Pakistan J. Zool., vol. 54(2), pp 761-769, 2022 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20191216081232

Adaptive Mutations in Nuclear Export Protein and Non-Structural 1 Protein of Avian Influenza A H9N2 Virus Circulating in Punjab, Pakistan

Rehman Shahzad[1], Saba Irshad[1]*, Malik Saddique Mehmood[1] and Faisal Amin[2]

[1]*Institute of Biochemistry and Biotechnology, University of the Punjab,5400-Lahore, Pakistan*
[2]*Grand Parent Laboratory, Lahore, Pakistan*

ABSTRACT

Avian influenza A virus subtype H9N2 has been in circulation since last two decades in poultry flocks of Pakistan. It is causing immense economic losses to the farmers. Avian influenza H9N2 virus were isolated from infected birds of different poultry farms in the Province of Punjab, Pakistan. Segment eight gene of the virus was amplified using RT-PCR and sequenced to analyze mutations in this viral segment. Phylogenetic tree analysis showed sequences from 2015 to 2017 form a single evolving clade. Valdar residues conservation scores by multiple sequence alignment showed the C terminal region of NEP protein is conserved while C terminal region of effector domain (ED) of NSI protein exhibit mutations. These mutations are enhancing the total hydrophobicity of the molecules. Hydrophobicity was calculated by using Kyte and Doolittle method. High hydrophobicity of NS1 protein is also posing a potential for H9N2 virus to adapt in host, which might be contributing to the increase in pathogenicity of the circulating H9N2 virus.

Article Information
Received 16 December 2019
Revised 13 May 2020
Accepted 16 September 2020
Available online 24 March 2021 (early access)
Published 21 January 2022

Authors' Contribution
RS designed and conducted the laboratory work. FA executed virus isolation. MSM helped in *in silico* work and manuscript preparation. SI supervised the work.

Key words
Avian influenza, NS1, Nuclear export protein (NEP), Sequencing, Sequence variants, Hydrophobicity

INTRODUCTION

Avian influenza (AIV)A is negative sense single stranded RNA virus which belongs to Orthomyxoviridae family (Wang *et al*., 2018). AIV H9N2 has been circulating in birds worldwide since 1966. The virus consists of lipid bilayer envelope derived from their host and nucleocapsid with inner shell of matrix proteins (Lazniewski *et al*., 2017). There are eight segments of viral genome encoding several proteins named as hemagglutinin (HA), neuraminidase (NA), proton channel protein M2, nucleoprotein (NP), complex of viral polymerases (PA, PB1, PB2), and matrix protein (M1) (Yamayoshi *et al*., 2016; Wu *et al*., 2014). Viral 8th segment about 840 nucleotides long encodes the two proteins, nuclear export protein (NEP) and nonstructural protein 1 (NS1) by alternating splicing mechanism (Hsu, 2018).

NA and HA proteins act as antigen of influenza A virus (Wu *et al*., 2014). The HA protein of AIV virus binds to terminal sialic acid residues of glycoprotein receptors on respiratory epithelial cells. The NA protein hydrolyzes terminal sialic acid from cell receptors to facilitate the release of viral progeny from the cells to spread the infection. Viral proteins are always in evolution to perform different functions; a prominent example is nuclear export protein (NEP) also referred as NS2. Basic function of NEP is the export of ribonucleoprotein (RNP) complex from nucleus to cell membrane where it assembles into viral particles. NEP also contributes in multiple biological path ways during influenza virus life cycle (Paterson and Fodor, 2012). Influenza A virus neutralizes host antiviral activities mainly interferons (IFNs) and other associated protein induced by (IFNs). AIV viral NS1 protein plays vital role to counteract such IFN base host antiviral activities (Krug, 2015). NS1 protein also acts as virulence determinant in viral infection (Kuo *et al*., 2018). The NS1 protein of AIV also participates in cell necroptosis via interacting the mixed-lineage kinase domain-like protein (MLKL) and increases the inflammation of cells (Goba *et al*., 2019). So NS1 protein can perform function both in nucleus and cytoplasm as well. NS1 could also generate nucleolar stress based on epigenetic change of rRNA gene promoter via interaction with nucleolin (Yan *et al*., 2017). Purpose of this study is to explore the genetic changes in NEP and NS1 protein as these proteins are responsible for human infection (Mahardika *et al*., 2019), and has always remained a priority target to discover the anti-influenza compounds (Kleinpeter *et al*., 2018).

In this study we have reported the different mutations in NEP and C terminal region of ED of NS1 protein H9N2 viruses isolated from diseased birds submitted in Grand

* Corresponding author: saba.ibb@pu.edu.pk
0030-9923/2022/0002-0761 $ 9.00/0

Parent (GP) Laboratory Lahore from different locations of Punjab, Pakistan during 2016.These amino acid mutations in NEP and NS1 proteins might have collaboratively enhanced the pathogenicity of H9N2 virus along with HA and NA proteins.

MATERIALS AND METHODS

Sample collection

Tracheal swabs were collected during 2016 at Grand Parent Diagnostic laboratory, Lahore from diseased/ dead birds of poultry flocks. Anigen Rapid AIV Ag Test Kit (RG1501MH) was used to detect the Avian Influenza Type A virus in samples according to manufacturer's instructions. Tracheal swabs of AIV positive samples were mixed thoroughly in 5mL of phosphate buffered saline(PBS) SIGMA-ALDRICH® with penicillin 2.0 mg/ ml, streptomycin 2.0 mg/ ml and gentamycin 50µg/mL and stored at -20 °C till further processing for virus isolation.

