Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2020.017

RESEARCH ARTICLE

# Mutational Analysis of Neuraminidase of Avian Influenza virus H9N2 Indicating the Cause of Hyper Pathogenicity in Poultry

Rehman Shahzad, Saba Irshad*, Anum Javaid and Tooba Yaqoob

Institute of Biochemistry and Biotechnology, Quaid e Azam campus, University of the Punjab, Lahore, Pakistan
*Corresponding author: saba.ibb@pu.edu.pk

ARTICLE HISTORY(19-391)

Received: August 29, 2019
Revised: December 27, 2019
Accepted: January 15, 2020
Published online: February 10, 2020

**Key words:**
H9N2
Neuraminidase (NA)
Pathogenicity
Protein model
Antigenic variations

**ABSTRACT**

H9N2 is a low pathogenic avian influenza virus that causes respiratory tract infection in poultry industry worldwide. Viral neuraminidase (NA) plays a vital role in pathogenicity due to its sialidase activity. In this study, H9N2 virus from diseased birds was isolated at GP laboratory, Lahore. Then viral NA gene was amplified, sequenced and analyzed using different bioinformatics tools. Results revealed that high pathogenicity of the isolated strains is due to G127S and S450L mutations in comparison to low pathogenic avian influenza virus and they have evolved from A/partridge/Pakistan/260/2015(H9N2) virus. The evolution of the isolated chicken virus from partridge virus shows its phylogenetic relationship. High pathogenicity of our isolates is attributed to the amino acid substitutions: G127S, I192V, V307M and S450L in the isolates with accession numbers MH105293, MH105294 & MH105295. Though H9N2 in Pakistani poultry is regarded as a low pathogenic influenza virus but our current study shows it to be highly pathogenic with high mortality >75%. By using SWISS MODEL software, NA protein model was also constructed, which is more stable than wild type with 119kJ/mol& 145kJ/mol energy values respectively. Furthermore, positions at G127S, I192V, V307M, S450L, D141N, V254I and V312I have significant antigenic variability, as well as more beta sheets than alpha helices affecting the protein structure, function and stability. This study will help the poultry vaccine manufacturers to design the customize *in silico* vaccine or neuraminidase inhibitors to meet the challenges arose in local poultry industry of Pakistan due to H9N2 viral infection.



**To Cite This Article:** Shahzad R, Irshad S, Javaid A and Yaqoob T, 2020. Mutational analysis of neuraminidase of Avian influenza virus H9N2 indicating the cause of hyper pathogenicity in poultry. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2020.017



## INTRODUCTION

H9N2 is an avian influenza virus which belongs to the *Orthomyxoviridae* family (Hutchinson *et al.,* 2010; Pflug *et al.,* 2017). It causes avian flu with low pathogenicity in various avian species such as duck, chicken, quail, partridge, pheasant, goose, pigeon, chukar, silky chicken, egret etc. and transmitted to pigs and even in humans (Huang *et al.*, 2013; Yu *et al.,* 2013; Wu *et al.,* 2018). Low pathogenic avian influenza viruses are spread over an extensive range of hosts from poultry species (Han *et al.,* 2019) and also humans in some Asian countries (Potdar *et al.,* 2019). H9N2 circulates in poultry globally (Jegede *et al.,* 2018) causing a huge economic loss to poultry industry. It is widespread in many countries across Asia, America, Europe and Africa including China, Pakistan, Saudi Arab, Iran, Hong Kong, Germany, Ireland, Italy, United States, United Kingdom South Africa etc. (Abolnik *et al.,* 2010; Lv, Wei *et al.,* 2015; Gíria, 2017).

H9N2 infection has been reported since 1990 in poultry with an abrupt increase in recent years (Alexander and Capua 2008; Capua and Alexander, 2009; Yang *et al.,* 2017). LPAIV H9N2 is pervasive in Pakistan poultry since 1996 and first outbreak was reported in 1998 (Lee *et al.,* 2016). In chickens, it is responsible for reduced egg production, immunity, and high mortality when associated with co-infection of other pathogens causing huge economic loss to the industry (Lee *et al.,* 2016). Although H9N2 is stated as a low pathogenic avian influenza virus but it is reported to escalate mortality due to co-infections (Wang *et al.,* 2019), (Zhang *et al.,* 2019).

Integral neuraminidase is a transmembrane, antigenic, surface glycoprotein studded on the viral membrane with less abundance. It is required for the release of virion from host cell after budding (Bouvier and Palese, 2008; Huang *et al.,* 2008; Klenk *et al.,* 2008). Neuraminidase is a tetrameric (240kDa) protein with 470 amino acid residues arranged in four spherical and identical subunits with central catalytic activity. It consists of mushroom like spikes and hydrophobic stalk attached to virion surface (Singh *et al.,* 2018). Neuraminidase prevents the binding of host cell sialic acid receptor and viral hemagglutinin (HA) through its sialidase activity. Thus, it plays role in replication, viral infectivity or pathogenicity, invading, budding, release and on the whole virus life cycle (Huang *et al.,* 2008; Lv, Wei *et al.,* 2015; Eichelberger *et al.,* 2018). Neuraminidase is one of the target proteins to treat influenza by using neuraminidase inhibitors which block its activity activity (Huang *et al.,* 2008; Singh *et al.,* 2018). Host antibodies and neuraminidase inhibitors inhibit viral replication by preventing the release of virus particles from infected virus producing host cells (Bouvier and Palese, 2008). Oseltamivir, peramivir and zanamivir are being used as neuraminidase inhibitors now a days. New inhibitors can be developed due to neuraminidase residues' conservancy. A118, A292, A371, Y408, D151 residues and other residues at glycosylation sites play important role in neuraminidase catalytic activity. Different mutations in these functional or structural catalytic active sites of neuraminidase, rapid evolution and genetic reassortments are emerging the resistant strains with less susceptibility to these inhibitors (Singh *et al.,* 2018).

