**Title: Genomic and biological characterization of Newcastle disease viruses isolated from migratory Mallards *(Anas platyrhynchos)***

**Authors:** Momena Habib[1], Tahir Yaqub[1], Jawad Nazir[1], Wasim Shehzad[1], Aziz-ul-Rahman[1], Tayyebah Sohail[1], Nadia Mukhtar[2], Arsalan Mehboob[1], Muhammad Munir[3], Muhammad Zubair Shabbir[1*]

**Affiliations:** [1]University of Veterinary and Animal Sciences Lahore 54600, Pakistan;

[2]Institute of Public Health, Lahore 54600, Pakistan;

[3]The Pirbright Ash Road, Pirbright, Woking, GU24 0NF United Kingdom

**Corresponding author:**

Dr. Muhammad Zubair Shabbir

shabbirmz@uvas.edu.pk

**Running Title:** Genetic characterization of Mallard originated NDVs

**Abstract:**

Given the global evolutionary dynamics of Newcastle disease viruses (NDVs), it is imperative to continue extensive surveillance, routine monitoring and characterization of isolates originating from natural reservoirs (waterfowls). In this report, we isolated and characterized two virulent NDV strains from clinically healthy Mallard (*Anas platyrhynchos*). Both isolates had a genome of 15,192 nucleotides encoding six genes in an order of 3′-NP-P-M-F-HN-L-5′. The biological characteristics (mean death time: 49.5-50 hr, $EID_{50}10^{8.5}$ $ml^{-1}$) and presence of typical cleavage site in the fusion (F) protein (112R-R-Q-K-R↓F117) confirmed velogenic nature of these isolates. Phylogenetic analysis classified both isolates as members of genotype VII within class-II. Furthermore, based upon hypervariable region of F gene (375 nt), isolates showed clustering within sub-genotype VIIi. Similarity index and parallel comparison revealed a higher nucleotide divergence from commonly used vaccine strains; LaSota (21%) and Mukteswar (17%). A comparative residues analysis with representative strains of different genotypes, including vaccine strains, revealed a number of substitutions at important structural and functional domains of the F and hemagglutinin-neuraminidase (HN) proteins. Together, the results highlight consistent evolution among circulating NDVs and, therefore, warrant extensive surveillance of the virus in waterfowls to better elucidate epidemiology, evolutionary relationships and their impacts on commercial and backyard poultry.

**Key words:** Newcastle disease viruses, Genotype VII, Mallard, Molecular characterization, Genome, Pakistan

## Introduction

Newcastle disease (ND) is a highly contagious and economically devastating viral disease of birds. It is caused by a virulent strain of avian paramyxovirus serotype 1 (APMV-1). The virus belongs to genus *Avulavirus* within family *Paramyxoviridae* [33]. It carries a single stranded, negative sense, and non-segmented RNA genome of 15.2 kb in length that encodes at least six structural proteins including nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large polymerase (L) [5]. Based upon genome size and nucleotide sequence at cleavage site of F protein, all NDV strains are divided into two distinct classes (Class I and Class II) [7, 15]. Class-I viruses, isolated from waterfowls and live-bird markets, are comprised of avirulent strains within single serotype. NDV strains within Class-II are comprised of both virulent and avirulent strains, originated from a range of avian species including wild birds and poultry and are classified in at least eighteen genotypes (I-XVIII) [17]. On the basis of clinical signs in chicken and inferred amino acid sequence of F protein cleavage motif, NDV strains are grouped into three main pathotypes: lentogenic, mesogenic and velogenic [42]. The HN and F proteins are surface glycoproteins and are major determinants of antigenicity and pathogenicity of the virus. The F protein is synthesized as an inactive precursor ($F_0$), and is cleaved by the host cellular protease into disulfide linked N-terminus $F_1$ and C-terminus $F_2$ subunits. The cleavage of F protein is a prerequisite for a virus (NDV) to enter the host cell and, therefore, is considered as a major determinant of NDV pathogenicity in chickens [10, 28] . Virulent strains usually contain either Lysin (K) or Arginine (R) residues in repetition along with phenylalanine (112R/K-R-Q-R/K-R↓F117) that is cleaved by ubiquitous intracellular proteases and, therefore, leads to extensive systemic infections. Contrary to this, avirulent strains contain monobasic residues in the cleavage motif (112G/E-K/R-Q-G/E-R↓L117) which can be cleaved

only by extracellular trypsin like proteolytic enzymes and, therefore, results in localized and asymptomatic infection [3, 10].

Waterfowls, a natural reservoir of NDVs, have potential to spread virus in the environment and their susceptible hosts, resulting in frequent disease outbreaks and subsequent economic losses [4, 26, 47, 51]. While their movement from North to South across international boundaries, shedding of virus takes place at their resting places that may serve as a potential contamination source to multiple avian species including backyard and commercial poultry [29, 43, 51]. Routine surveillance and characterization of waterfowl-carrying pathogens (e.g. NDVs) has an immense importance to curtain disease spread in those areas which are considered endemic or naïve from infection. Nevertheless, there is a paucity of data on NDVs in waterfowl (Mallard ducks) and is limited to partial F gene based genomic analysis [6, 19, 30, 34, 48] which is insufficient to draw a reliable epidemiological conclusions. The NDV is endemic in Pakistan and causes enormous economic losses to the poultry industry, thereby necessitating the investigation of circulating viruses at a higher resolution. Here, we assessed genetic and biological characteristics of two virulent NDVs isolated from clinically healthy mallard during avian influenza (AIV) surveillance in migratory birds in Pakistan.

## Materials and Methods

### Sample collection

From June 2015 to September 2016, cloacal and oropharyngeal swabs were collected aseptically from clinically healthy mallard (*Anas platyrhynchos,* n= 213) at Chashma barrage. The barrage (32º 25′ N, 71º 22′ E) is built on the River Indus and serves as one of the major wildlife sanctuary for aquatic and terrestrial habitat under the provision of Punjab Wildlife Act, 1974. Precisely, comprised of 0.327 Mha, it is located in the provinces of Punjab and Khyber Pakhtun

Khwa (KPK) provinces of Pakistan. Major part of sanctuary lies in the Punjab province (district Mianwali) while a small proportion lies in Tehsil Lakki Marwat of Dera Ismail Khan district in KPK province. The barrage is considered as a wetland of international importance that accommodates a large variety of migratory and indigenous birds each year [1]. All samples were transferred into a separate cryovials (2.0 ml) containing 1.5 ml brain heart infusion medium with antimicrobials (Penicillin 2000IU/ml, Fungizone 1.5µg/ml and Gentamicin 200µg/ml) [38]. The cryovials were placed in chilled cooler with ice packs, transported to diagnostic laboratory and stored at -80 ºC until further processing.