Isolation of virus

Embryonated chicken eggs (ECE), 9-11 days old provided by Big Bird hatchery Raiwind, Pakistan were used for isolation of H9N2 virus. Frozen samples were thawed and centrifuged at 4000 rpm for 30 min at room temperature. The supernatant (200µl/egg) was inoculated into ECE through the allantoic sac route using 1.0 mL syringe. ECE were incubated at 37 °C for 72 h. To check any mortality of embryos, eggs were candled at regular interval of 12 h until the completion of 72 h' incubation. After the incubation eggs were chilled at 4 °C. Then allanto-amniotic fluid (AAF) was harvested using the sterile syringes. Presence of AIV in AAF was confirmed by using the hemagglutination (HA) assay (Alexander and Chettle, 1977).

Confirmation of H9N2 by RT-PCR

Diagnostic RT-PCR was applied to confirm the presence of H9N2 virus. Qiagen Viral RNA Extraction Mini Kit (Cat: 52906) was used to extract the viral RNA. Thermo Scientific Revert Aid First Strand cDNA kit (Cat # K1822) and Uni-12 oligo (AGCAAAAGCAGG) was utilized to perform cDNA synthesis from viral genomic RNA. Dream Taq green master mix (2X) Thermo Fischer Scientific™ along with H9N2 specific primers was used to prepare RT-PCR reaction mixture. Primer sequences are mentioned in the Table I.

Amplification and sequencing of genomic segment eight

Gene specific primers (Table I) reported by Hoffmann were used for amplification of viral genomic segment eight using the T100™ thermocycler (Bio-Rad). The first cycle of the PCR program consisted of initial denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 Sec, 58 °C for 30 Sec, and 72 °C for 3 min, and final elongation at 72 °C for 10 min (Hoffmann *et al*., 2001). The amplified and purified genes were sequenced by1st Base41 Science Park Rd, Singapore 117610, using the Sanger dideoxy sequencing method. Sequences were submitted to NCBI GenBank with the nucleotides and proteins accession numbers given in the Table II.

Computational analysis

Then MEGA X (Molecular evolution and genetic analysis) version 10.0.5 software was used to construct the Neighbor-Joining phylogenetic tree using MEGA X (version 10.0.5) software by applying the Tamura-Nei model and 1000 bootstrap replicates values. Online server Clustal Omega was used for multiple sequence alignment (MSA) analysis. The Sequence Manipulation Suite Copyright © (Stothard, 2000)was used to calculate the Valdar residue conservation scores and grand average hydropathy (GRAVY) of protein sequences. GRAVY of a protein is calculated by adding the hydropathy values of each individual amino acid then dividing by the number of amino acids residues in the protein sequence or length.

RESULTS AND DISCUSSION

Identification and confirmation of H9N2 virus

HA assay of H9N2 suspected samples was performed in a 96 well plate. Only six samples produced strong agglutination with RBCs. Samples screened by HA assay were tested for H9N2 virus by RT-PCR. Separate PCR reactions were performed for H9 and N2 segments. All six samples produced the specific band of 276 and 149 base pairs for H9 and N2 respectively (Fig. 1). H9N2 RT-PCR positive samples were proceeded for amplification of genomic segment 8 according to (Hoffmann *et al*., 2001) procedure. PCR product of segment 8 was purified by using the Gene All Expin™ kit followed by1% agarose gel electrophoretic run Figure 2.

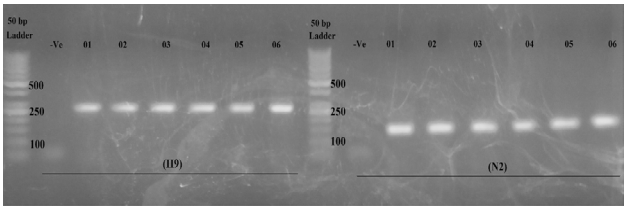

Fig. 1. Agarose gel (2%) electrophoresis for six H9N2 RT-PCR positive samples. First lane 50 base pair DNA ladder, second lane is negative control followed by six positive samples of H9N2 virus.

Table I. Primers used for multiplex RT-PCR and for amplification of genomic segment eight of H9N2 Virus.

Primer	Oligonucleotide sequence	Length	Annealing	Product bp	Reference
H9-F	GTAGAGGGCTATTTGGIGC	19	57 °C	276	Tahir *et al*., 2016
H9-R	CGTCTTGTATTTGGTCATCA	20	57 °C		
N2-F	ATGTTATCAATTTGCACTTGGGCAG	25	40 °C	149	Huang *et al*., 2013
N2-R	CATGCTATGCACACTTGTTTGGTTC	25	40 °C		
NS-F	TATTCGTCTCAGGGAGCAAAAGCAGGGG	28	58 °C	840	Hoffmann *et al*., 2001
NS-R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	35	58 °C		

Table II. Sequences numbered 1 to 6isolated during this and sequence 7 is wild type strains and its NEP and NS1 protein sequences were used as reference.