The objective of present study was to isolate H9N2 viruses from infected chickens and to analyze the neuraminidase gene by sequencing & bioinformatics analysis to study molecular evolution, antigenic variations, pathogenic potential and protein modelling to reveal various mutations on structure of neuraminidase protein. This study is bioinformatically advantageous to poultry industries and *in silico* vaccine or neuraminidase inhibitors developments companies.

## MATERIALS AND METHODS

**Sample collection and preliminary tests:** Initially thirty tracheal samples from the diseased chickens were collected from GP laboratory of Grand Parent Poultry Private Limited® Lahore, Pakistan. The samples were subjected to rapid immune migration (RIM ™) test kit, followed by propagation in penicillin containing PBS solution to grow in nine-day old chick embryos narrowing down the sample number for RNA isolation and further experimentation to ten. Allantois fluid was collected from dead embryos' allantois cavity after candling. Spot test and Haem-agglutination (HA titre) were performed, detecting H9N2 Avian influenza viruses in three of the isolates.

**Viral RNA isolation and cDNA synthesis:** Further, RNA was extracted using QIAamp viral RNA Mini kit. For this; 5.6µl of RNA carrier, 200µl AL lysis buffer, 180µl cell culture sample, 20µl proteinase K, 250µl of 95% ethanol, 500µl washing buffer (AW1), 500µl AW2 (Washing buffer 2) and 60µl elution buffer (AE) were used. Following cDNA synthesis by reverse transcriptase polymerase chain reaction (RT-PCR), using Thermo scientific RevertAid First strand cDNA synthesis kit catalogue K1622 Lot 00442759.

**Multiplex PCR and gel exclusion:** cDNA was amplified by multiplex PCR using primers as mentioned in Table 1. 1.5% agarose gel was used for the above multiplexed cDNA isolates, followed by NA gene amplification using Hoffman's primers and temperature profile (Hoffmann *et al.,* 2001). Then NA was eluted, and bands were isolated by gel exclusion.

**Table 1:** Primers used in multiplex PCR for H9N2

| Primer name | Oligonucleotide Sequence | Length |
|---|---|---|
| Forward primer (AI) | GTAGAGGGCTATTTGGIGC | 19 |
| Reverse primer (H9) | CGTCTTGTATTTGGTCATCA | 20 |

**Sequencing:** Purified DNA samples were sent to Axil Scientific Pvt. Ltd (Singapore Science Park II, Singapore) for sequencing. All the nucleotide sequences were submitted in GenBank and accession numbers were allotted to these sequences.

**Bioinformatics analysis:** Amino acid sequences of the neuraminidase (NA) were deduced through nucleotide translation tool EMBL-EBI EMBOSS Transeq tool. Nucleotide sequences with their respective deduced amino acid sequences of NA segments from NCBI nucleotide BLAST results were retrieved on 17th march, 2018 (accession numbers & protein IDs mentioned in Table. 2). Followed by phylogenetic tree construction, multiple sequence alignment through CLUSTAL Omega Software (https://www.ebi.ac.uk/Tools/msa/clustalo) (Sievers *et al.,* 2011) and protein modeling of selected mutant and wildtype strains using SWISS-MODEL (https://swissmodel.expasy.org/), SAVES (http://servicesn.mbi.ucla.edu/SAVES), Discovery studio, FoldX and HOPE Server software (http://www.cmbi.ru.nl/hope/).

## RESULTS

Rapid immune test kit (Rapid immuno-migration (RIM TM) detects the presence of virus against the sensitized molecules attached to the selected analyte present in the sample. the principle of rapid test kit is based on the migration of analyte along a surface. The formation of the complex gives colored band for test and control. Thus, it detected Avian influenza virus in all samples. Further immunological testing revealed three isolates as positive for H9N2 viruses which was confirmed by polymerase chain reaction and Agarose gel electrophoretic analysis of cDNA showing bands for H9N2 (Fig. 1a) and NA gene was amplified for sequencing (Fig. 1b).

**Multiple sequence alignment:** Multiple sequence alignment revealed the conserved residues (G346, V307, T212 etc.) and substituted residues (D141N, I192V, S450L etc.) when our sample H9N2 positive conserved isolates were compared with first three H9N2 strains having 99% identity on BLAST as depicted in Table 3

and Fig. 2. Mainly four substitutions G127S, I192V, V307M and S450L in all our three isolates MH105293, MH105294 &MH105295 were observed, making them highly pathogenic in comparison to those first three low pathogenic viruses KU042920.1, KU042912.1 & KU042904.1 of the BLAST results. These MH105293, MH105294 & MH105295 accession numbers are allotted to our isolates by GenBank. Selected wild type MF280170.1 and mutant MH105295 strains were also aligned to find antigenic variations.

**Phylogenetic relationship:** Phylogeny of isolates revealed their evolutionary relationship in Fig. 3. They are clustered on adjacent branches of phylogenetic tree instead of dispersing far apart showing their close resemblance and similarity. They have evolved from MF280170.1 (A/partridge/Pakistan/260/2015(H9N2)) as their closest neighbor.

**Antigenic predictions:** In comparison of antigenic variations; G346, T212, S153, S77, T43 and L10 on the glycosylation sites, and residues at HB site (368 & 370) were shown to be conserved in our isolates. Novel mutations observed were T62I, A65V, G127S, D141N, I192V, V254I, V307M, V312I and S450L that mutating and evolving our isolates from their ancestor. No amino acid deletions at positions 46-50 were observed in mutant model sequence, significant for poultry adaptation (Butt *et al.,* 2010). Seven mutations were marked on monomer protein models.