**Virus isolation, biological titration and pathogenicity assessment**

Approximately 1.0 ml of each sample was filtered through 0.22µm syringe filter (EMD Millipore Millex™, Millipore Billerica MA, USA). A 0.2 ml of filtrate was inoculated in 9 day-old embryonated chicken eggs and processed as per protocol described previously [37]. The harvested allantoic fluid was tested for NDV by standard hemagglutination (HA), hemagglutination inhibition (HI) assays using specific antisera [2, 21] and F-gene based polymerase chain reaction (PCR) [36]. The mean infectious dose (EID50 $ml^{-1}$) and pathogenicity (mean death time, MDT) were assessed separately. The MDT of each isolate was assessed by a serial ten-fold dilution of infectious allantoic fluid where 0.1 ml of each dilutions ($10^{-1}$ to $10^{-10}$) was inoculated into allantoic cavity of the embryonated chicken eggs using 10 eggs per dilution [8, 41].

**Genome isolation, sequencing and phylogenetic analysis**

Viral RNA was extracted from allantoic fluid as per manufacturer's guidelines (QIAamp viral RNA mini kit, Qiagen®, Germany). The extracted genome (RNA) was subjected for complete genome sequencing using 22 pairs of primer reported previously [36]. Amplified PCR products

were purified as per manufacturer's procedure (Wizard® SV Gel and PCR Clean-Up System, Promega, Co., Madison, WI, USA) and sequenced with the same primer pairs in both directions using ABI PRISM Genetic analyzer 3130x1 version (Applied Biosystems, Foster City, CA, USA).

The consensus sequence of both isolates was assembled by Geneios® version 8.1.6 [25]. Representative strains of NDV, reported previously from Asia and other parts of world, were retrieved from GenBank database. These datasets were aligned through ClustalW methods in BioEdit® version 5.0.6 [20]. Deduced residue analysis of representative strains of each genotype was also analyzed using BioEdit. A phylogenetic consensus tree of complete F, HN and hypervariable region of the F genes was constructed by neighbor-joining method with 1000 bootstrap replicates through MEGA® version 6.0 software [52]. Pairwise Sequence Comparisons (PASC) was performed against whole virus genomes of genotypes (I-VIII and X) available in GenBank using MEGA6.0 software. To predict 3D structure of the F and HN protein of studied isolates, amino acid sequences were submitted to I-TASSER® (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) [59], and further analysis for substitutions were performed in PyMol® software (https://www.pymol.org/).

**Accession numbers**

The complete genome sequences of both NDV strains were submitted to the GenBank database and are available under accession numbers; KY967611 (Mallard-I/UVAS/Pak/2016) and KY967612 (Mallard-II/UVAS/Pak/2016).

**Results and discussion**

**Identification, genome sequence and evolutionary analysis**

We characterized two NDV strains originating from migratory birds, the mallard. Both isolates showed a titer of $\log 2^9$/50µl for HA in harvested allantoic fluid and showed an inhibition activity in HI assay. The F gene-based targeted amplification further confirmed both isolates as Newcastle disease viruses [2, 36, 37]. The mean death time (MDT) of both isolates was found to be 49.5-50 h with ± 4.4 standard deviation, that is considered typical for velogenic strains of NDVs [11]. The mean infectious dose (EID50 $ml^{-1}$) of both isolates was comparable and found to be $10^{8.5}$ $ml^{-1}$. The isolated viruses had a characteristic sequence of F protein cleavage motif (112R/K-R-Q-R/K-R↓F117). Taken together, these results revealed virulence nature of studied isolates.

Genome length of isolates was found to be 15,192 nucleotides and followed the "rule of six" with six structural proteins in an order of 3′-NP-P-M-F-HN-L-5′, a characteristic feature of paramyxoviruses [9, 27]. Complete F gene-based phylogenetic analysis showed clustering of under-study isolates within class-II and genotype VII with viruses reported previously from goose (AF431744 and DQ227246) **(**Fig. 1A**)**. The HN gene-based analysis also showed a clustering pattern similar to F gene where studied isolates clustered with genotype-VII-originated chicken isolate (HQ697259) (Fig. 1B). Although transmission experiments are required to assess the potential of isolates, clustering of study isolates with viruses originating from different hosts showed a potential inter-species transmission. In continuation to the observation made by previous studies in Pakistan [35, 37, 45, 46, 56], these findings indicate dominant circulation of genotype VII in the country. Analysis of hypervariable region of F gene (375 nt, between 4597-4972 nt of original genome) showed grouping of study isolates to sub-genotype VIIi (Fig. 1C), closely related to isolate reported from chicken in Indonesia (HQ697258-60) [57]. In short, the phylogenetic analysis suggested a close relatedness of study isolates to NDV strains reported

153 from Indonesia and China. This is imperative owing to the close genetic association with isolates
154 of Indonesian and Chinese origin from genotype VII (Chicken/BYP/Pakistan/2010) and highly
155 pathogenic avian influenza strain H5N1 reported previously in Pakistan [18, 55]. Genotype VII is
156 composed of genetically diverse groups of viruses that have resulted in a number of epidemics in
157 the Middle East, Asia, Africa and South America [39, 46, 50]. Added to this, the sub-genotype
158 VIIi has been isolated from multiple avian species including poultry and pet-birds in Asia,
159 Western Europe and Middle East [40, 58], and has the potential of panzootic intercontinental
160 spread [16]. There could be several reasons for intercontinental transmission of avian pathogens
161 including illegal transport of contaminated and infected materials across the borders. However,
162 the role of waterfowl in dissemination of these viruses cannot be ignored [36]. Therefore, there is
163 a need for extensive surveillance of wild birds to elucidate exposure to regions that are
164 previously considered naïve to NDV infections.
165 Nucleotide and amino acid sequences were compared between under-study isolates and
166 representative strains of different genotypes. Similarity indices revealed 91% nucleotide identity
167 between studied isolates and NDV strain ZJ1 (genotype VII). The highest homologies were
168 observed between investigated isolates and ZJ1 (AF431744) for M and L genes with 93%
169 nucleotides identity. A lowest nucleotide identity was observed with vaccine strain [LaSota
170 (79%) and Mukteswar (83%)] (Table 1). Though it requires challenge-protection studies to be
171 conducted, the genetic gap between field and vaccine isolates raises concerns for vaccine
172 efficacy, and also highlights the continuous evolutionary nature of NDV across different regions
173 of the world.
174 **Pathotype characterization based on the F- and HN-protein analysis**

The presence of three basic amino acid residues at position 113 (arginine), 115 (lysine) and 116 (arginine) and phenylalanine at position 117 indicate the virulent nature of our characterized isolates. The corresponding residues at $F_2$-protein and the N-terminus of $F_1$-protein of these isolates had cleavage motif (112RRQKR↓F117) similar to what has previously been documented for virulent NDVs [10, 35]. The six potential glycosylation sites including 85NRT87, 191NNT193, 366NTS368, 447NIS449, 471NNS473 and 541NNT543 were identified in F protein of these studied isolates [32]. Moreover, 12 cysteine (C) residues at position 27, 76, 199, 338, 347, 362, 370, 394, 399, 401, 424 and 523 were also observed. The glycosylation sites and cysteine residues are thought to be conserved [54] and have a known role in stability and maintenance of structure and virulence of F protein [44]. However, we observed variations in residue composition for glycosylation sites, as well as in number and position of cysteine residues. The C-residue at position 27 was exclusive to study isolates while other representatives of APMV-1 had at position 25 (Fig. 2).