Isolates	Abbreviation	NCBI accession number	Protein ID / NS2 +NS1
(A/chicken/Pakistan/101/2016(H9N2))	101/2016	MG230435	ATS91874 + ATS91873
(A/chicken/Pakistan/102/2016(H9N2))	102/2016	MG230436	ATS91876 + ATS91875
(A/chicken/Pakistan/103/2016(H9N2))	103/2016	MG230437	ATS91878 + ATS91877
(A/chicken/Pakistan/104/2016(H9N2))	104/2016	MG230438	ATS91880 + ATS91879
(A/chicken/Pakistan/107/2016(H9N2))	105/2016	MG230439	ATS91882 + ATS91881
(A/chicken/Pakistan/115/2016(H9N2))	115/2016	MG230440	ATS91884 + ATS91883
(A/Avian/Pakistan/984/2015(H9N2))	984/2015	MH180645	AVX27241+AVX 27240

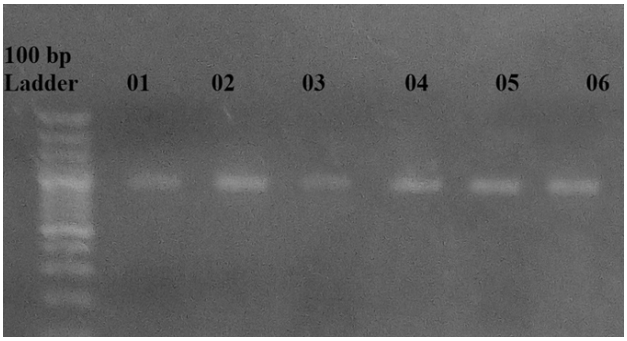

Fig. 2. Agarose gel (1%) electrophoresis for amplified and purified segment 8 of six H9N2 virus. First laneis 100 base pair DNA ladder, followed by six lanes with purified segment 8 of 840 base pairs.

Phylogenetic tree

Nucleotide sequences data of segment eight of avian influenza A virus H9N2 from NCBI GenBank was retrieved to construct phylogenetic tree with 1000 bootstrap values using freeware MEGA X (Molecular evolution and genetic analysis) version 10.0.5 developed by Pennsylvania State University. Phylogenetic tree has been divided into two clades, A and Bas demonstrated in Figure 3. The sequences from 2004 to 2015 form clade A and sequences from 2015 to 2017 clustered in clade B. The sequences of strains isolated during this study are labeled with square boxes in the phylogenetic tree and linked to A/Avian/Pakistan/984/2015(H9N2) strain. The phylogenetic analysis showed that majority of H9N2 isolates clusterd in clade A belongs to Iran, Israel and India. Clade B containing H9N2 isolates reported from Pakistan demonstrated that evolutionary changes in H9N2 virus prevailing in poultry flocks have adopted the virus to persist in the country since 2015.This indicates a potential threat for Pakistani poultry and there should be strong concern in developing some vaccines or inhibitors to cease the virulent transmission.

Multiple sequence alignment (MSA) analysis

Sequences of NSI and NS2 proteins were aligned and compared with the wild type (ancestor)strain A/Avian/Pakistan/984/2015(H9N2) sequences (NCBI-Accesssion AVX 27240-41). For this purpose online server Clustal Omega was used and mutations were highlighted with red color on sequences. From alignment analysis it is evident that NEP protein has remained conserved in all isolates only few mutations were observed, even the NEP protein (Accession number: ATS91874) of a strain 101/2016 is completely conserved like ancestor (Fig. 4). NS1 protein also exhibited conservation of amino acids from 1 to 71 which is N terminal RNA-binding domain (RBD). While C terminal region of ED of NS1 has acquired greater number of substitutions, highlighted in Figure 5.