**Protein modelling:** Protein IDs AVT44450, AVT44451 & AVT44452 were allotted to three novel isolates MH105293, MH105294 & MH105295 respectively. One of the three amino acid sequences of our isolates, "MH105295" i.e. more conserved was selected through sequence alignment and used for protein modeling. Through SWISS MODEL software, protein models were predicted for MH105295 as 'mutant' with qmean score -0.88 and ASB31536.1 as 'wild type' with qmean score -0.33. No apparent structural dissimilarity was observed. Wild type protein model with 98.65% residues, while mutant model with 98.19% residues having average 3D-1D score >=0.2 on SAVES software were verified. Ramachandran plot of mutant model showed 95.3% favorability of model (Fig.4). Alignment of mutant and

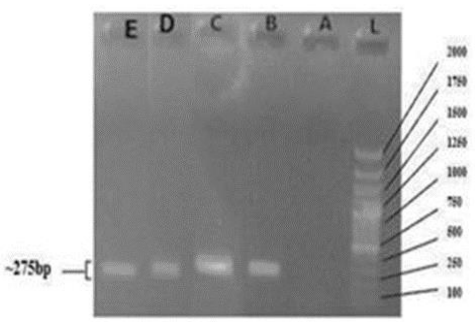
**Table 2:** Accession # of NA nucleotide sequences for phylogenetic tree and their respective Protein IDs; Red color showing accession numbers and protein IDs of our isolates; brown color showing accession # and protein IDs of H9N2 virus taken as ancestor of our isolates and as wildtype for protein modelling, ***** accession # and protein IDs of low pathogenic H9N2 virus having highest similarity index in BLAST results and used in comparison of high and low pathogenicity with our isolates

| Accession number | Protein ID | Year | Accession number | Protein ID | Year | Accession number | Protein ID | Year |
|---|---|---|---|---|---|---|---|---|
| KU042920.1* | ALP31335.1* | 2015 | KF800944.1 | AHA98350.1 | 2011 | FJ464616.1 | ACJ68657.1 | 2008 |
| KU042912.1* | ALP31323.1* | 2015 | JX456188.1 | AFP99300.1 | 2010 | FJ605502.1 | ACL79895.1 | 2005 |
| KU042904.1* | ALP31311.1* | 2015 | CY038452.1 | ACP50699.1 | 2007 | FJ464627.1 | ACJ68778.1 | 2006 |
| MF280168.1 | ASB31534.1 | 2015 | KX759096.1 | AOO54548.1 | 2005 | FJ464619.1 | ACJ68690.1 | 2007 |
| MF280169.1 | ASB31535.1 | 2015 | KF975482.1 | AHG26523.1 | 2010 | FJ464613.1 | ACJ68625.1 | 2006 |
| MF280170.1 | ASB31536.1 | 2015 | KF188348.1 | AGO17935.1 | 2005 | KM519975.1 | AIT38522.1 | 2008 |
| MF280171.1 | ASB31537.1 | 2015 | KF800942.1 | AHA98348.1 | 2011 | KM519967.1 | AIT38510.1 | 2008 |
| KY028778.1 | ASL68964.1 | 2016 | KF800945.1 | AHA98351.1 | 2011 | KF188360.1 | AGO17931.1 | 2005 |
| KF975479.1 | AHG26520.1 | 2010 | CY038468.1 | ACP50721.1 | 2008 | GQ120546.1 | ACR48929.1 | 2007 |
| KF975476.1 | AHG26517.1 | 2010 | CY038460.1 | ACP50710.1 | 2008 | FJ464625.1 | ACJ68756.1 | 2007 |
| KF975475.1 | AHG26516.1 | 2010 | JQ419729.1 | AFA51696.1 | 2009 | FJ464623.1 | ACJ68734.1 | 2007 |
| KF975477.1 | AHG26518.1 | 2010 | JX456189.1 | AFP99301.1 | 2011 | FJ464615.1 | ACJ68646.1 | 2007 |
| KF975478.1 | AHG26519.1 | 2010 | CY038476.1 | ACP50732.1 | 2008 | KM922686.1 | AIW60837.1 | 2008 |
| KF975480.1 | AHG26521.1 | 2010 | JX456190.1 | AFP99302.1 | 2011 | KF188300.1 | AGO17967.1 | 1999 |
| KF975484.1 | AHG26525.1 | 2010 | CY038484.1 | ACP50743.1 | 2007 | GQ120545.1 | ACR48928.1 | 2008 |
| KY028776.1 | ASL68962.1 | 2015 | CY038404.1 | ACP50633.1 | 2006 | GQ120539.1 | ACR48922.1 | 2008 |
| KF188404.1 | AGO17903.1 | 2005 | JQ419727.1 | AFA51694.1 | 2009 | FJ464624.1 | ACJ68745.1 | 2007 |
| JN540075.1 | AEM97780.1 | 2009 | KX759092.1 | AOO54544.1 | 2004 | AF508577.1 | AAQ04866.1 | 1999 |
| JN540059.1 | AEM97760.1 | 2006 | JQ419726.1 | AFA51693.1 | 2009 | CY108939.1 | AFA43946.1 | 2004 |
| JX465625.1 | AFQ60222.1 | 2010 | CY038436.1 | ACP50677.1 | 2006 | KF188261.1 | AGO17990.1 | 2005 |
| JN540067.1 | AEM97770.1 | 2008 | KF975481.1 | AHG26522.1 | 2010 | KF188326.1 | AGO17957.1 | 2005 |
| KX759098.1 | AOO54550.1 | 2005 | KX759090.1 | AOO54542.1 | 2004 | KF188338.1 | AGO17945.1 | 2005 |
| CY038428.1 | ACP50666.1 | 2006 | CY038412.1 | ACP50644.1 | 2005 | GQ120535.1 | ACR48918.1 | 2008 |
| JQ307202.1 | AEX30532.1 | 2011 | FJ464629.1 | ACJ68800.1 | 2006 | MH105293 | AVT44450 | 2017 |
| KF800943.1 | AHA98349.1 | 2011 | KF975483.1 | AHG26524.1 | 2010 | MH105294 | AVT44451 | 2017 |
| JQ307201.1 | AEX30531.1 | 2011 | FJ464628.1 | ACJ68789.1 | 2006 | MH105295 | AVT44452 | 2017 |

