 313, 368, 370 and 371 located in HB loop are associated with pathogenicity of virus (Ali *et al*., 2017).

This research work covers three objectives, first is phylogenetic analysis of H9N2 virus isolated from Punjab province. Second is mutational analysis of NA gene of recently isolated H9N2 virus. Third is comparative analysis of potential pathogenic amino acids residues of neuraminidase isolated from poultry during this study to the previously reported from poultry and human as well. This comparison is important to understand the potential risk factors associated with poultry and human health as well.

MATERIALS AND METHODS

Sample collection and virus isolation

Tracheal swabs of dead birds suspected for IAV were collected and processed from 2016 to 2019 at GP laboratory Lahore. Anigen Rapid AIV Ag Test Kit (RG1501MH) was used to screen the IAV virus in samples according to manufacturer's instructions. Tracheal swabs of nineteen IAV positive flocks were further processed for virus isolation in embryonated chicken eggs (ECE). Samples (5 tracheal swabs per flock) were mixed thoroughly in 5mL of phosphate buffer saline (PBS) SIGMA-ALDRICH® with penicillin 2.0 mg/mL, streptomycin 2.0 mg/ mL and gentamycin 50 µg/mL and centrifuged at 4000 rpm for 30 minutes at room temperature. Nine days old ECE provided by Big Bird hatchery Raiwind Pakistan were used for isolation of virus. The supernatant (200µl/egg) was inoculated into ECE through the allantoic sac route using 1.0 mL syringe. ECE were incubated for 72 h at 37 °C. Eggs were candled every 12 h to observe any embryo mortality. All the ECE were chilled overnight at 4 °C after completion of incubation. Hemagglutination (HA) test was used to check the presence of IAV virus in harvested amnio-allantoic fluid (AAF) according to prescribed procedure (Ali *et al*., 2017).

Multiplex RT-PCR to confirm H9N2 virus

Multiplex RT-PCR was applied for confirmation of H9N2 virus and to rule out the presence of H5 and H7 viruses. Qiagen Viral RNA Extraction Mini Kit (Cat: 52906) was used to extract the viral RNA. Thermo Scientific RevertAid First Strand cDNA kit (Cat # K1822) and Uni-12 oligos (AGCAAAAGCAGG) were utilized to perform cDNA synthesis from viral genomic RNA. RT-PCR reaction mixture was prepared using DreamTaq green master mix (2X) ThermoFischer Scientific™ along with specific primers. Primer sequences are mentioned in Table I.

Amplification and sequencing of neuraminidase gene

Bio-Rad T100™ thermocycler was used to amplify the neuraminidase gene of nineteen H9N2 viruses (Hoffmann *et al*., 2001). The PCR conditions consisted of initial denaturation at 95 °C for 3 min, followed by 30 cycles at 94 °C for 40 seconds, 58 °C for 40 seconds and 72 °C for 7 min and final extension at 72 ˚C for 10 min. PCR product of NA gene was purified using the GeneAll Expin™ kit, followed by 1% agarose gel electrophoretic run. The amplified NA segments were sequenced by 1st Base 41 Science Park Rd, Singapore 117610, using the Sanger dideoxy sequencing method.

Table I. Primers used for multiplex RT-PCR and for amplification of NA gene of H9N2 Virus.

Primer	Oligonucleotide sequence	Length	Annealing temp.	Product size in bp	Reference
AIV-F	GTAGAGGGCTATTTGGIGC	19	57 °C	661	Tahir *et al*., 2016
H5-R	TGCAAATTCTGCATTGTAAC	20			
H7-R	CTGACTGGGTGCTCTTGTA	19		140	
H9-R	CGTCTTGTATTTGGTCATCA	20		276	
N2-F	ATGTTATCAATTTGCACTTGGGCAG	25	40 °C	149	Huang *et al*., 2013
N2-R	CATGCTATGCACACTTGTTTGGTTC	25			
NA-F	TATTGGTCTCAGGGAGCAAAAGCAGGAGT	29	58 °C	1413	Hoffmann *et al*., 2001
NA-R	ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	36			

Computational analysis

MEGA X software package (version 10.0.5) was used to draw Neighbor-Joining phylogenetic tree with Tamura-Nei method. Online server ExPASy was applied to translate the nucleotide sequences. BioEdit version 7.2.6 was used to make comparative analysis of currently isolated H9N2 strains NA proteins with previously reported sequences from Pakistan and China. Online software NetNGlyc 1.0 Server was used to calculate the glycosylation sites in neuraminidase amino acid sequences.