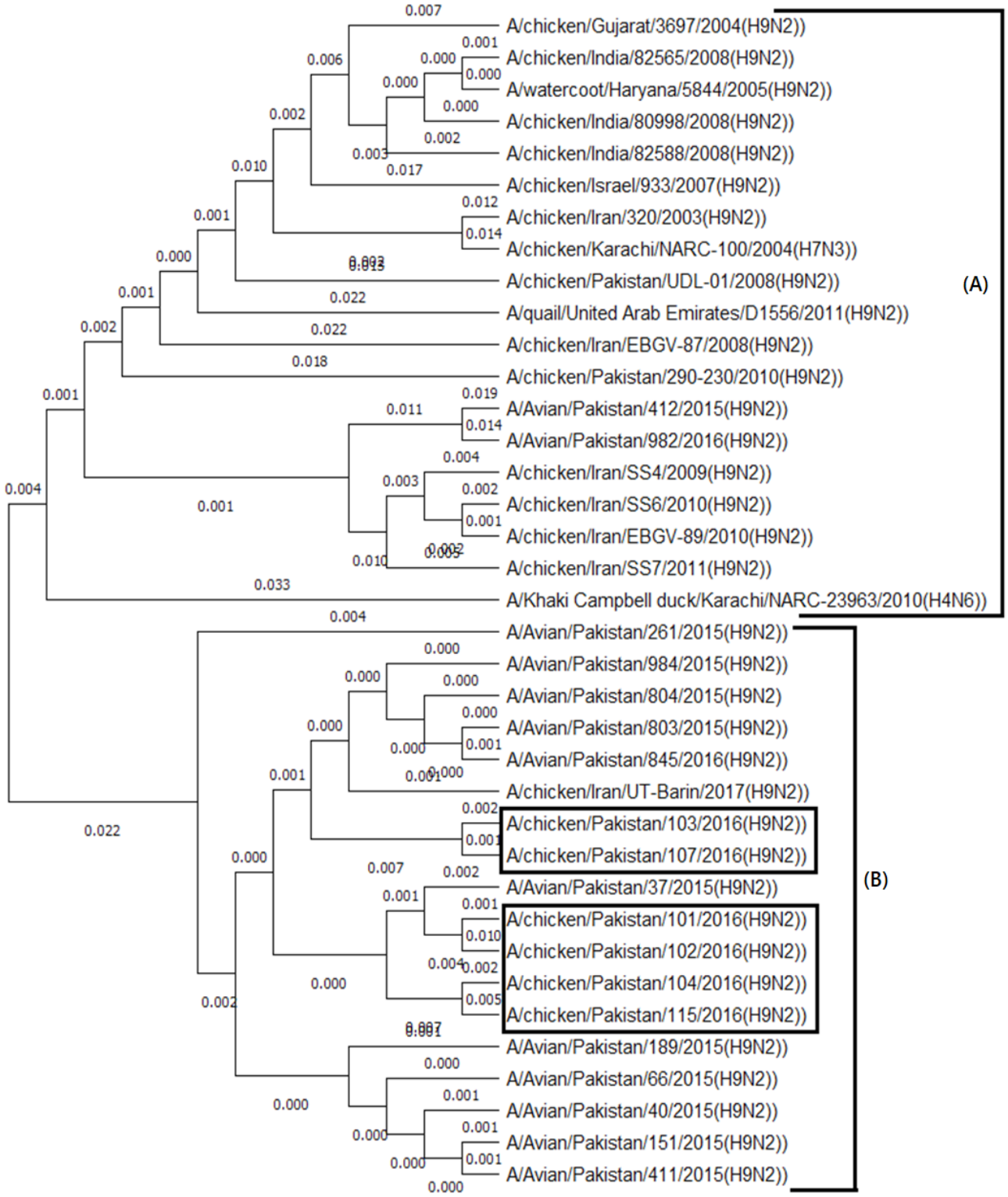

Fig. 3. Neighbor-Joining phylogenetic tree constructed using MEGA X (version 10.0.5) software with Tamura-Nei model and 1000 bootstrap values.

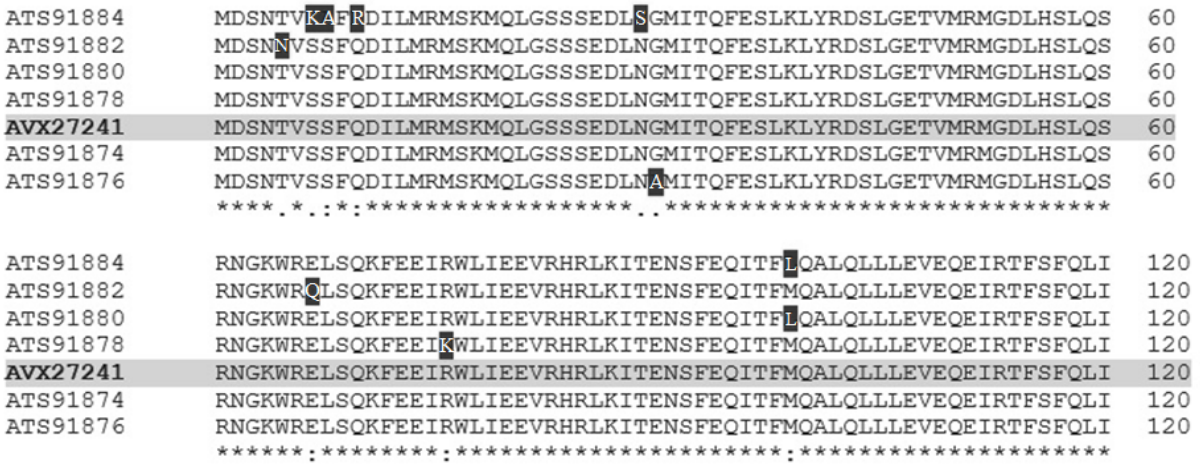

Fig. 4. Multiple Sequence Alignment (MSA) analysis by online server Clustal Omega: NEP sequnces alligned and compared with the sequence of wild type AVX 27241(grey colour), mutations are highlighted in red colour.

