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

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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**

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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).

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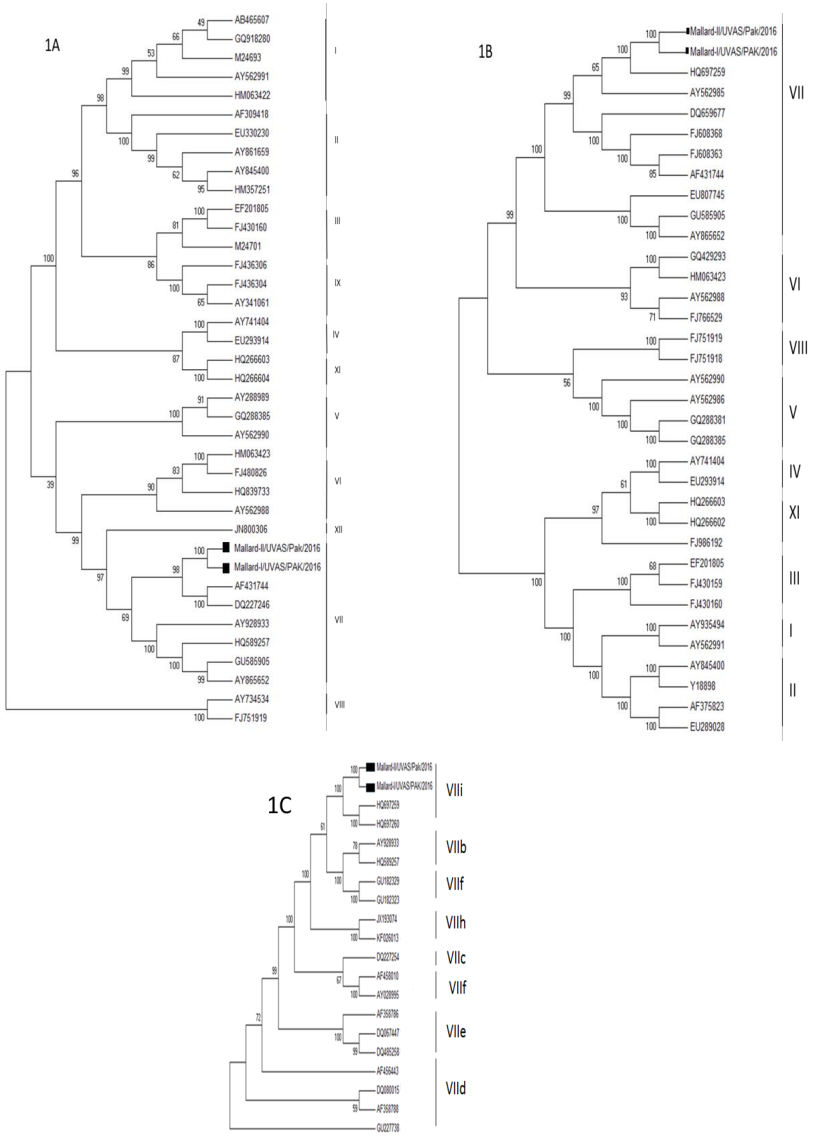
Table 1: The percent identity between nucleotide and amino acid sequences of complete genome and individual ORF of each gene of Mallard-II/UVAS/Pak/2016 (KY967612) isolate compared with representative NDV strains from other genotypes