RESULTS

Identification and confirmation of H9N2 virus

All the nineteen samples subjected to virus isolation in ECEs showed positive HA activity in amnio-allantoic fluid. All these HA positive viruses were confirmed as H9N2 through RT-PCR using separate PCR reactions which produced bands of 276 and 149 base pairs for H9 and N2 genes, respectively. RT-PCR for H5 and H7 were found negative for all isolates which ensured the absence of these viruses in poultry flocks. All nineteen RT-PCR positive samples proceeded for amplification and sequencing of NA gene produced expected 1413 bp product size. NA gene sequences were submitted to NCBI GenBank, and their accession numbers are given as: MF959751-54, MH105293-99, MK348237-40 and MK425202-05.

Phylogenetic analysis

The evolutionary tree was inferred using the Neighbor-Joining method using employing MEGA X software. NA nucleotide sequences were obtained from NCBI database. The analysis involved total 82 nucleotide sequences including 19 sequences isolated for this study. The optimal tree with the sum of branch length 1.41266013 is shown (Fig. 1). The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. There were a total of 1894 positions in the final dataset. All ambiguous positions were removed for each sequence pair. Y280 lineage includes the H9N2 strains isolated from human, and G1 lineage covers the human and poultry isolates of H9N2 virus from Pakistan and other middle east countries. Here G1 is divided into B1 and B2 sub- lineages. B1 sub-lineage contains isolates previously reported from Pakistan and some. All isolates collected for this study clustered in B2 sub-lineage highlighted in red color bar (Fig. 1).

A comparison of potential pathogenic residues in NA amino acid sequences of recently isolated H9N2 virus with the previously reported sequences was performed (Table II). B2* represents the NA sequences in B2 sub-lineage that are ancestors of H9N2 strains isolated during this study. Stalk domain, HB site and HB loop are three potential regions involved in pathogenicity of H9N2 virus. Comparison demonstrated that HB loop has remained conserved in all isolates while HB site is mostly conserved and stalk domain contains two mutations at Gln39Arg and Lys47Glu. These two mutations are constant in all isolated viruses of B2 sub-lineage.

NetNGlyc 1.0 Server calculated seven glycosylation sites mostly at positions 44, 61, 69, 146, 200, 234 and 402 in NA amino acid sequences as described (Table III). NetNGlyc 1.0 Server provides a potential positive threshold confidence with value >0.5. All isolates of H9N2 virus collected during this study and earlier reported from Pakistan contain seven glycosylation sites mostly at mentioned positions. Exception is only two isolates A/Chick/Pak/303/2018 and A/Chick/Pak/436/2019 have only six glycosylation sites instead of seven. Glycosylation at position 69 is absent in strain A/Chick/Pak/303/2018 and position 61 is found absent in strain A/Chick/Pak/436/2019.

DISCUSSION

Phylogenetically, AIV subtype H9N2 have been classified into three lineages i.e. North American lineage, human/swine lineage and the Eurasian lineage. There is further subdivision in the Eurasian lineage represented as Y280 lineage, Korean Lineage and G1 lineage. The H9N2 viruses prevalent in Pakistan poultry have origin in G1 lineage (Abid *et al*., 2017). G1 lineage is subdivided into human origin H9N2 and Middle East group B. Pakistani H9N2 isolates belongs to Middle East group B, clustered at BI and B2 sub-lineages (Ali *et al*., 2017). All isolates of H9N2 virus of this study have origin either from strainA/chicken/ Pakistan/ 10A/ 2015 or A/pigeon/ Pakistan/25A/2015 of B2 sub-lineage. So it is evident from phylogenetic analysis that since 2015, H9N2 viruses from B2-sub-lineage have extended all over Pakistan, whereas in other countries of Asia e.g. Indonesia and China different subgroups of IAV are prevailing in the poultry industry (Novianti *et al*., 2019; Han *et al*., 2019). Recently it has been reported that Y280 like H9N2 virus are predominating in China affecting both mammalian and human hosts (Zou *et al*., 2019) whereas till to date not any single strain of H9N2 of Y280 lineage has been reported from Pakistan. Reasons for widespread B2 sub-lineage in Pakistan poultry would be change in antigenicity of virus due to mutation which resulted in viral escape from host immune system or wide spread use of vaccine has posed immune pressure on virus to adopt in host cells (Sun *et al*., 2019).