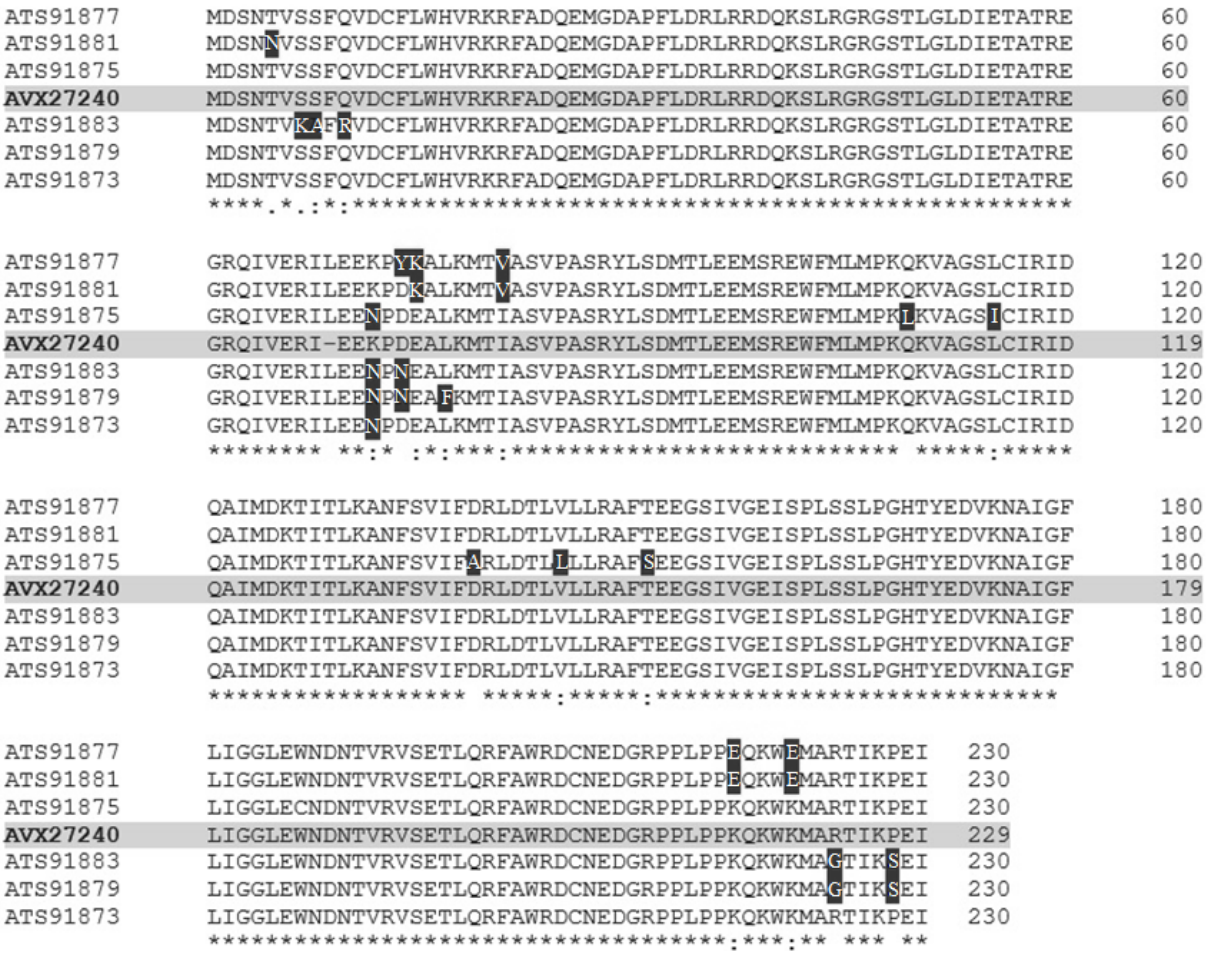

Fig. 5. Multiple Sequence Alignment (MSA) analysis by online server Clustal Omega: NSI sequences alligned and compared with wild type AVX 27241 (grey colour) mutations are labelled in red colour.

NEP conservation and mutations

Valdar residues conservation scores show that NEP proteins of all sex isolates are strongly conserved. The C terminal helix has the most conserved regions which interact with the M1 protein (Akarsu *et al*., 2003). Whereas N terminal region in NEP of A/chicken/Pakistan/115/2016(H9N2) has some mutations, which account for adaptation of pathogenic avian influenza virus in their host cells with increased replication (Mänz *et al*., 2012). Threonine and glutamine at positions 33 and 34 are conserved in all isolates, these amino acids enhance the interaction of NEP with CRM1 protein with greater affinity (Boukharta *et al*., 2019). N terminal region of NEP protein also contains leucine rich two nuclear export signal motifs (NES) at positions 11 to 21 and 22 to 45 (Huang *et al*., 2013). In sequences for this work five leucine residues at positions 21, 28, 38, 40 and 45 are strongly conserved. These residues are involved in identification and binding of cellular CRM1-RanGTP to the NES and is very important for the transport of viral ribonucleo- protein (vRNPs) out of the infected nucleus (Akarsu *et al*., 2003). These motifs in isolated strains also exhibit strong conservation without a single mutation and could be the ideal target regions for designing of novel antiviral inhibitors to face the challenges of evolving strains of AIV H9N2 virus in Pakistan.

Potential mutations in NS1 protein

The size of NS1 protein of influenza A H9N2 virus is 230 amino acids with two functional domains linked by short linker region. N terminal RNA-binding domain (RBD) consist of amino acids from 1 to 73 and residues from 85 to end form C terminal effector-domain (ED) (Hale *et al*., 2008). It has been reported that the sequences of NSI proteins of most AIV virus strains are strongly conserved but variations occur in C terminal region of ED (Krug and Gracia, 2013). A single amino acid arginine (R) at position 38 is prerequisite for dsRNA binding but other adjoining basic amino acids also contribute in this binding process (Cheng *et al*., 2009). We have observed R38 in RBD has remain conserved in all six isolates. It has been reported that S42 residue of the NS1 protein of H1N1 strain is the key amino acid residues in modulating the host IFN response by inhibiting the activation of IRF3 and facilitate virus replication (Cheng *et al*., 2018). S42 residue in NS1 has been conserved in all six isolates H9N2 virus. Some strains of H9N2 from Iran have been reported to have mutation M81I, two isolates A/chicken/Pakistan/103/2016(H9N2) and A/chicken/Pakistan/107/2016(H9N2) in this study also exhibit the same mutation at same position.