Considering the importance of structure and function of F protein in the pathobiology of the NDV, the comparative residue analysis revealed seven neutralizing epitopes in under-study isolates that included at residues D72, E74, A75, K78, A79, and L343 and a stretch of amino acid positioned at 157SIAATNEAVHEVTDG171. We found no variation/substitution in neutralizing epitope of F-protein in study isolate and these sites were found to be conserved as reported previously [31, 60] (Fig. 2). However, we observed a number of substitutions in residue sequence at different sites that included one in signal peptide (1-31aa) at position 25 (Y→C), two in fusion peptide (117-142aa) at position 121 (V→I) and 125 (I→V/A), one in hydrophilic region a (HRa, 143-185) at 171 (S→A), two in HRb (268-299) at 272 (Y→N) and 288 (N→T), two in HRc region (471-500) at 494 (R→K) and 482 (T→E/A) and three in trans-membrane

198 domain (501-521) at 506 (A→V), 513 (F→V) and 513 (G→V). Though these substitutions may
199 have an effect, it requires further functional and biological investigations to fully evaluate the
200 importance of these substitutions in virus pathogenesis. Besides, a number of substitutions were
201 also found in non-conserved regions that were exclusive to isolates characterized in this study
202 (Fig. 2).
203 The deduced amino acid length for HN protein was found to be 571 residues, a feature
204 characteristic of virulent strains of NDV [31, 53]. The isolates reported here had conserved 13
205 cysteine residues at position 123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531 and 542,
206 11 sialic acid receptor binding sites at positions 174R, 175I, 258E, 299Y, 317Y, 401E, 416R,
207 498R, 526Y, 516R and 547E, and four residues responsible for neuraminidase activity at
208 positions 174R, 175I, 416R and 498R as reported previously [12, 32] (Fig. 3). Nevertheless, four
209 substitutions were noticed in transmembrane region (21 to 49 aa) at position 33 (M→T/I), 34
210 (I→V), 35 (M→V/I) 36 (I→T) and six in stalk region at position 50 (T→A), 57 (T→V/A), 58
211 (D→G), 61 (I→T), 77 (S→N) and 81 (I→V) (Fig. 3). The HN protein has seven important
212 antigenic sites (1, 2, 3, 4, 12, 14 and 23) that are involved in formation of a continuum in three
213 dimensional conformation of HN molecules [23]. We found three substitutions at position 263
214 (K→N), 514 (V→I) and 569 (N→D) within these antigenic sites. Substitutions in some of these
215 regions have been individually evaluated previously and are known to have a role in resulting
216 escape mutants and subsequent vaccine failure [14, 22, 24]. Four conserved glycosylation sites
217 were identified at position 119NNS121, 433NKT435, 481NHT483 and 538NKT540, whereas
218 one glycosylation site at position 508 was absent in both reported isolates (Mallard-
219 I/UVAS/Pak/2016, Mallard-II/UVAS/Pak/2016). The latter glycosylation site is considered non-
220 conserved among paramyxoviruses [13].

221 Since, both isolates carried similar residues-pattern (99.9% identity) for F and HN protein, only
222 one isolate (KY967612) was used in 3D structure prediction, and subsequent comparative
223 analysis with the vaccine strain, LaSota (AF077761). Significant substitutions in the fusion
224 peptide, hydrophilic regions and trans-membrane domain were observed for F protein (Fig. 4A).
225 Two substitutions in HRa region, positioned at 145 (N→K) and 176 (S→A), five in major trans-
226 membrane domain at 506 (A→V), 509 (V→I), 513 (F→V), 516 (V→I) and 520 (G→I), and two
227 in cytoplasmic tail at 552 (R→K) and 553(A→M) were observed. A few substitutions were also
228 noticed in globular head and stalk, trans-membrane domain, heptad repeat regions and antigenic
229 sites of HN protein (Fig. 4B). It is supposed that mutations in such particular regions could effect
230 on neuraminidase and fusion activity of protein [23, 49].

231 **Conclusion**

232 We determined genetic and biological characteristics of NDV strains isolated from asymptomatic
233 mallard ducks. Both isolates were found to be velogenic and clustered within class-II, genotype
234 VII and specifically sub-genotype VIIi. A number of substitutions were observed at site
235 considered important for structural and functional integrity of F and HN protein. Continuous
236 monitoring and surveillance programs, therefore, are suggested to effectively manage disease
237 along with potential evaluation of presented isolates for infectivity to commercial and backyard
238 poultry.

239 **Acknowledgment**

240 We acknowledge team members of Pak-UK collaborative research project entitled “Combating
241 avian influenza through systemic analysis of antigenic drift, genetic variation and development
242 of diagnostic tools and vaccines” (Grant reference: BB/L018853 funded by BBSRC/UK) for
243 providing study isolate and necessary data.

## Author's contribution

**Conceived and designed the work:** MH, TY, JN, MZS

**Performed the laboratory procedures and relevant methods:** MH, AR, MM, JN, TY, TS, MZS

**Data analysis:** MH, AR, MM, MZS

**Necessary laboratory resources and consumables:** TY, WS, JN, MZS

**Draft writing and editing:** MH, AR, MM, MZS

## Figure Legends

Fig. 1: Phylogenetic tree based on (**A**) complete F-gene (**B**) complete HN-gene (**C**) hypervariable region of the F-gene of mallard originated NDVs and previously characterized isolates representing different genotypes. The phylogenetic tree is constructed by neighbor-joining method in MEGA ver. 6.0 software. Bootstrap values (1000 bootstraps) are shown next to the branches. The study isolates are marked with solid black square box.

Fig. 2: Amino acid sequence alignment of complete F-gene of study isolates and other NDV representative strains from different genotypes (I-VIII and X) within Class II including vaccine strain. Important regions, both in epitope structure and function, are boxed with black. Substitutions are highlighted with red box. Positions of cysteine residues are indicated with red dot on top of residue position. Glycosylation sites are marked with green line at its position.

Fig. 3: Amino acid sequence alignment of complete HN-gene of study isolates and other NDV representative strains from different genotypes (I-VIII and X) within Class II including vaccine strain. Important regions, both in epitope structure and function, are boxed with black.

Substitutions are highlighted with red box. Positions of cysteine residues are indicated with red dot on top of residue position. Glycosylation sites are marked with green line at its position.

Fig. 4: Schematic presentation of predicted three-dimensional structure of F (**A**) and HN (**B**) proteins. The substitutions at particular sites are highlighted with different colors in comparison to the vaccine strain (LaSota; AY845400)

## Compliance with ethical standards

### Conflict of Interest

All authors declared no conflict of interest with data presented in this manuscript

### Ethics statement

All animal handling and sample processing procedures were carried out in strict accordance of institutional guidelines and regulations related to Animal Welfare and Health. The used procedures were approved by the Ethical Review Committee for use of Laboratory Animals (ERCULA) of the University of Veterinary and Animal Sciences, Lahore, Pakistan (Permit Number: ORIC/DR-70).