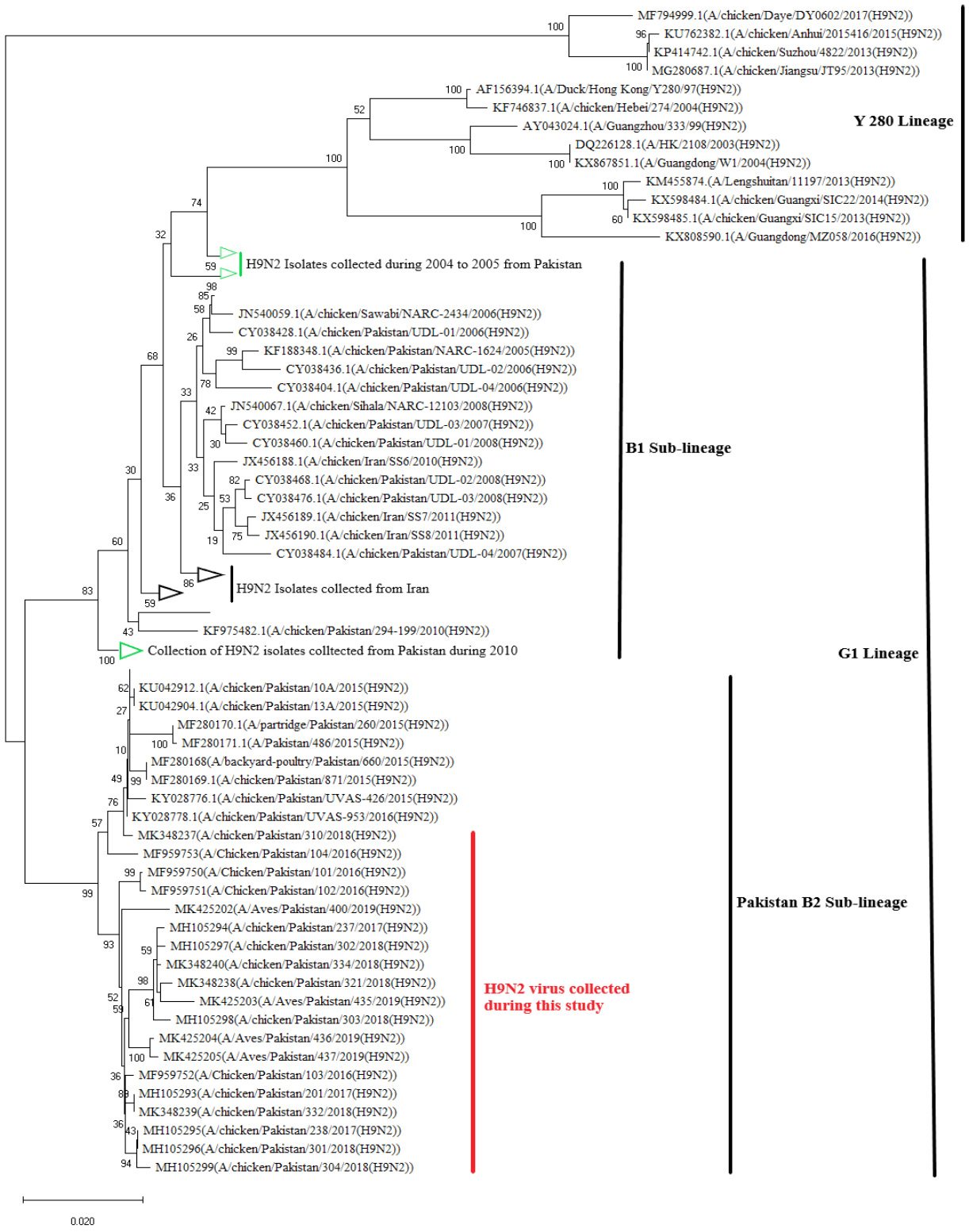

Fig. 1. Neighbor-Joining phylogenetic tree was constructed using MEGA X software (version 10.0.5) with Tamura-Nei method and 1000 bootstrap replicates values.

Table II. Comparison of potential pathogenic residues of currently isolated H9N2 viral NA protein with previously reported sequences. One letter code used in the table represent the following amino acids.