A substitution D139A in strain A/chicken/Pakistan/102/2016 and substitution P228S in A/chicken/Pakistan/104/2016 and A/chicken/Pakistan/115/2016 have been observed. It was reported that these two substitutions does not alter the anti- cellular defense mechanism and strains retained their virulence potential (Boukharta *et al*., 2015). Sequence ATS91875 contain leucine instead of glutamine at position 109. ATS91877 and ATS91881 contain glutamic acid 221 instead of lysine 221 which contribute in nucleolus localization signal (NoLS2) (Han *et al*., 2010). Nuclear export signal (NES) amino acids 138 to 147 of ATS91875 contain two mutations at 139 aspartic acid to 139 Alanine and 145Valine to 145Leucine (Keiner *et al*., 2010). Domain ARTIK (223 to 227) has shifted to AGTIK in sequences ATS91879 and ATS91883which is reported to cause defects in the transcription of antiviral gene by interacting with the polymerase-II associated factors (Hsu, 2018). However apart from mutations a number of regions has remained conserved in NS1 proteins of all isolated strains. These conserved mutated regions of NS1 from different clades could be used to generate recombinant NS1 antigen for the development of diagnostic test for differentiation of different clades of AIV (Lorch *et al*., 2019).

GRAVY

More positive values of GRAVY indicate the greater the hydrophobicity of protein molecules (Kyte and Doolittle, 1982). GRAVY-values for NEP and NS1 proteins are provided in Tables III and IV. Due to mutations a gradual increase in positive GRAVY values were observed for both NS2 and NS1 proteins only the exception is NEP protein of strain 107/2016 due to insertion of asparagine with hydropathy index (HI: -3.5) an amino acid with basic side chain instead of threonine (HI -0.7) at position 05 and glutamine replacing glutamic acid at position 67, both amino acids have the same H1 (-3.5). All mutations in NEP and NS1 protein would have resulted in enhanced stability due to more hydrophobicity. As hydrophobicity is most important factor in protein folding and aggregation and contributes to protein stability (Durell and Ben-Naim, 2017). A comprehensive understanding of origins of the hydrophobicity in protein molecules and its role in protein aggregation and folding has remained an open problem (Camilloni *et al*., 2016). The hydrophobicity of nonstructural proteins facilitates the interaction with membranous markers during various stages of AIV virus replication cycle (Al-Saadi and Jones, 2019). Alpha helical bundle of matrix proteins of orthomyxo virus are also stabilized by hydrophobic residues which help in membrane association and self-polymerization (Zang *et al*., 2017).

Table III. Mutations of amino acid in isolated NEP protein in comparison to close ancestor (A/Avian/Pakistan/984/2015(H9N2)) and their calculated GRAVY value, according to the (Kyte and Doolittle,1982).

Isolate	Protein Ids	Mutation in NEP									GRAVY
		Positions of amino acids									
984/2015	**AVX27241**	**T5**	**S7**	**S8**	**Q10**	**N29**	**G30**	**E67**	**R76**	**M99**	**-0.405**
101/2016	ATS91874	-	-	-	-	-	-	-	-	-	-0.405
102/2016	ATS91876	-	-	-	-	-	A	-	-	-	-0.387
103/2016	ATS91878	-	-	-	-	-	-	-	K	-	-0.400
104/2016	ATS91880	-	-	-	-	-	-	-	-	L	-0.39
107/2016	ATS91882	N	-	-	-	-	-	Q	-	-	-0.429
115/2016	ATS91884	-	K	A	R	S	-	-	-	L	-0.38

Table IV. Amino acid mutations in isolated NS1 proteins in comparison to close ancestor (A/Avian/Pakistan/984/2015(H9N2)) and their calculated GRAVY value, according to the (Kyte and Doolittle,1982).

Isolate/ protein Ids	Mutation in NS1																		GRAVY
	Positions of amino acids																		
984/2015 AVX27240	**5T**	**7,8 SS**	**10Q**	**69 del**	**72 K**	**74D**	**75E**	**77L**	**81I**	**109Q**	**115L**	**139D**	**145V**	**151T**	**217K**	**221K**	**224R**	**228P**	**- 0.409**
101/2016 ATS91873	-	-	-	L	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.389
102/2016 ATS91875	-	-	-	L	N	-	-	-	-	L	I	A	L	S	-	-	-	-	-0.318
103/2016 ATS91877	-	-	-	L		Y	K	-	V	-	-	-	-	-	E	E	-	-	-0.380
104/2016 ATS91879	-	-	-	L	N	N	-	F	-	-	-	-	-	-	-	-	G	S	-0.371
107/2016 ATS91881	N	-	-	L		-	K	-	V	-	-	-	-	-	E	E	-	-	-0.402
115/2016 ATS91883	-	KA	R	L	N	N	-	-	-	-	-	-	-	-	-	-	G	S	-0.373

CONCLUSION

Mutations in segment eight are leading to adaptive evolution of NEP and NS1 proteins of H9N2 virus endemic in Pakistan, which might be leading to enhanced stability associated with increased in hydrophobicity. This is also posing a potential in virus for adaptation in their host. There is a dire need to constantly study the evolution of non-structural proteins of H9N2 virus prevailing in all regions of country. The structural and functional analysis of non-structural proteins will be helpful in the designing of antiviral inhibitors to meet the future challenges of H9N2 virus in poultry flocks of Pakistan.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Akarsu, H., Burmeister, W.P., Petosa, C., Petit, I., Müller, C.W., Ruigrok, R.W. and Baudin, F., 2003. Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2). *EMBO J.*, **22**: 4646-4655. https://doi.org/10.1093/emboj/cdg449