## References


1. Akbar M, Mushtaq-Ul-Hassan M, Mahmood-Ul-Hassan M, Hassan M (2009) Waterfowl diversity at Chashma barrage (Wildlife Sanctuary Mianwali) and Marala headworks (Game Reserve Sialkot), Pakistan during 1996-2005. International Journal of Agriculture and Biology 11:188-192
2. Akhtar S, Muneer MA, Muhammad K, Tipu MY, Rabbani M, Shabbir MZ (2016) Genetic characterization and phylogeny of pigeon paramyxovirus isolate (PPMV-1) from Pakistan. SpringerPlus 5:1295
3. Aldous E, Alexander D (2001) Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). Avian Pathology 30:117-128
4. Alexander D (1995) The epidemiology and control of avian influenza and Newcastle disease. Journal of comparative pathology 112:105-126
5. Alexander D, Senne D (2008) Newcastle disease, other avian paramyxoviruses, and pneumovirus infections, p 75–98. Diseases of poultry, 12th ed Blackwell Publishing, Ames, IA

6. Bahl A, Pomeroy B, Mangundimedjo S, Easterday B (1977) Isolation of type A influenza and Newcastle disease viruses from migratory waterfowl in the Mississippi flyway. Journal of the American Veterinary Medical Association 171:949-951
7. Ballagi-Pordany A, Wehmann E, Herczeg J, Belak S, Lomniczi B (1996) Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. Archives of virology 141:243-261
8. Bilal ESA, Elnasri IM, Alhassan AM, Khalifa KA, Elhag JI, Ahmed SO (2014) Biological Pathotyping of Newcastle Disease Viruses in Sudan 2008–2013. Journal of veterinary medicine 2014
9. Calain P, Roux L (1993) The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. Journal of virology 67:4822-4830
10. Collins M, Bashiruddin J, Alexander D (1993) Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. Archives of virology 128:363-370
11. Commission IOoEBS, Committee IOoEI (2008) Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees. Office international des épizooties
12. Connaris H, Takimoto T, Russell R, Crennell S, Moustafa I, Portner A, Taylor G (2002) Probing the sialic acid binding site of the hemagglutinin-neuraminidase of Newcastle disease virus: identification of key amino acids involved in cell binding, catalysis, and fusion. Journal of virology 76:1816-1824
13. Crennell S, Takimoto T, Portner A, Taylor G (2000) Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. Nature Structural & Molecular Biology 7:1068
14. Crennell S, Takimoto T, Portner A, Taylor G (2000) Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. Nature Structural & Molecular Biology 7:1068-1074
15. Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, Lomniczi B (2006) Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus research 120:36-48
16. Dimitrov KM, Bolotin V, Muzyka D, Goraichuk IV, Solodiankin O, Gerilovych A, Stegniy B, Goujgoulova GV, Silko NY, Pantin-Jackwood MJ (2016) Repeated isolation of virulent Newcastle disease viruses of sub-genotype VIId from backyard chickens in Bulgaria and Ukraine between 2002 and 2013. Archives of virology 161:3345-3353
17. Dimitrov KM, Ramey AM, Qiu X, Bahl J, Afonso CL (2016) Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). Infection, genetics and evolution 39:22-34
18. Fiebig L, Soyka J, Buda S, Buchholz U, Dehnert M, Haas W (2011) Avian influenza A (H5N1) in humans: new insights from a line list of World Health Organization confirmed cases, September 2006 to August 2010.
19. Friend M, Trainer D (1970) Serologic evidence of Newcastle disease in captive mallards and swans. Journal of wildlife diseases 6:130-135
20. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: Nucleic acids symposium series. [London]: Information Retrieval Ltd., c1979-c2000., pp 95-98
21. Health WOfA (2012) Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees. Biological Standards Commission, World Organization for Animal Health Paris, France
22. Iorio RM, Syddall RJ, Glickman RL, Kiel AM, Sheehan JP, Bratt MA (1989) Identification of amino acid residues important to the neuraminidase activity of the HN glycoprotein of Newcastle disease virus. Virology 173:196-204

23. Iorio RM, Syddall RJ, Sheehan JP, Bratt MA, Glickman RL, Riel AM (1991) Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition. Journal of virology 65:4999-5006
24. Iorio RM, Field GM, Sauvron JM, Mirza AM, Deng R, Mahon PJ, Langedijk JP (2001) Structural and functional relationship between the receptor recognition and neuraminidase activities of the Newcastle disease virus hemagglutinin-neuraminidase protein: receptor recognition is dependent on neuraminidase activity. Journal of virology 75:1918-1927
25. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647-1649
26. Kim LM, King DJ, Curry PE, Suarez DL, Swayne DE, Stallknecht DE, Slemons RD, Pedersen JC, Senne DA, Winker K (2007) Phylogenetic diversity among low-virulence Newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates. Journal of virology 81:12641-12653
27. Kolakofsky D, Roux L, Garcin D, Ruigrok RW (2005) Paramyxovirus mRNA editing, the 'rule of six'and error catastrophe: a hypothesis. Journal of General Virology 86:1869-1877
28. Lamb RA, Paterson RG, Jardetzky TS (2006) Paramyxovirus membrane fusion: lessons from the F and HN atomic structures. Virology 344:30-37
29. Lee E-K, Jeon W-J, Kwon J-H, Yang C-B, Choi K-S (2009) Molecular epidemiological investigation of Newcastle disease virus from domestic ducks in Korea. Veterinary microbiology 134:241-248
30. Lindh E, Ek-Kommonen C, Väänänen V-M, Alasaari J, Vaheri A, Vapalahti O, Huovilainen A (2012) Molecular epidemiology of outbreak-associated and wild waterfowl-derived Newcastle disease virus strains in Finland, including a novel class I genotype. Journal of clinical microbiology:JCM. 01427-01412
31. Liu X, Wan H, Ni X, Wu Y, Liu W (2003) Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985-2001. Archives of virology 148:1387-1403
32. Maminiaina OF, Gil P, Briand F-X, Albina E, Keita D, Andriamanivo HR, Chevalier V, Lancelot R, Martinez D, Rakotondravao R (2010) Newcastle disease virus in Madagascar: identification of an original genotype possibly deriving from a died out ancestor of genotype IV. PLoS one 5:e13987
33. Mayo M (2002) A summary of taxonomic changes recently approved by ICTV. Archives of virology 147:1655-1656
34. Miller PJ, Dimitrov KM, Williams-Coplin D, Peterson MP, Pantin-Jackwood MJ, Swayne DE, Suarez DL, Afonso CL (2015) International biological engagement programs facilitate Newcastle disease epidemiological studies. Frontiers in public health 3
35. Miller PJ, Haddas R, Simanov L, Lublin A, Rehmani SF, Wajid A, Bibi T, Khan TA, Yaqub T, Setiyaningsih S (2015) Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. Infection, genetics and evolution 29:216-229
36. Munir M, Linde A-M, Zohari S, Ståhl K, Baule C, Holm K, Engström B, Berg M (2010) Complete genome analysis of an avian paramyxovirus type 1 strain isolated in 1994 from an asymptomatic black-headed gull (Larus ridibundus) in southern Sweden. Avian diseases 54:923-930
37. Munir M, Abbas M, Khan MT, Zohari S, Berg M (2012) Genomic and biological characterization of a velogenic Newcastle disease virus isolated from a healthy backyard poultry flock in 2010. Virology journal 9:46
38. Pantin-Jackwood MJ, Miller PJ, Spackman E, Swayne DE, Susta L, Costa-Hurtado M, Suarez DL (2014) Role of poultry in the spread of novel H7N9 influenza virus in China. Journal of virology 88:5381-5390