Lineage		Strain	Accession number	Stalk domain			HB site		HB loop			
				38-39	46-50	62-64	372	402-403	313	368	370	381
Y280 Linage		A/Duck/Hong Kong/Y280/97(H9N2)	AF156394	KQ	SNNQV	---	S	NW	E	E	S	T
		A/Guangzhou/333/99(H9N2)	AY043024	KQ	SDNQV	ITE	S	NS	E	K	S	G
		A/HK/2108/2003(H9N2)	DQ226128	KQ	SNNQV	ITE	S	NS	D	K	S	G
		A/Langhian/11197/2013(H9N2)	KM455874	RQ	SNNQV	---	S	DW	D	N	S	N
		A/Guangdong/MZ058/2016(H9N2)	KX808590	GQ	SNNQV	ITE	S	DW	D	N	S	N
		A/Hong Kong/1073/99(H9N2)	AJ404629	N-	SNNQA	ITE	S	NW	D	K	S	G
		A/Hong Kong/33982/2009(H9N2)	KF188318	KQ	SDNQA	ITE	S	NS	D	N	S	N
G-1 Lineage	B1 sub-lineage (Pakistan)	A/chicken/Pakistan/2/1999(H9N2)	KF188300	KQ	SNNQV	---	T	NW	D	K	S	G
		A/chicken/Pakistan/NARC-1617/2005(H9N2)	KF188326	KQ	SKNQV	ITE	A	NW	D	K	S	D
		A/chicken/Pakistan/UDL-01/2005(H9N2)	CY038412	KQ	SKNQV	ITE	A	NW	D	K	S	G
		A/chicken/Pakistan/UDL-02/2005(H9N2)	CY038420	KQ	SVQCD	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/UDL-04/2007(H9N2)	CY038484	SQ	SKNHV	IAK	T	NR	D	K	S	G
		A/chicken/Pakistan/UDL-03/2007(H9N2)	CY038452	NQ	SKNHV	ITE	A	NR	D	K	S	G
		A/chicken/Pakistan/UDL-02/2008(H9N2)	CY038468	NQ	SKNHV	ITK	A	NR	D	K	S	G
		A/chicken/Attack/NARC-14994/2009(H9N2)	JN540075	KQ	SKNHV	ITE	A	NR	D	K	S	G
	B2 *	A/chicken/Pakistan/10A/2015(H9N2)	KU042912	NR	SENHV	ITE	T	NR	D	K	S	G
		A/pigeon/Pakistan/25A/2015(H9N2)	KU042920	NR	SENHV	ITE	T	NR	D	K	S	G
		A/Chicken/Pakistan/101/2016(H9N2)	MF959751	NR	SENHV	ITE	T	NG	D	K	S	G
		A/chicken/Pakistan/102/2016(H9N2)	MF959752	NR	SENHV	ITE	T	NG	D	K	S	G
		A/chicken/Pakistan/103/2016(H9N2)	MF959753	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/104/2016(H9N2)	MF959754	NR	SENHV	ITE	T	NR	D	K	S	G
	H9N2 virus isolated for this study (B2- linage)	A/chicken/Pakistan/201/2017(H9N2)	MH105293	NW	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/237/2017(H9N2)	MH105294	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/238/2017(H9N2)	MH105295	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/301/2018(H9N2)	MH105296	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/302/2018(H9N2)	MH105297	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/303/2018(H9N2)	MH105298	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/304/2018(H9N2)	MH105299	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/310/2018(H9N2)	MK348237	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/321/2018(H9N2)	MK348238	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/332/2018(H9N2)	MK348239	NW	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/334/2018(H9N2)	MK348240	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/400/2019(H9N2)	MK425202	NR	SENHV	MTE	T	NR	D	K	S	G
		A/chicken/Pakistan/435/2019(H9N2)	MK425203	NR	SENHV	ITE	T	NR	D	K	S	G
		A/chicken/Pakistan/436/2019(H9N2)	MK425204	NR	SENHV	IKE	T	NR	D	K	S	G
		A/chicken/Pakistan/437/2019(H9N2)	MK425205	NR	SENHV	ITE	T	NR	D	K	S	G

K, Lysin; N, Asparagine; R, Arginine; Q, Glutamine; W, tryptophan; E, Glutamic acid; S, Serine; T, Threonine; G, Glycine; H, Histidine; V, Valine and D, Aspartic acid.

Table III. Glycosylation sites in H9N2 neuraminidase amino acids sequence.