Alexander, D.J. and Chettle, N.J., 1977. Procedures for the haemagglutination and the haemagglutination inhibition tests for avian infectious bronchitis virus. *Avian Pathol.*, **6**: 9-17. https://doi.org/10.1080/03079457708418208

Alsaadi, J.E.A. and Jones, I.M., 2019. Membrane binding proteins of coronaviruses. *Future Virol.*, **14**: 275-286. https://doi.org/10.2217/fvl-2018-0144

Boukharta, M., Azlmat, S., Elharrak, M. and Ennaji, M.M., 2015. Multiple alignment comparison of the non-structural genes of three strains of equine influenza viruses (H3N8) isolated in Morocco. *BMC Res. Notes*, **8**: 471. https://doi.org/10.1186/s13104-015-1441-0

Boukharta, M., Kasmi, Y., Zakham, F., El Amri, H. and Ennaji, M.M., 2019. Prediction of genetic mutations of equine influenza virus related to adaptive determination nuclear export ribonucleoprotein complex. *J. Basic appl. Zool.*, **80**: 25. https://doi.org/10.1186/s41936-019-0099-x

Camilloni, C., Bonetti, D., Morrone, A., Giri, R., Dobson, C.M., Brunori, M., Gianni, S. and Vendruscolo, M., 2016. Towards a structural biology of the hydrophobic effect in protein folding. *Sci. Rep.*, **6**: 28285. https://doi.org/10.1038/srep28285

Cheng, A., Wong, S.M. and Yuan, Y.A., 2009. Structural basis for dsRNA recognition by NS1 protein of influenza A virus. *Cell Res.*, **19**: 187. https://doi.org/10.1038/cr.2008.288

Cheng, J., Zhang, C., Tao, J., Li, B., Shi, Y. and Liu, H., 2018. Effects of the S42 residue of the H1N1 swine influenza virus NS1 protein on interferon responses and virus replication. *Virol. J.*, **15**: 57. https://doi.org/10.1186/s12985-018-0971-1

DahiruRogo, L., Rezaei, F., Shafiei-Jandaghi, N.Z., Ghavami, N., Fatemi-Nasab, G. and Mokhtari-Azad, T., 2015. Analysis of amino acid changes in NS protein of influenza A/(H3N2) virus in Iranian isolates. *Future Virol.*, **10**: 1195-1206. https://doi.org/10.2217/fvl.15.90

Durell, S.R. and Ben-Naim, A. 2017. Hydrophobic-hydrophilic forces in protein folding. *Biopolymers*, **107**: e23020. https://doi.org/10.1002/bip.23020

Gaba, A., Xu, F., Lu, Y., Park, H.S., Liu, G. and Zhou, Y., 2019. The NS1 protein of influenza A virus participates in necroptosis by interacting with MLKL and increasing its oligomerization and membrane translocation. *J. Virol.*, **93**: e01835-18. https://doi.org/10.1128/JVI.01835-18

Hale, B.G., Randall, R.E., Ortín, J. and Jackson, D., 2008. The multifunctional NS1 protein of influenza A virus. *J. Gen. Virol.*, **89**: 1-47. https://doi.org/10.1099/vir.0.2008/004606-0

Han, H., Cui, Z.Q., Wang, W., Zhang, Z.P., Wei, H.P., Zhou, Y.F. and Zhang, X.E., 2010. New regulatory mechanisms for the intracellular localization and trafficking of influenza A virus NS1 protein revealed by comparative analysis of A/PR/8/34 and A/Sydney/5/97. *J. Gen. Virol.*, **91**: 2907-2917. https://doi.org/10.1099/vir.0.024943-0

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G. and Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A virus. *Arch. Virol.*, **146**: 2275-2289. https://doi.org/10.1007/s007050170002

Hsu, A.C.Y., 2018. Influenza virus: A master tactician in innate immune evasion and novel therapeutic interventions. *Front. Immunol.*, **9**: 743. https://doi.org/10.3389/fimmu.2018.00743

Huang, S., Chen, J., Chen, Q., Wang, H., Yao, Y., Chen, J. and Chen, Z., 2013. A second CRM1-dependent nuclear export signal in the influenza A virus NS2 protein contributes to the nuclear export of viral ribonucleoproteins. *J. Virol.*, **87**: 767-778. https://doi.org/10.1128/JVI.06519-11

Huang, Y., Khan, M. and Măndoiu, I.I., 2013. Neuraminidase subtyping of avian influenza viruses with primer hunter-designed primers and quadruplicate primer pools. *PLoS One*, **8**: e81842. https://doi.org/10.1371/journal.pone.0081842

Keiner, B., Maenz, B., Wagner, R., Cattoli, G., Capua, I. and Klenk, H.D., 2010. Intracellular distribution of NS1 correlates with the infectivity and interferon antagonism of an avian influenza virus (H7N1). *J. Virol.*, **84**: 11858-11865. https://doi.org/10.1128/JVI.01011-10

Kleinpeter, A.B., Jureka, A.S., Falahat, S.M., Green, T.J. and Petit, C.M., 2018. Structural analyses reveal the mechanism of inhibition of influenza virus NS1 by two antiviral compounds. *J. Biol. Chem.,* **293**: 14659-14668. https://doi.10.1074/jbc.RA118.004