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.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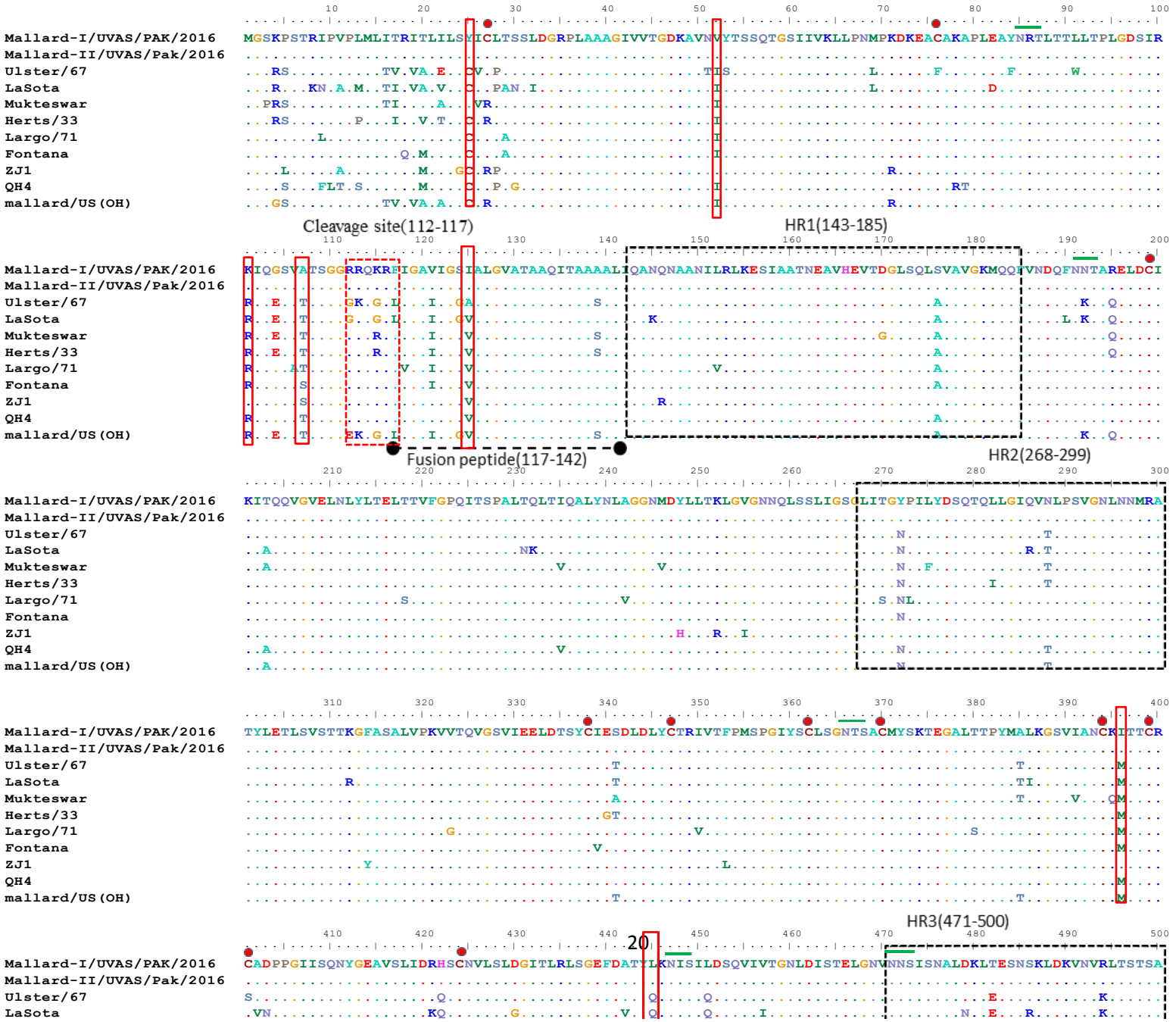
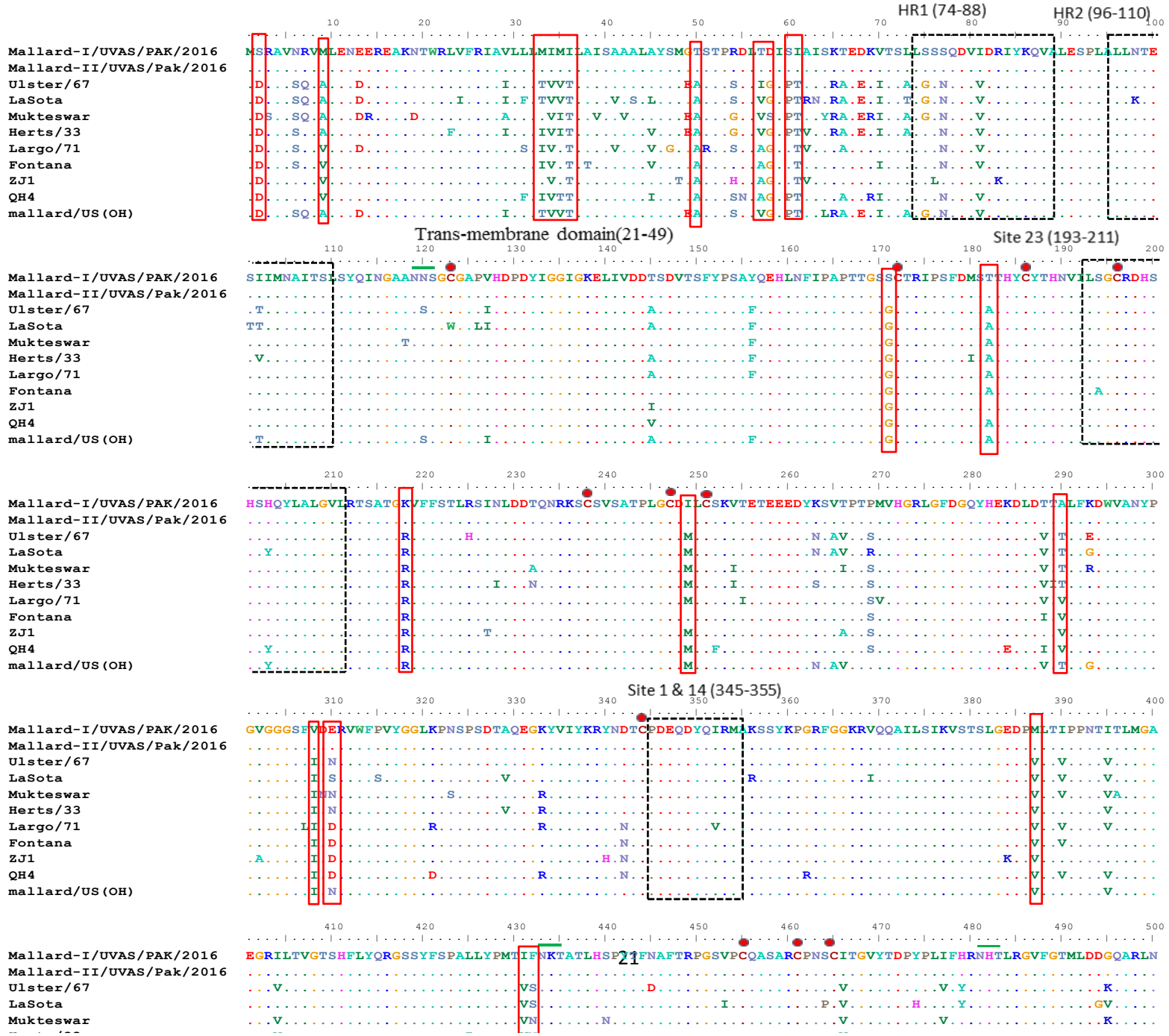
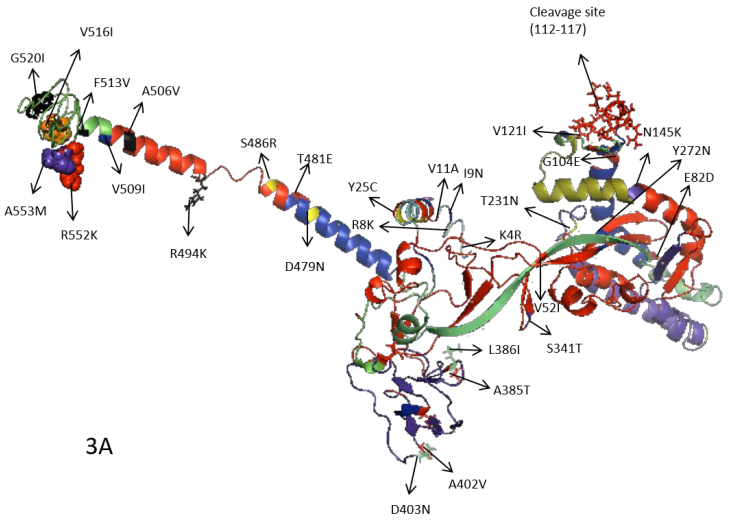
FJ430160
M24701
FJ436306
FJ436304
AY341061
AY741404
EU293914
HQ266603
HQ266604
AY288989
GQ288385
AY562990
HM063423
FJ480826
HQ839733
AY562988
JN800306
Mallard-II/UVAS/Pak/2016
Mallard-I/UVAS/PAK/2016
AF431744
DQ227246
AY928933
HQ589257
GU585905
AY865652
AY734534
FJ751919
I
II
III
IX
IV
XI
V
VI
XII
VII
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)
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G520I
F513V
A506V
S486R
T481E
V509I
A553M
R552K
R494K
D479N
Y25C
V11A
I9N
R8K
K4R
V121I
N145K
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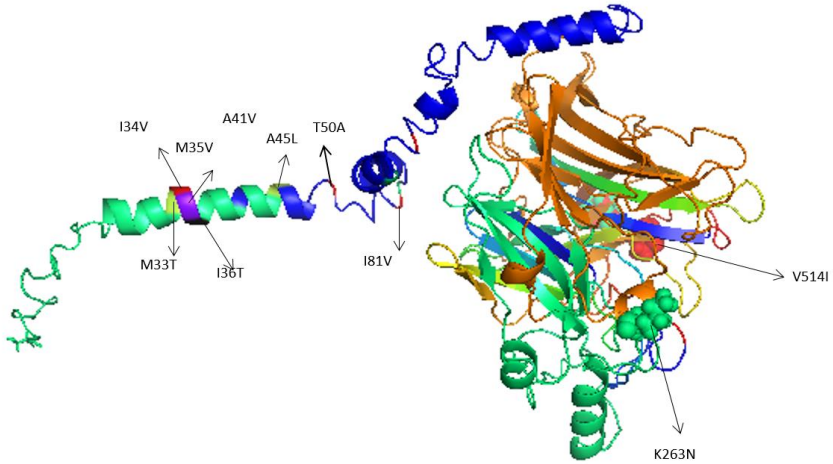
D403N