39. Perozo F, Marcano R, Afonso CL (2012) Biological and phylogenetic characterization of a genotype VII Newcastle disease virus from Venezuela: efficacy of field vaccination. Journal of clinical microbiology 50:1204-1208
40. Radwan M, Darwish S, El-Sabagh I, El-Sanousi A, Shalaby M (2013) Isolation and molecular characterization of Newcastle disease virus genotypes II and VIId in Egypt between 2011 and 2012. Virus genes 47:311-316
41. Reed LJ, Muench H (1938) A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS12. American journal of epidemiology 27:493-497
42. Römer-Oberdörfer A, Werner O, Veits J, Mebatsion T, Mettenleiter TC (2003) Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. Journal of General Virology 84:3121-3129
43. Sakai K, Sakabe G, Tani O, Watanabe Y, Jahangir A, Nakamura M, Takehara K (2007) Characterization of Newcastle disease virus isolated from northern pintail (Anas acuta) in Japan. Journal of Veterinary Medical Science 69:1307-1311
44. Samal S (2012) Role of fusion protein in Newcastle disease virus pathogenesis. University of Maryland, College Park
45. Shabbir MZ, Abbas M, Yaqub T, Mukhtar N, Subhani A, Habib H, Sohail MU, Munir M (2013) Genetic analysis of Newcastle disease virus from Punjab, Pakistan. Virus genes 46:309-315
46. Shabbir MZ, Malik AN, Wajid A, Rehmani SF, Munir M (2013) Newcastle Disease Virus: Disease Appraisal with Global and Pakistan Perspectives.
47. Shengqing Y, Kishida N, Ito H, Kida H, Otsuki K, Kawaoka Y, Ito T (2002) Generation of velogenic Newcastle disease viruses from a nonpathogenic waterfowl isolate by passaging in chickens. Virology 301:206-211
48. Stanislawek W, Wilks C, Meers J, Horner G, Alexander D, Manvell R, Kattenbelt J, Gould A (2002) Avian paramyxoviruses and influenza viruses isolated from mallard ducks (Anas platyrhynchos) in New Zealand. Archives of virology 147:1287-1302
49. Stone-Hulslander J, Morrison TG (1999) Mutational analysis of heptad repeats in the membrane-proximal region of Newcastle disease virus HN protein. Journal of virology 73:3630-3637
50. Suarez D, Miller P, Koch G, Mundt E, Rautenschlein S (2012) Newcastle Disease, other Avian Paramyxoviruses, and Avian Metaneumovirus Infections. Diseases of Poultry, 13th eds Swayne DE, Glisson JR, McDougald LR, Nolan LK, Suarez D, Nair V Iowa USA Wiley-Blackwell
51. Takakuwa H, ITO T, Takada A, Okazaki K, Kida H (1998) Potentially virulent Newcastle disease viruses are maintained in migratory waterfowl populations. Japanese Journal of Veterinary Research 45:207-215
52. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution 30:2725-2729
53. Tsai H-J, Chang K-H, Tseng C-H, Frost KM, Manvell RJ, Alexanderc DJ (2004) Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996. Veterinary microbiology 104:19-30
54. Umali DV, Ito H, Suzuki T, Shirota K, Katoh H, Ito T (2013) Molecular epidemiology of Newcastle disease virus isolates from vaccinated commercial poultry farms in non-epidemic areas of Japan. Virology journal 10:330
55. Uyeki TM (2009) Human infection with highly pathogenic avian influenza A (H5N1) virus: review of clinical issues. Clinical infectious diseases 49:279-290
56. Wajid A, Dimitrov KM, Wasim M, Rehmani SF, Basharat A, Bibi T, Arif S, Yaqub T, Tayyab M, Ababneh M (2017) Repeated isolation of virulent Newcastle disease viruses in poultry and captive non-poultry avian species in Pakistan from 2011 to 2016. Preventive Veterinary Medicine 142:1-6

435 57. Xiao S, Paldurai A, Nayak B, Samuel A, Bharoto EE, Prajitno TY, Collins PL, Samal SK (2012)
436 Complete genome sequences of Newcastle disease virus strains circulating in chicken
437 populations of Indonesia. Journal of virology 86:5969-5970
438 58. Yang C-Y, Shieh HK, Lin Y-L, Chang P-C (1999) Newcastle disease virus isolated from recent
439 outbreaks in Taiwan phylogenetically related to viruses (genotype VII) from recent outbreaks in
440 western Europe. Avian diseases:125-130
441 59. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y (2015) The I-TASSER Suite: protein structure and
442 function prediction. Nature methods 12:7-8
443 60. Yusoff K, Nesbit M, McCartney H, Meulemans G, Alexander D, Collins M, Emmerson P, Samson A
444 (1989) Location of neutralizing epitopes on the fusion protein of Newcastle disease virus strain
445 Beaudette C. Journal of General Virology 70:3105-3109