Krug, R.M. and García-Sastre, A., 2013. The NS1 protein: a master regulator of host and viral functions. In: *Textbook of influenza,* 2nd edn. John Wiley and Sons, Ltd, USA. pp. 114-132. https://doi.org/10.1002/9781118636817.ch7

Krug, R.M., 2015. Functions of the influenza A virus NS1 protein in antiviral defense. *Curr. Opin. Virol.*, **12**: 1-6. https://doi.org/10.1016/j.coviro.2015.01.007

Kuo, R.L., Chen, C.J., Tam, E.H., Huang, C.G., Li, L.H., Li, Z.H., Su, P.C. and Wu, C.C., 2018. Interactome analysis of NS1 protein encoded by influenza A H7N9 virus reveals an inhibitory role of NS1 in host mRNA maturation. *J. Proteome Res.*, **17**: 1474-1484. https://doi.org/10.1021/acs.jproteome.7b00815

Kyte, J. and Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. *J. mol. Biol.*, **157**: 105-132. https://doi.org/10.1016/0022-2836(82)90515-0

Lazniewski, M., Dawson, W.K., Szczepińska, T. and Plewczynski, D., 2017. The structural variability

of the influenza A hemagglutinin receptor-binding site. *Brief. Funct. Genom.*, **17**: 415-427.

Li, X., Qi, W., He, J., Ning, Z., Hu, Y., Tian, J. Jiao, P., Xu, C., Chen, J., Richt, J. and Ma, W., 2012. Molecular basis of efficient replication and pathogenicity of H9N2 avian influenza viruses in mice. *PLoS One*, **7**: e40118. https://doi.org/10.1371/journal.pone.0040118

Lorch, M.S., Collado, M.S., Argüelles, M.H., Rota, R.P., Spinsanti, L.I., Lozano, M.E. and Goñi, S.E., 2019. Production of recombinant NS1 protein and its possible use in encephalitic flavivirus differential diagnosis. *Protein Expr. Purif.*, **153**: 18-25. https://doi.org/10.1016/j.pep.2018.08.008

Mahardika, G.N., Suartha, N., Kencana, G.A.Y., Suardana, I.B.K. and Budayanti, N.S., 2019. *Molecular dissection of the structural and nonstructural proteins of spanish-1918 Influenza, pandemic-2009, and bird flu viruses.* bioRxiv, pp. 585778. https://doi.org/10.1101/585778

Mänz, B., Brunotte, L., Reuther, P. and Schwemmle, M., 2012. Adaptive mutations in NEP compensate for defective H5N1 RNA replication in cultured human cells. *Nat. Commun.*, **3**: 802. https://doi.org/10.1038/ncomms1804

Paterson, D. and Fodor, E., 2012. Emerging roles for the influenza A virus nuclear export protein (NEP). *PLoS Pathog.*, **8**: e1003019. https://doi.org/10.1371/journal.ppat.1003019

Stothard, P., 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques*, **28**: 1102-1104. https://doi.org/10.2144/00286ir01

Tahir, M.S., Mehmood, D., Sultan, A.U., Saeed, M.H., Khan, A.R., Ansari, F. Salman, M.M. and Majeed, K.A., 2016. A modified strategy of multiplex RT-PCR for simultaneous detection of H5, H7, and H9 subtypes of avian influenza virus based on common forward oligo. *J. appl. Poult. Res.*, **25**: 322-327. https://doi.org/10.3382/japr/pfw014

Wang, S., Zhang, L., Zhang, R., Chi, X., Yang, Z., Xie, Y. Shu, S., Liao, Y. and Chen, J.L., 2018. Identification of two residues within the NS1 of H7N9 influenza A virus that critically affect the protein stability and function. *J. Vet. Res.*, **49**: 98. https://doi.org/10.1186/s13567-018-0594-y

Wu, Y., Wu, Y., Tefsen, B., Shi, Y. and Gao, G.F., 2014. Bat-derived influenza-like viruses H17N10 and H18N11. *Trends Microbiol.*, **22**: 183-191. https://doi.org/10.1016/j.tim.2014.01.010

Yamayoshi, S., Watanabe, M., Goto, H. and Kawaoka, Y., 2016. Identification of a novel viral protein expressed from the PB2 segment of influenza A virus. *J. Virol.,* **90**: 444-456. https://doi.org/10.1128/JVI.02175-15

Yan, Y., Du, Y., Wang, G. and Li, K., 2017. Non-structural protein 1 of H3N2 influenza A virus induces nucleolar stress via interaction with nucleolin. *Sci. Rep*., **7**: 17761. https://doi.org/10.1038/s41598-017-18087-2

Yang, H., Carney, P.J., Mishin, V.P., Guo, Z., Chang, J.C., Wentworth, D.E., Gubareva, L.V. and Stevens, J., 2016. Molecular characterizations of surface proteins hemagglutinin and neuraminidase from recent H5Nx avian influenza viruses. *J. Virol.*, **90**: 5770-5784. https://doi.org/10.1128/JVI.00180-16

Zhang, W., Zheng, W., Toh, Y., Betancourt-Solis, M.A., Tu, J., Fan, Y. Vakharia, V.N., Liu, J., McNew, J.A., Jin, M. and Tao, Y.J., 2017. Crystal structure of an orthomyxo virus matrix protein reveals mechanisms for self-polymerization and membrane association. *Proc. natl. Acad. Sci.*, **114**: 8550-8555. https://doi.org/10.1073/pnas.1701747114