Sites #	Positions	Sequences	Potential	N-Glyc results
1	44 – 47	NSSE	0.6779	+ +
2	61 – 63	NITE	0.7416	+ +
3	69 – 72	NGTI	0.7189	+ +
4	146 – 149	NGTT	0.7092	+ +
5	200 – 203	NATA	0.3470	- -
6	234 – 237	NGTC	0.7558	+ + +
7	402 – 405	NRSG	0.3279	- -

NA amino acids sequence comparison of G1 and Y 280 lineages present in Table II has demonstrated that Asp, Lys, Ser and Gly residues at positions 313, 368, 370 and 381 in HB loop have remained conserved in both lineages. Reason for this conservation of amino acids at positions 268 to 370 has been dictated with *in vitro* experiments in mice cells that this region of different IAVs has ability to propagate in lungs (Gilbertson *et al*., 2017). In B2-sub-lineage 372Thr is present in all isolates instead of Ala372 or Ser372 of B1 and Y280 lineage. It has been demonstrated that Ser370 and Ser372 present in HB site of Y280 and G1 lineages play an important role in the binding of sialic acid residues (McAuley *et al*., 2019). The function of 372Thr in HB site of H9N2 viruses of B2-sublineage is still unknown. A substitution with unknown function at site Ser372Ala similar to human H9N2 influenza isolates but different from avian H9N2 isolates has been reported in G1 Avian influenza H9N2 from Egypt (Mosaad *et al*., 2017). HB site at position 402-403 contains Asn-Trp or Asp-Trp residues in Y280 and human origin H9N2 G1 lineages. Whereas B2 sub-lineage contains Asn-Arg at positions 402-403 but two isolates of this study A/Chicken/Pakistan/101/2016 and A/Chicken/Pakistan/102/2016 has Arg403Gly mutation. Recently a similar substitution Asn- Arg in HB site at position 402-403 has been reported from Egypt (Mosaad *et al*., 2017).

Stalk domain of Y280 lineage contain Lys-Gln at 38-39 position which are substituted to Asn-Gln at same position in B1 sub-lineage. It has been reported that stalk domain of NA gene of H9N2 is conserved (Kandeil *et al*., 2017; Mosaad *et al*., 2017). From alignment data, it has been observed that Asn-Arg residues at 38-39 position are present in strains of B2 sub-lineage, a distinctive feature observed in this group. Two isolates of this study A/chicken/Pakistan/201/2017 and A/chicken/Pakistan/332/2018 contain Asn-Trp residues at 38-39 position which is a unique deviation from B2 sub-lineage. A sequence of SNNQV from 46 to 50 in stalk domain of Y280 linage is shifted to SKNQV (human) and SKNHV in BI sub-lineages. Whereas in B2 sub-lineage sequence SENHV is found conserved in all H9N2 isolates from 2015 to 2019, a unique substitution in B2 as compare to B1 sub-lineage in this region is Lys47Glu is observed in all our nineteen isolates (Table II). A reverse mutation for these amino acids has been reported in H9N2 polymerase acidic enzyme as Glu31Lys with the enhanced viral replication in the vero cells than in the chicken eggs (Lee *et al*., 2017). Comparative analysis demonstrated that new mutations are emerging in B2 sub-lineage and there is need for constant surveillance of evolving genome of H9N2 virus prevailing in the country.

CONCLUSIONS

Here we conclude that H9N2 virus of B2 sub-lineage is prevailing in poultry industry of Pakistan. Comparison of potential pathogenic regions of NA proteins has revealed that no human specific pathogenic site has been observed in neuraminidase enzyme of H9N2 viruses isolated from poultry flocks in during the last 12 years of genetic evolution. These findings strengthen the probability that, H9N2 viruses of B2 sub-lineage in Pakistan may not have human health related potential risk. Despite the conservancy of catalytic part of NA protein, its somatic regions are in evolution to adopt the virus in their host. This kind of evolution might be indirectly enhancing the virulence of H9N2 virus in the host cells which could result in increased economic losses to poultry farmers. Vaccines with currently circulating H9N2 virus B2 sub lineage could be more efficacious and better cop future challenges due to continuous evolution of these viruses. We emphasized that further investigations are needed to comprehend the pathogenicity associated with evolving proteins of H9N2 virus endemic in Pakistan.

Statement of conflict of interest

The authors have declared no conflict of interest.

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