3A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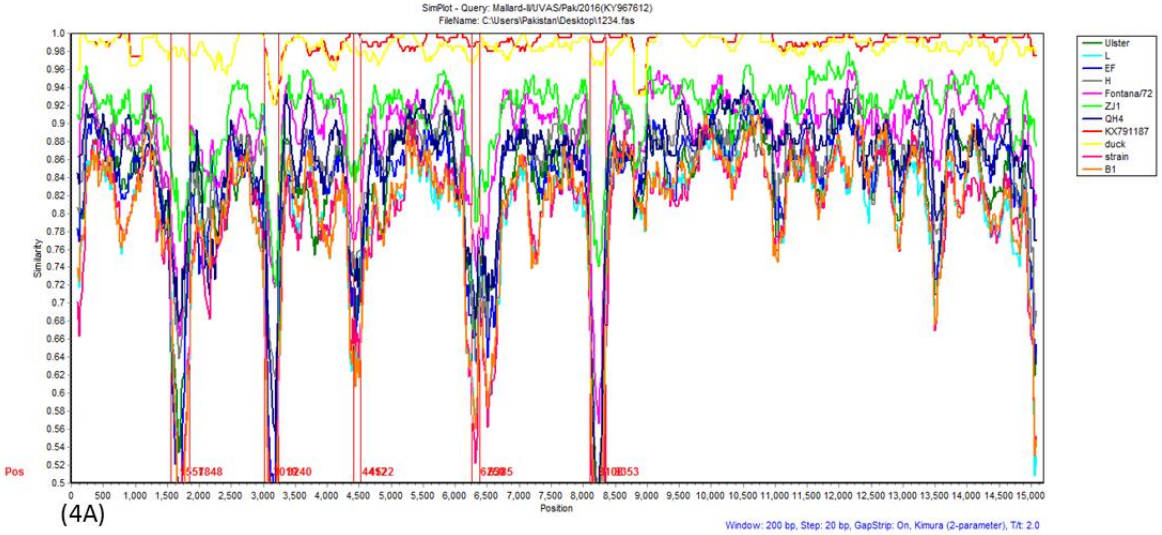
A41V
M35V
A45L
T50A
M33T
I36T
I81V
V514I
K263N