**Table 3:** Comparative analysis of substitutions and conservancy in stalk region, drug- binding pocket region and hemadsorbing sites of neuraminidase of our H9N2 influenza A virus 2017 isolates and three 2015 viruses; Red color showing our H9N2 recent isolates, while blue color denoting H9N2 LPAIV to which our isolates have been compared. Dashes (-) indicated similar or conserved amino acid residues

| Viruses | Stalk region | | | | | | Hemadsorbing site | | | Drug binding pocket region | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Deletions | | | Substitutions | | | | | | | | | | | | | |
| | 38 | 39 | 46-50 | 38 | 39 | 56 | 368 | 370 | 118-119 | 127 | 192 | 221 | 258 | 307 | 435 | 440 | 431-433 |
| A/pigeon/Pakistan/25A/2015(H9N2)* | No | No | No | N | R | V | K | S | RE | G | I | N | R | V | R | S | PQE |
| A/chicken/Pakistan/10A/2015(H9N2) | No | No | No | - | - | - | - | - | - | | | | - | - | G | - | - |
| A/chicken/Pakistan/13A/2015(H9N2 | No | No | No | - | - | - | - | - | - | | | | - | - | G | - | - |
| A/chicken/Pakistan/201/2017(H9N2) | No | No | No | - | W | - | - | - | - | S | V | S | - | M | - | - | - |
| A/chicken/Pakistan/237/2017(H9N2) | No | No | No | - | - | - | - | - | - | S | V | | K | M | - | - | - |
| A/chicken/Pakistan/238/2017(H9N2) | No | No | No | - | - | I | - | - | - | S | V | | - | M | - | - | - |

*H9N2 virus used as a reference strain. Abbreviations: N- Asparagine, R-Arginine, V-Valine, K-Lysine, S-Serine, E-glutamic acid, G-Glycine, I-Isoleucine, P-Proline, Q-Glutamine.



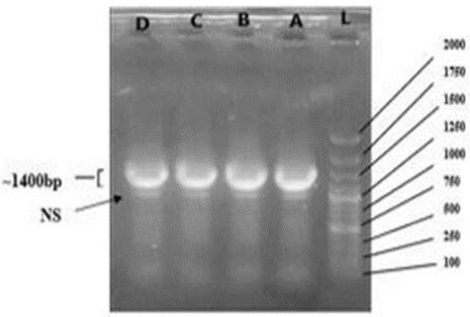




**Fig.1a:** Agarose gel electrophoresis of cDNA for H9N2 subtype detection;(L) Ladder 100bp plus; (A) negative control for H9N2; (C, D, E) H9N2 isolates showing band at ~275bp.