446

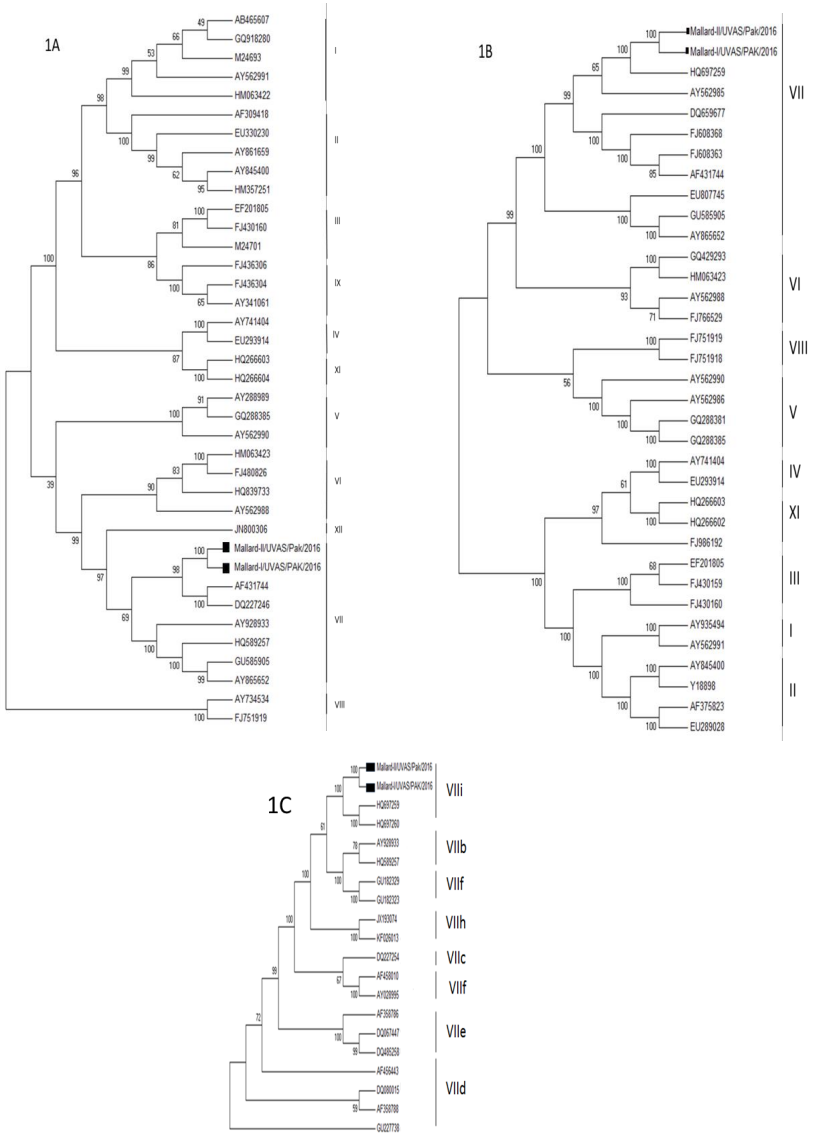
447

448

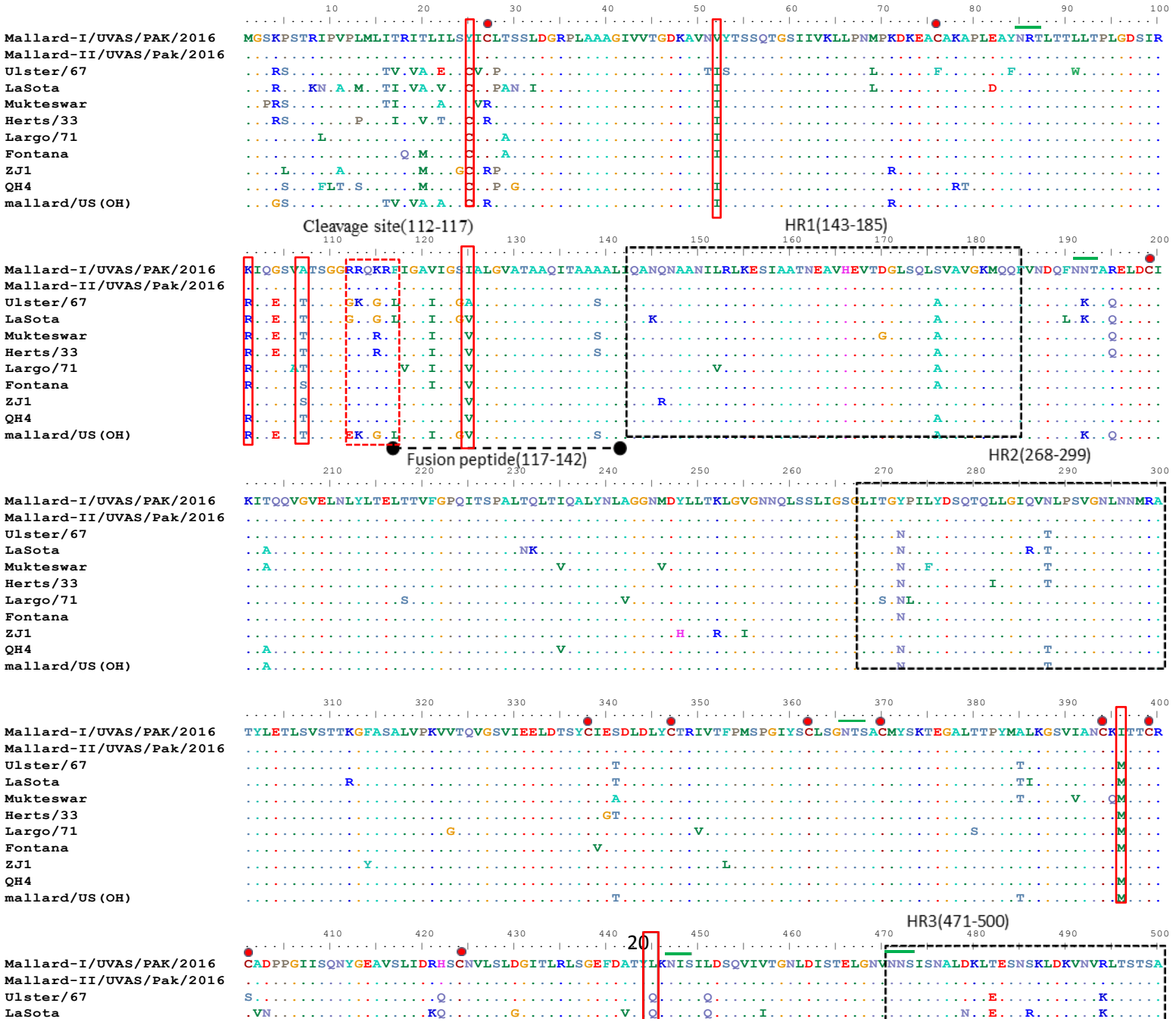
Table 1: The percent identity between nucleotide and amino acid sequences of complete genome and individual ORF of each gene of Mallard-II/UVAS/Pak/2016 (KY967612) isolate compared with representative NDV strains from other genotypes