3B

459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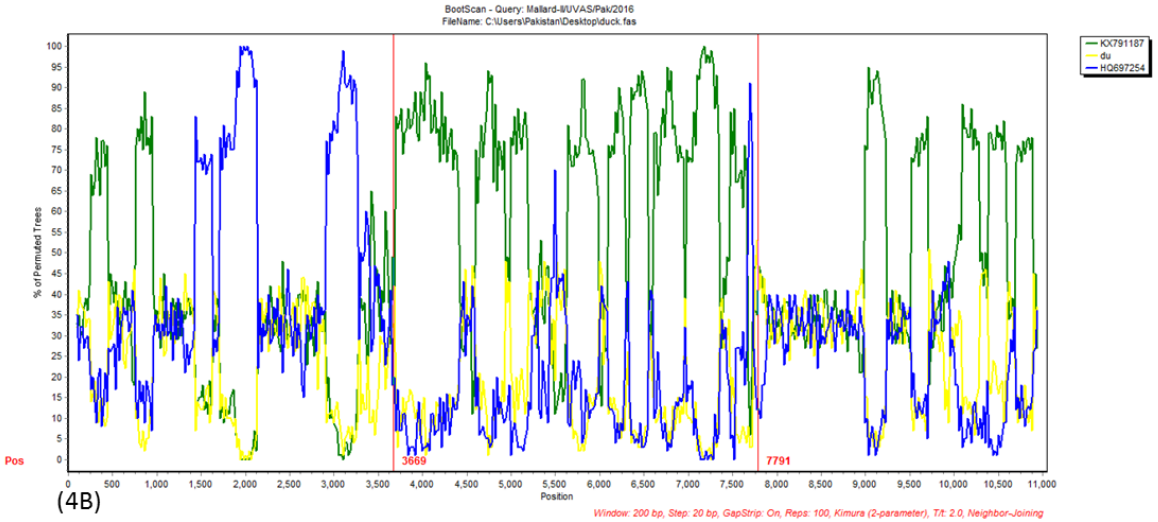
Similarity
Position
Pos
Ulster
L
EF
H
Fontana/72
ZJ1
QH4
KX791187
duck
strain
B1
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2.0

(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)

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