**Fig.1b:** Agarose gel electrophoresis of H9N2 isolates; showing bands at 1400bp of NA gene of H9N2 in (A, B, CF, D) wells for gene exclusion and sequencing (L) Ladder, (NS) Non-Structure gene band.

```
ALP31323.1      MNPNQKIIALGSASLTIATVCLLIQIAILATTMTLHFNRNEYTNSSENHVVHCDPVIIER   60
ALP31311.1      MNPNQKIIALGSASLTIATVCLLIQIAILATTMTLHFNRNEYTNSSENHVVHCDPVIIER   60
ALP31335.1      MNPNQKIIALGSASLTIATVCLLIQIAILATTMTLHFNRNEYTNSSENHVVHCDPVIIER   60
MH105293        MNPNQKIIALGSASLTIATVCLLIQIAILATTMTLHFNWNEYTNSSENHVVHCDPVIIER   60
MH105294        MNPNQKIIALGSASLTIATVCLLIQIAILATTMTLHFNRNEYTNSSENHVVHCDPVIIER   60
MH105295        MNPNQKIIALGSASLTIATVCLLIQIAILATTMTLHFNRNEYTNSSENHVVHCDPIIIER   60
                ************************************ ****************:****

ALP31323.1      NITEVVHLNGTIIKKESCPRAAEYKNWLKPQCRITGFVPFSKDNSIRLSAGGDIWITREP   120
ALP31311.1      NITEVVHLNGTIIKKESCPRAAEYKNWLKPQCRITGFVPFSKDNSIRLSAGGDIWITREP   120
ALP31335.1      NITEVVHLNGTIIKKESCPRAAEYKNWLKPQCRITGFVPFSKDNSIRLSAGGDIWITREP   120
MH105293        NITEVVHLNGTIIKKESCPRAAEYKNWLKPQCRITGFVPFSKDNSIRLSAGGDIWITREP   120
MH105294        NITEVVHLNGTIIKKESCPRAAEYKNWLKPQCRITGFVPFSKDNSIRLSAGGDIWITREP   120
MH105295        NITEVVHLNGTIIKKESCPRAAEYKNWLKPQCRITGFVPFSKDNSIRLSAGGDIWITREP   120
                ************************************************************

ALP31323.1      YVSCGLGKCYQFALGQGTTLNNKHSNGTTHDRSPYRTLLMSELGVPFHLGTKQVCIAWSS   180
ALP31311.1      YVSCGLGKCYQFALGQGTTLNNKHSNGTTHDRSPYRTLLMSELGVPFHLGTKQVCIAWSS   180
ALP31335.1      YVSCGLGKCYQFALGQGTTLNNKHSNGTTHDRSPYRTLLMSELGVPFHLGTKQVCIAWSS   180
MH105293        YVSCGLSKCYQFALGQGTTLNNKHSNGTTHDRSPYRTLLMSELGVPFHLGTKQVCIAWSS   180
MH105294        YVSCGLSKCYQFALGQGTTLNNKHSNGTTHDRSPYRTLLMSELGVPFHLGTKQVCIAWSS   180
MH105295        YVSCGLSKCYQFALGQGTTLNNKHSNGTTHDRSPYRTLLMSELGVPFHLGTKQVCIAWSS   180
                ******.*****************************************************

ALP31323.1      SSCHDGRAWLHICVTGDDRNATASIIYDGMLTDSIGSWSQNILRTQESECVCINGTCTVV   240
ALP31311.1      SSCHDGRAWLHICVTGDDRNATASIIYDGMLTDSIGSWSQNILRTQESECVCINGTCTVV   240
ALP31335.1      SSCHDGRAWLHICVTGDDRNATASIIYDGMLTDSIGSWSQNILRTQESECVCINGTCTVV   240
MH105293        SSCHDGRAWLHVCVTGDDRNATASIIYDGMLTDSIGSWSQSILRTQESECVCINGTCTVV   240
MH105294        SSCHDGRAWLHVCVTGDDRNATASIIYDGMLTDSIGSWSQNILRTQESECVCINGTCTVV   240
MH105295        SSCHDGRAWLHVCVTGDDRNATASIIYDGMLTDSIGSWSQNILRTQESECVCINGTCTVV   240
                **********:**************************.******************

ALP31323.1      MTDGSASGRADTRILFIREGRIIHISPLSGSAQHVEECSCYPRYPEVRCVCRDNWKGSNR   300
ALP31311.1      MTDGSASGRADTRILFIREGRIIHISPLSGSAQHVEECSCYPRYPEVRCVCRDNWKGSNR   300
ALP31335.1      MTDGSASGRADTRILFIREGRIIHISPLSGSAQHVEECSCYPRYPEVRCVCRDNWKGSNR   300
MH105293        MTDGSASGRADTRILFIREGRIIHISPLSGSAQHVEECSCYPRYPEVRCVCRDNWKGSNR   300
MH105294        MTDGSASGRADTRILFIKEGRIIHISPLSGSAQHVEECSCYPRYPEVRCVCRDNWKGSNR   300
MH105295        MTDGSASGRADTRILFIREGRIIHISPLSGSAQHVEECSCYPRYPEVRCVCRDNWKGSNR   300
                ****************:*******************************************

ALP31323.1      PVLYVNVIDYNIDSSYVCSGLVGDTPRSDDSSSSSNCRDPNNERGGPGVKGWAFDDGNDV   360
ALP31311.1      PVLYVNVIDYNIDSSYVCSGLVGDTPRSDDSSSSSNCRDPNNERGGPGVKGWAFDDGNDV   360
ALP31335.1      PVLYVNVIDYNIDSSYVCSGLVGDTPRSDDSSSSSNCRDPNNERGGPGVKGWAFDDGNDV   360
MH105293        PVLYVNMIDYNIDSSYVCSGLVGDTPRSDDSSSSSNCRDPNNERGGPGVKGWAFDDGNDV   360
MH105294        PVLYVNMIDYNIDSSYVCSGLVGDTPRSDDSSSSSNCRDPNNERGGPGVKGWAFDDGNDV   360
MH105295        PVLYVNMIDYNIDSSYVCSGLVGDTPRSDDSSSSSNCRDPNNERGGPGVKGWAFDDGNDV   360
                ******:*****************************************************

ALP31323.1      WMGRTIEKDSRTGYETFKVIGGWTMANSKSQINRQVIVDSGNRSGYSGIFSVEGKTCINR   420
ALP31311.1      WMGRTIEKDSRTGYETFKVIGGWTMANSKSQINRQVIVDSGNRSGYSGIFSVEGKTCINR   420
ALP31335.1      WMGRTIEKDSRTGYETFKVIGGWTMANSKSQINRQVIVDSGNRSGYSGIFSVEGKTCINR   420
MH105293        WMGRTIEKDSRTGYETFKVIGGWTMANSKSQINRQVIVDSGNRSGYSGIFSVEGKTCINR   420
MH105294        WMGRTIEKDSRTGYETFKVIGGWTMANSKSQINRQVIVDSGNRSGYSGIFSVEGKTCINR   420
MH105295        WMGRTIEKDSRTGYETFKVIGGWTMANSKSQINRQVIVDSGNRSGYSGIFSVEGKTCINR   420
                ************************************************************

ALP31323.1      CFYVELIRGRPQETGVWWTSNSIIVFCGTSGTYGTGSWPDGADISFMPI  469
ALP31311.1      CFYVELIRGRPQETGVWWTSNSIIVFCGTSGTYGTGSWPDGADISFMPI  469
ALP31335.1      CFYVELIRGRPQETRVWWTSNSIIVFCGTSGTYGTGSWPDGADISFMPI  469
MH105293        CFYVELIRGRPQETRVWWTSNSIIVFCGTLGTYGTGSWPDGADISFMPI  469
MH105294        CFYVELIRGRPQETRVWWTSNSIIVFCGTLGTYGTGSWPDGADISFMPI  469
MH105295        CFYVELIRGRPQETRVWWTSNSIIVFCGTLGTYGTGSWPDGADISFMPI  469
                ************** ************** ******************
```

**Fig. 2:** Multiple sequence alignment of 6 amino acid sequence; including 3 LPAI H9N2 strains (ALP31323.1, ALP31311.1, ALP31335.1) with maximum similarity index in BLAST results and 3 our novel isolates (MH105293, MH105294, MH105295). (*) showing conserved residues, (:) showing conservation between residues of strongly similar properties, (.) showing conservation between residues of weakly similar properties.

wild type sequences showed conservancy of most residues. Monomers of protein models were visualized and analyzed through Discovery studio software (Fig. 5a & 5b). Seven mutations affecting the folding, residues' interactions, activity and function of mutant protein are observed. G127S, D141N, I192V, V254I, V307M, V312I, and S450L are distributed throughout the monomer structure, mostly on the corners, instead of at central core of protein, with less alpha helices and more beta sheets. The energy value recorded for wildtype protein was 131.83kcal/mol while that for mutant protein was 119.05kcal/mol through FoldX software to analyze the stability of models.