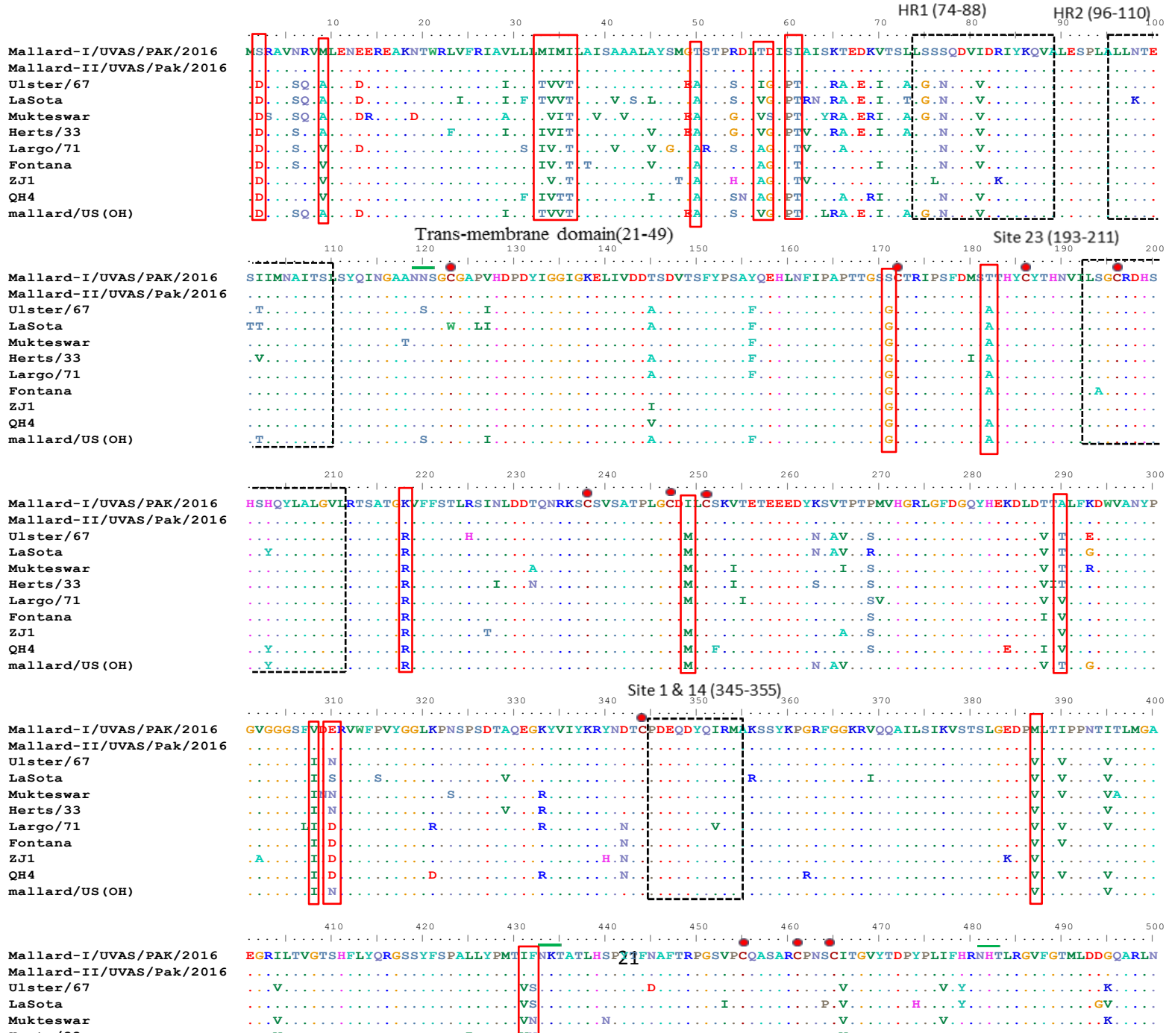
| **Reference strain** | **G-I (AY562991)** | **G-II (AY845400I)** | **G-III (EF201805)** | **G-IV (AY741404)** | **G-V (AY562990)** | **G-VI (AY562988)** | **G-VII (AF431744)** | **G-VIII (FJ751919)** | **G-X (GQ288377)** |
|---|---|---|---|---|---|---|---|---|---|
| Study isolate | KY967612 | KY967612 | KY967612 | KY967612 | KY967612 | KY967612 | KY967612 | KY967612 | KY967612 |
| Comparison | nt(%)a.a(%) | nt(%)a.a(%) | nt(%)a.a(%) | nt(%)a.a(%) | nt(%)a.a(%) | nt(%)a.a(%) | nt(%)a.a(%) | nt(%)a.a(%) | nt(%)a.a(%) |
| NP | 85 87 | 81 84 | 84 86 | 88 89 | 87 89 | 89 90 | 92 92 | 86 88 | 80 86 |
| P | 81 84 | 80 83 | 82 84 | 84 86 | 86 87 | 87 89 | 90 91 | 83 85 | 84 82 |
| M | 82 84 | 80 83 | 84 86 | 86 83 | 87 88 | 91 91 | 93 93 | 87 88 | 80 83 |
| F | 85 87 | 81 84 | 85 87 | 88 89 | 86 88 | 91 92 | 92 93 | 87 89 | 83 85 |
| HN | 83 85 | 77 81 | 83 85 | 86 87 | 87 88 | 89 90 | 92 92 | 85 86 | 79 83 |
| L | 85 87 | 83 85 | 86 88 | 87 89 | 89 90 | 91 92 | 93 93 | 88 89 | 84 86 |
| Complete genome | 82 - | 79 - | 83 - | 85 - | 86 - | 89 - | 91 - | 85 - | 80 - |

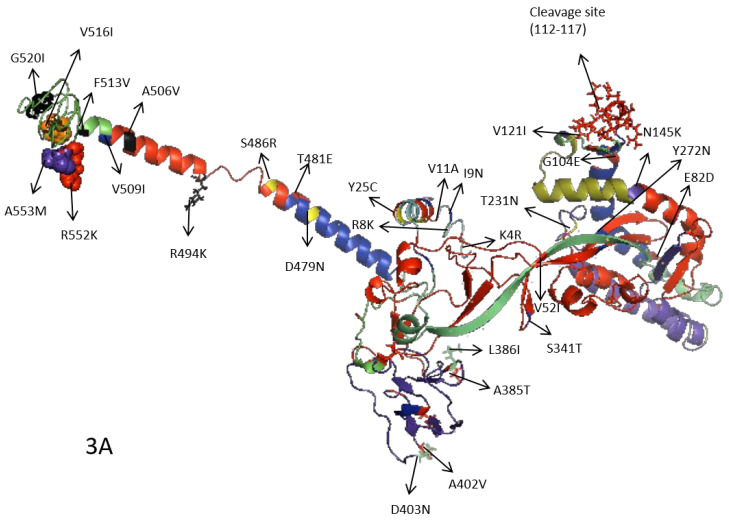
**Note:** Since, both study isolates (KX967611 and KX967612) were found to be 99.9% identical, a comparison between study isolate (KX967612) and NDVs representative strain from different genotypes is given.


1A
AB465607
GQ918280
M24693
AY562991
HM063422
AF309418
EU330230
AY861659
AY845400
HM357251
EF201805
FJ430160
M24701
FJ436306
FJ436304
AY341061
AY741404
EU293914
HQ266603
HQ266604
AY288989
GQ288385
AY562990
HM063423
FJ480826
HQ839733
AY562988
JN800306
Mallard-II/UVAS/Pak/2016
Mallard-I/UVAS/PAK/2016
AF431744
DQ227246
AY928933
HQ589257
GU585905
AY865652
AY734534
FJ751919
I
II
III
IX
IV
XI
V
VI
XII
VII
VIII
1B
Mallard-II/UVAS/Pak/2016
Mallard-I/UVAS/PAK/2016
HQ697259
AY562985
DQ659677
FJ608368
FJ608363
AF431744
EU807745
GU585905
AY865652
GQ429293
HM063423
AY562988
FJ766529
FJ751919
FJ751918
AY562990
AY562986
GQ288381
GQ288385
AY741404
EU293914
HQ266603
HQ266602
FJ986192
EF201805
FJ430159
FJ430160
AY935494
AY562991
AY845400
Y18898
AF375823
EU289028
VII
VI
VIII
V
IV
XI
III
I
II
1C
VIIi
VIIb
VIIf
VIIh
VIIc
VIIf
VIIe
VIId