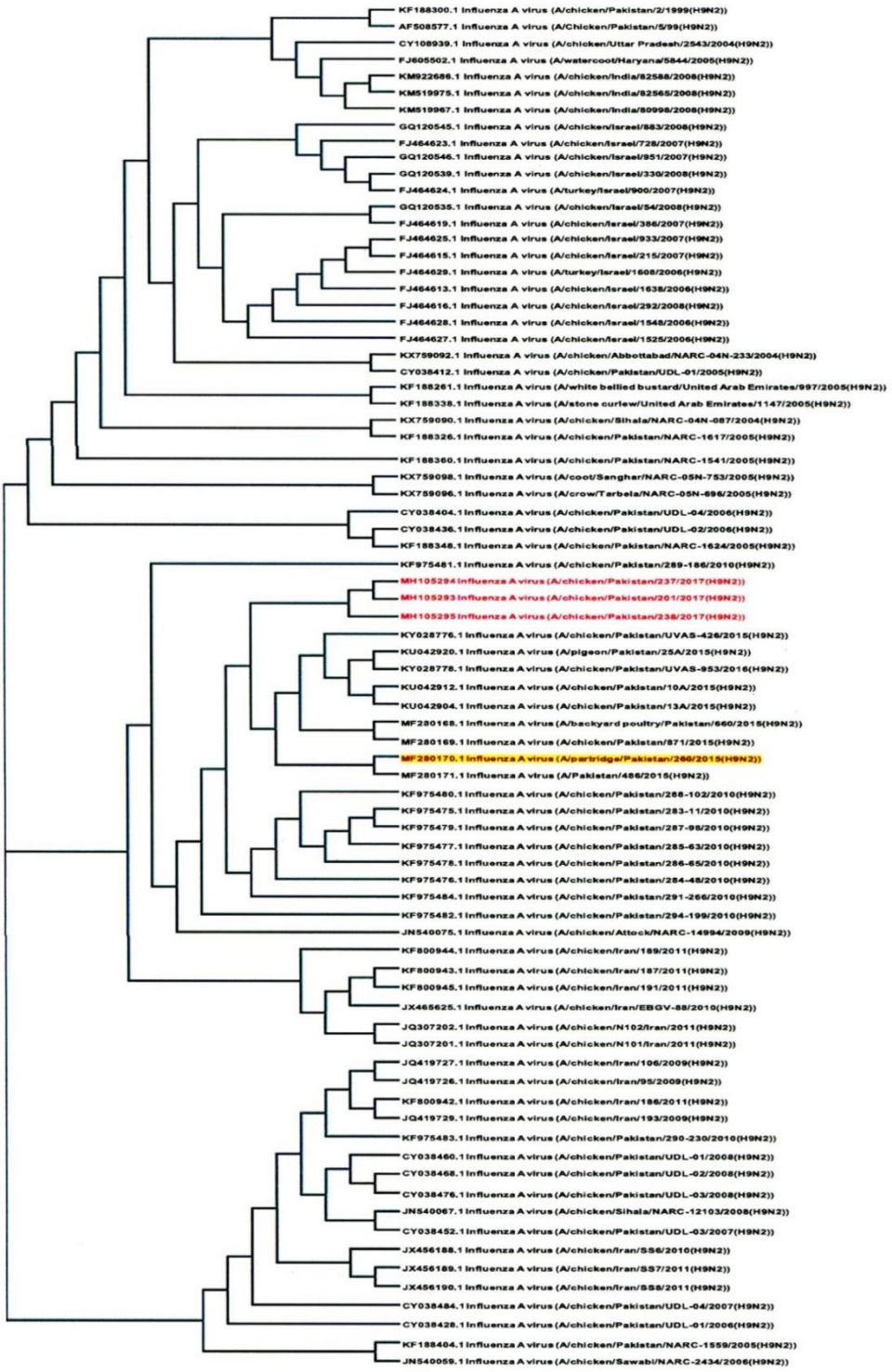



**Fig. 3:** Phylogenetic tree of 78 H9N2 strains; sequences marked with red color showing our 2017 isolates and brown color (A/partridge/ Pakistan/ 260/ 2015 (H9N2)) with yellow highlighted showing the ancestor of these three isolates.

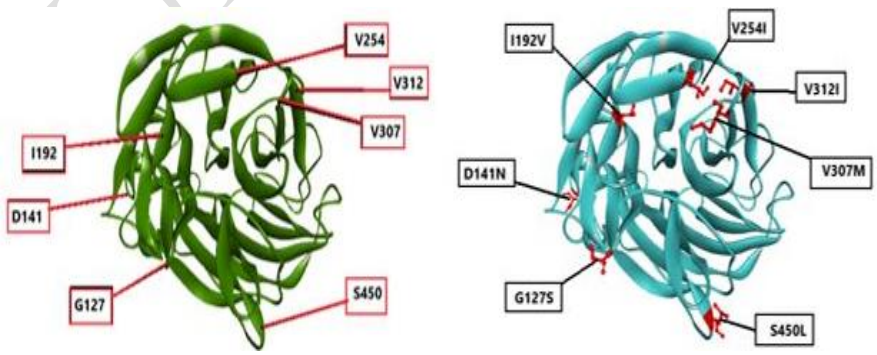



**Fig. 5a:** Wild type monomer model built by Discovery Studio software; (green color showing conserved regions, with flat beta sheets & coiled alpha helices, Red boxes showing amino acids present at particular positions being mutated in mutant model, S-Serine, G-Glycine, D-Aspartic acid, V-Valine, I-Isoleucine)

**Fig. 5b:** Mutant monomer model built by Discovery Studio Software;(Blue color showing conserved regions and red colored regions showing mutations, with flat beta sheets & coiled alpha helices in mutant model, Black boxes showing mutations at particular positions, L-leucine, S- Serine, G-Glycine, N-Asparagine, D-Aspartic acid, V-Valine, I-Isoleucine)

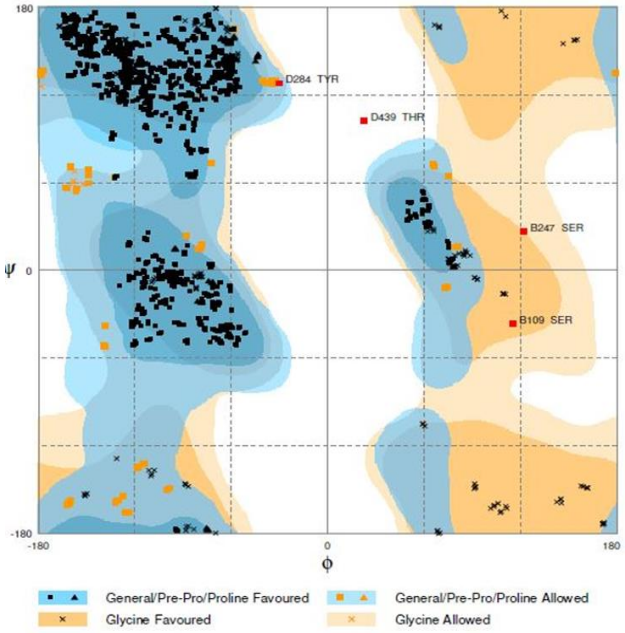



**Fig. 4:** Ramachandran plot of mutant model; Number of residues in favored region (~98.0% expected): 1467 (95.3%)**,** Number of residues in allowed region (~2.0% expected): 69 (4.5%).

## DISCUSSION

H9N2 avian influenza is prevalent in many countries, causing a prodigious economic loss to poultry industry by infecting the poultry (Gíria, 2017). Moreover, crossing the species barrier to instigate mild human infections (Lv, Wei *et al.,* 2015). Since, the viral genome is in segmented form it creates recombinant viruses with enhanced pandemic potentiality contributing grave dangers to poultry health (Song *et al.,* 2019; Wang, Li *et al.,* 2019). It is endemic in the poultry of Pakistan, since 1996 H9N2 AIV isolated in 2015 from different avian species of China and Pakistan were described low pathogenic (Sun and Liu 2015; Lee *et al.,* 2016). However, H9N2 in this current study from chicken in Pakistan have shown to be highly pathogenic with high mortality >75% in poultry. The potential cause of this enhanced pathogenicity is investigated in this study.

Mutations in hemadsorbing (HB) sites (368 & 370) of NA were reported to be like human pandemics of H3N2 & H2N2 (Matrosovich *et al.,* 2001; Aamir *et al.,* 2007; Butt *et al.,* 2010). While, in this study, this HB site (368 & 370) has not been mutated and remained conserved. D381G, K313E, L370S and E368D mutations in NA head region were associated with aerosol transmission (Lv, Wei *et al.,* 2015). Similarly, in our study, 313D, 368K, 370S, 381G were observed. The positions 370 & 381 had similar amino acid residues of the airborne characteristic and making them high pathogenic. Deletions at 399 position in HB site and 63-65 in stalk region was observed in strains isolated from ostriches (Wang *et al.,* 2018). Deletions in NA stalk regions at the positions 38 & 39 and no deletions at positions 46-50 were reported significant for poultry adaptation (Butt *et al.,* 2010), but no such deletions were observed here, instead R39W substitution was observed in MH105293.

The phylogenetic analysis showed similarity among NA of our mutant H9N2 viruses as they clustered on the same class. Mutant H9N2 viruses have been originated from A/partridge/Pakistan/260/2015(H9N2).

The evolution of H9N2 avian influenza viruses into different distinct antigenic- groups is associated with the antigenic drift leading to their pathogenic prevalence and immunization failure even by using vaccination (Sun and Liu 2015) also reassortments among/between different sub-types (Lv *et al.,* 2015).

Complete substitution of some amino acid residues at specific positions in our novel sequences have evolved them into a virus with different NA activity, functionality and structure leading to increased pathogenicity and this species transmission from partridge to chickens i.e. poultry adaptation.

NAs of human A/HK/1073/99, quail Qa/HK/G1/97 and Ck/Pk/2/99 possessed conserved seven amino acid residues G346, V307, T212, S153, S77, T43 and L10 on their glycosylation sites (Cameron *et al.,* 2000; Butt *et al.,* 2010). In this analysis of antigenic variations of isolates and ancestor, same conserved regions were observed except for V307 as here a substitution of V307M was observed.

The potent drug binding sites/pockets of neuraminidase protein are 371, 292, 274, 152, 151, 119, and 118 (Wu, Lin *et al.,* 2018). Mutant and wild type strains amino acid sequence alignment marked no mutations in hemadsorbing site and these antiviral drug binding pockets with conservancy in their enzyme active sites, showing mutant protein as resistant against sialidase inhibitors, thus more stable and virulent protein contributing increased virulence and NA activity of mutant strain. Residues at these drug binding sites observed are R371, R292, H274, R152, D151, E119 and R191.

R292 K mutation significantly reduced the susceptibility to a neuraminidase inhibitor oseltamivir by 2,523-fold and moderately reduced that to zanamivir. While, E119D mutation decreased the susceptibility to zanamivir by 415 fold and that to oseltamivir remained same. R292 K mutation distorts the oseltamivir binding (Kode *et al.,* 2019). And in our present study, such mutations were not observed to contribute in decreasing the susceptibility to neuraminidase inhibitors.

G127S mutation present in NA head region of neuraminidase protein monomer model can abolish protein flexibility, so disturbing protein function. D141N, I192V, V254I, V307M, V312I and S450L are also located in NA head region and disturb the protein function and activity; D141N & I192V through loss in interaction with other residues while V254I, V307M, V312I and S450L through bigger size and bumps, and S450L increases the hydrophobicity of protein (Venselaar *et al.,* 2010).

Structural amino acid residues of neuraminidase protein are E425, D293, E277, I222, D198, S179, W178, R156, E119 while, Functional residues are Y406, R371, R292, E276, R224, R152, D151, R118 which responsible for this enzyme's catalytic activity and remain conserved in all influenza A subtypes (Singh *et al.,* 2018). In this study of mutant monomer model, all the above mentioned functional and structural residues are found to be conserved. Increased energy value of mutant protein shows its increased stability in comparison to wild type protein through which it has been evolved.

**Conclusions:** *In silico* analysis of neuraminidase structure revealed novel mutations leading to high pathogenicity of recent H9N2 isolates with more stable protein model. This study is helpful for determining H9N2 viruses' pathogenicity and antigenic variations.