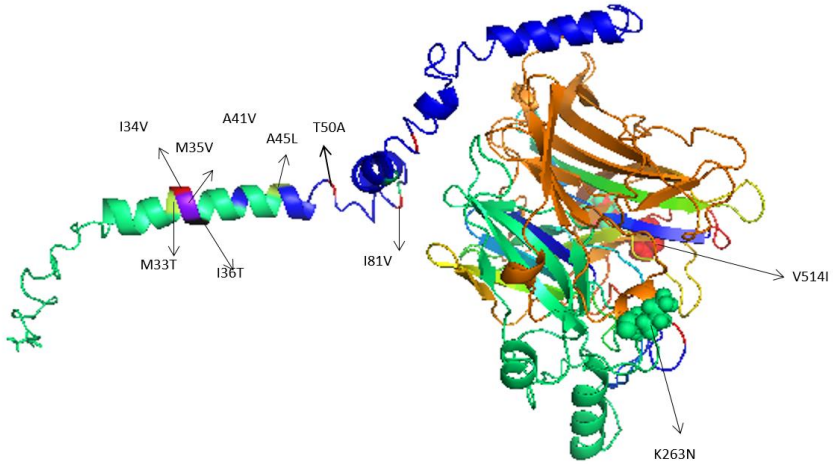
456


Mallard-I/UVAS/PAK/2016
Mallard-II/UVAS/Pak/2016
Ulster/67
LaSota
Mukteswar
Herts/33
Largo/71
Fontana
ZJ1
QH4
mallard/US(OH)
Cleavage site(112-117)
HR1(143-185)
Fusion peptide(117-142)
HR2(268-299)
HR3(471-500)


HR1 (74-88)
HR2 (96-110)
Trans-membrane domain(21-49)
Site 23 (193-211)
Site 1 & 14 (345-355)
Mallard-I/UVAS/PAK/2016
Mallard-II/UVAS/Pak/2016
Ulster/67
LaSota
Mukteswar
Herts/33
Largo/71
Fontana
ZJ1
QH4
mallard/US(OH)

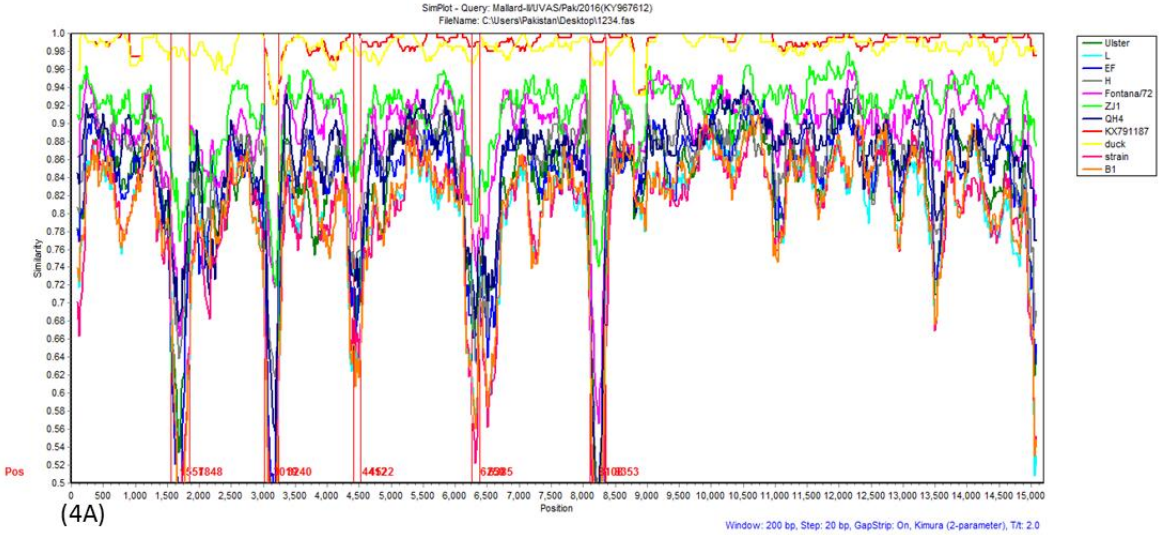

Cleavage site
(112-117)
V516I
G520I
F513V
A506V
S486R
T481E
V509I
A553M
R552K
R494K
D479N
Y25C
V11A
I9N
R8K
K4R
V121I
N145K
G104E
Y272N
E82D
T231N
V52I
S341T
L386I
A385T
A402V
D403N

3A

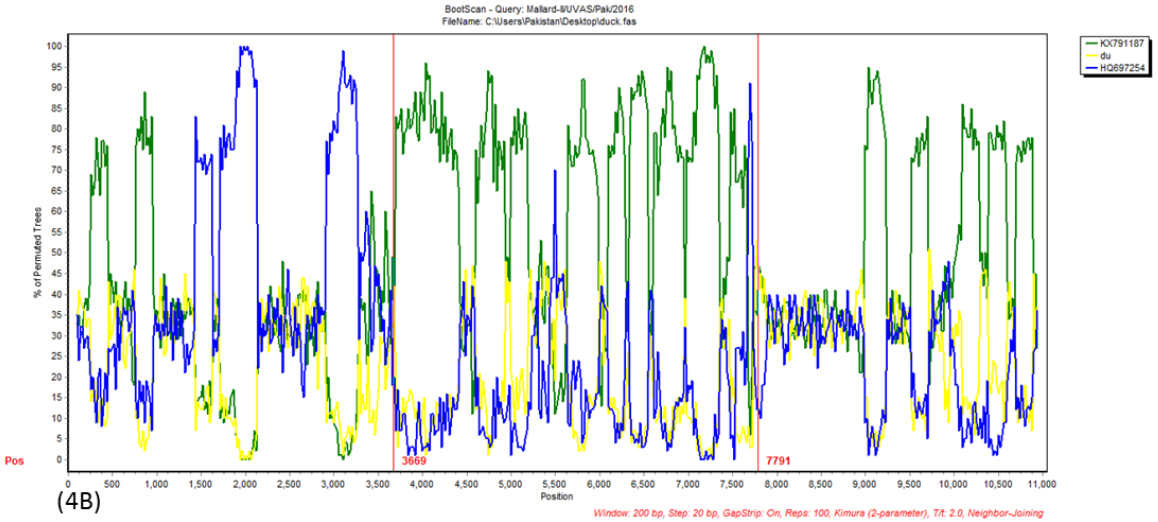

I34V
A41V
M35V
A45L
T50A
M33T
I36T
I81V
V514I
K263N

3B

459


SimPlot - Query: Mallard-II/UVAS/Pak/2016(KY967612)
FileName: C:\Users\Pakistan\Desktop\1234.fas
Similarity
Position
Pos
Ulster
L
EF
H
Fontana/72
ZJ1
QH4
KX791187
duck
strain
B1
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2.0

(4A)


BootScan - Query: Mallard-II/UVAS/Pak/2016
FileName: C:\Users\Pakistan\Desktop\duck.fas
% of Permuted Trees
Position
Pos
3669
7791
KX791187
du
HO697254
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2.0, Neighbor-Joining

(4B)

460

461