**Acknowledgements:** We are thankful to Grand Parent Laboratory Private Limited® Lahore, Pakistan for providing us samples and their platform to work for H9N2 virus isolation. We further acknowledge Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore for giving us an opportunity to do research.

**Authors contribution:** RS, AJ and TY executed the experiments and analyzed the results. AJ and RS prepared the manuscript and SI supervised the project.

## REFERENCES

Aamir U, Wernery U, *et al.,* 2007. Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. Virology 361:45-55.

Abolnik C, Gerdes GH, *et al.,* 2010. Phylogenetic analysis of influenza A viruses H6N8, H1N8, H4N2, H9N2, H10N7 isolated from wild birds, ducks and ostriches in South Africa from 2007 to 2009. Avian Dis 54:313-22.

Alexander D and Capua I, 2008. Avian influenza in poultry. World's Poult Sci J 64:513-32.

Bouvier NM and Palese P, 2008. The biology of influenza viruses. Vaccine 26:D49-D53.

Butt AM, Siddique S, *et al.,* 2010. Avian influenza A H9N2: computational molecular analysis and phylogenetic characterization of viral surface proteins isolated between 1997 and 2009 from the human population. Virology J 7:319.

Cameron K, Gregory V, *et al.,* 2000. H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. Virology 278:36-41.

Capua I and Alexander D, 2009. Avian influenza infection in birds: a challenge and opportunity for the poultry veterinarian. Poult Sci 88:842-6.

Eichelberger MC, Morens DM, *et al.,* 2018. Neuraminidase as an influenza vaccine antigen: a low hanging fruit, ready for picking to improve vaccine effectiveness. Current OpinImmunol 53:38-44.

Gíria MT, 2017. Polymerase basic protein 1 PB1 as a molecular determinant of fitness and adaptation in influenza a virus. http://hdl.handle.net/10451/27140.

Han L, He W, *et al.,* 2019. The evolution and molecular characteristics of H9N2 avian influenza viruses in Jiangxi of China. J Med Virol 91:711-6.

Hoffmann E, Stech J, *et al.,* 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146:2275-89.

Huang IC, Li W, *et al.,* 2008. Influenza A virus neuraminidase limits viral superinfection. J Virol 82:4834-43.

Huang R, Wang AR, *et al.,* 2013. Seroprevalence of avian influenza H9N2 among poultry workers in Shandong Province, China. Europ J Clin MicrobiolInfec Dis 32:1347-51.

Hutchinson EC, von Kirchbach JC, *et al.,* 2010. Genome packaging in influenza A virus. J Gen Virol 91:313-28.

Jegede A, Fu Q, *et al.,* 2018. H9N2 avian influenza virus retained low pathogenicity after serial passage in chickens. Canad J Vet Res 82:131-8.

Klenk HD, Matrosovich M, *et al.,* 2008. Avian Influenza: Molecular mechanisms of pathogenesis and host range. Anim Virus: Mol Biol 253. In: Mettenleiter TC, Sobrino F, editors. Anim Virus: Mol Biol. Wymondham, United Kingdom: Caister Academic Press; 2007. Pp:253-303.

Kode SS, Pawar SD, *et al.,* 2019. Selection of avian influenza A H9N2 virus with reduced susceptibility to neuraminidase inhibitors oseltamivir and zanamivir. Virus Res 265:122-6.

Lee DH, Swayne DE, *et al.,* 2016. H9N2 low pathogenic avian influenza in Pakistan 2012-2015. Vet Record Open 3:e000171.

Lv J, Wei L, *et al.,* 2015. Amino acid substitutions in the neuraminidase protein of an H9N2 avian influenza virus affect its airborne transmission in chickens. Vet Res 46:44.

Matrosovich MN, Krauss S, *et al.,* 2001. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology 281:156-62.

Pflug A, Lukarska M, *et al.,* 2017. Structural insights into RNA synthesis by the influenza virus transcription-replication machine. Virus Res 234:103-17.

Potdar V, Hinge D, *et al.,* 2019. Laboratory-confirmed avian influenza A H9N2 virus infection, India, 2019. Emer Infec Dis 25:2328.

Sievers F, Wilm A, *et al.,* 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Systems Biol 7:539.

Singh N, Anjum N, *et al.,* 2018. Combating influenza: natural products as neuraminidase inhibitors. Phytochem Rev18:69-107

Song Y, Zhang Y, *et al.,* 2019. Genetic characteristics and pathogenicity analysis in chickens and mice of three H9N2 avian influenza viruses. Viruses 11:1127.

Sun Y and Liu J, 2015. H9N2 influenza virus in China: a cause of concern. Protein Cell 6:18-25.

Venselaar H, Beek TA, *et al.,* 2010. Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. BMC Bioinfo 11:548.

Wang D, Wang J, *et al.,* 2018. Characterization of avian influenza H9N2 viruses isolated from ostriches Struthio camelus. Sci Reports 8:2273.

Wang Z, Li Z, *et al.,* 2019. Enhanced cross-lineage protection induced by recombinant H9N2 avian influenza virus inactivated vaccine. Vaccine 37:1736-42.

Wu Y, Lin J, *et al.,* 2018. The molecular characteristics of avian influenza viruses H9N2 derived from air samples in live poultry markets. Infec, Gen Evol 60:191-6.

Yang W, Punyadarsaniya D, *et al.,* 2017. Mutations during the adaptation of H9N2 avian influenza virus to the respiratory epithelium of pigs enhance sialic acid binding activity and virulence in mice. J Virol 91:e02125-02116.

Yu Q, Liu L, *et al.,* 2013. Risk perceptions for avian influenza virus infection among poultry workers, China. Emer infec Dis 19:313.

Zhang Q, Zuo Z, *et al.,* 2019. Contaminated feed-borne bacillus cereus aggravates respiratory distress post avian influenza virus H9N2 infection by inducing pneumonia. Sci Reports 9:7231.