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1 **Phylogenomics and Infectious Potential of Avian Avulaviruses specie-type 1 Isolated**
2 **from Healthy Green-winged Teal (*Anas carolinensis*) from a Wetland Sanctuary of**
3 **Indus River**

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22 **Running Title:** Genetic characterization and evolutionary analysis of AAvV 1

SUMMARY

Given the importance of *Avian avulaviruses* (AAvVs) in commercial poultry, continuous monitoring and surveillance in natural reservoirs (waterfowls) is imperative. Here, we report full genomic and biological characterization of two virulent AAvVs isolated from apparently asymptomatic Green-winged teal (*Anas carolinensis*). Genetic characterization [(genome length, coding potential and presence of typical cleave motif ($_{112}$RRQKR↓F$_{117}$)] and biological assessment (HA: log2^9, mean death time: 49.2-50 hrs, $10^{-6.51}$ EID$_{50}$/0.1mL and 1.5 Intracerebral pathogenicity index or ICPI value) revealed virulence of both isolates. Phylogenetic analysis of complete genome and hypervariable region of the fusion (*F*) gene revealed clustering of both isolates within class-II strains in close-association with domestic poultry-origin AAvVs representing genotype VII and sub-genotype VIIi. The inferred residue analysis of *F* and haemagglutinin-neuraminidase genes showed a number of substitutions in critical domains compared to reference strains of each genotype (I-XVIII). The isolates showed a high nucleotide resemblance (99%) with strain isolated previously from backyard poultry, however showed a variable similarity (16.1% to 19.3%) with most commonly used vaccine strains; Mukteswar (EF201805) and LaSota (AF077761). In accordance to pathogenicity assessment and horizontal transmission, the clinical and histopathological observations in experimental chicken indicated velogenic viscerotropic nature of AAvV 1 isolates. Taken together, study concludes evolutionary nature of AAvVs and their potential role in disease occurrence, necessitating continuous surveillance of migratory/aquatic fowls to better elucidate infection epidemiology and potential impacts on commercial poultry.

Key words: Virulent AAvV 1; Green-winged teal; Biological characteristics; Genotypic characterization; Complete genome

Abbreviation: AAvVs = Avian avulaviruses; AAvV 1= Avian avulavirus 1; HA= Haemagglutination assay; ICPI= Intra-cerebral pathogenicity index; MDT= Mean death time;

48 EID50= Embryo infective dose; ND= Newcastle disease; *F*= Fusion; *HN*= Haemagglutinin-
49 neuraminidase; KPK= Khyber Pakhtun Khwa; HI= Haemagglutination inhibition; RT-PCR=
50 Reverse transcriptase polymerase chain reaction; NCBI= National centre for biotechnology
51 information; ORF= Open reading frame; HR= Hydrophobic heptad repeat region; GARD=
52 Genetic algorithm for recombination detection.

53 **INTRODUCTION**

54 Avian avulaviruses (AAvVs) are enveloped, mono-partite, negative sense and single-stranded
55 RNA viruses, and are classified into the genus *Avulavirus* and family *Paramyxoviridae* (6).
56 Newcastle disease (ND), caused by *Avian Avulavirus* 1 (previously known as avian
57 paramyxovirus 1), is a highly contagious disease of multiple avian species including
58 commercial poultry, and are posing significant economic impacts worldwide (5). The whole
59 genome of AAvV-1 is either 15186, 15192 or 15198 nucleotides in length and encodes six
60 structural proteins in an order of 3′-NP-P-M-F-HN-L-5′ (5, 26). Based on the pathogenicity,
61 AAvV 1 are categorized into velogenic, mesogenic, lentogenic or avirulent (8). The presence
62 of mono- or poly-basic amino acids in fusion (F) protein is considered a key determinant of
63 virulence (13). Based on clustering patterns, all AAvV 1 strains can be classified into two
64 classes within a single serotype; Class-I contains at least nine genotypes of avirulent AAvVs
65 and Class-II consists of at least eighteen genotypes of virulent AAvVs (15). Based on partial
66 sequence of the *F* gene (375bp between 4597-4972 nts), genotypes VI and VII can be further
67 classified into eight (a-h) and eleven (a–k) further sub-genotypes, respectively (17, 32-34, 36,
68 49), which depict a high level of genetic heterogeneity and distribution of multiple strains of
69 AAvVs in the environment (22, 41, 66).

70 Being a natural reservoir for AAvVs, aquatic and/or wild waterfowls have potential to
71 shed virus in the environment for an extended period of time and, therefore, could be potential
72 sources of disease transmission to highly susceptible and commercially valuable hosts such as

73 chickens (49). Virulent AAvVs have previously been reported from clinically healthy green-

74 winged teal (39, 44); nevertheless, there is a paucity of higher resolution characterization of

75 genome, evolutionary dynamics and occurrence of potential recombination events across the

76 length of viral genome. While aquatic bird-origin AAvV strains are reported, the

77 characterization is mainly attributed to partial sequencing of hypervariable region of *F* gene

78 (24, 25, 27). Additionally, biological assessments of AAvVs isolated from teal have not been

79 assessed before. Therefore, owing to proven roles in virus transmission and potential threats to

80 commercial vulnerable poultry, it is imperative to investigate the evolutionary and infectious

81 potential of these viruses from waterfowl. In this study, we determined genetic and biologic

82 assessments of two AAvVs isolated from clinically healthy green-winged teal from Pakistan.

83 **MATERIALS AND METHODS**

84 **Ethics statement**

85 This study was carried out in strict accordance with the recommendations of the Guide

86 for the Care and Use of Laboratory Animals by National Institutes of Health and Animal

87 Research Council (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-

88 laboratory-animals.pdf). The swab sampling, embryonated eggs inoculation and other essential

89 protocols were approved by the Ethical Review Committee for the Use of Laboratory Animals

90 (ERCULA) of University of Veterinary and Animal Sciences, Lahore vide permit number

91 ORIC/DR-70 dated March 15, 2015.

92 **Sample collection and virus isolation**

93 During 2015-2016, a total of 217 Green-winged teal (*Anas carolinensis*) were captured

94 with mist-net during an avian influenza surveillance program at Chashma Barrage, Pakistan

95 (60). The barrage (32º 25´ N, 71º 22´ E) is built on the River Indus and serves as one of the

96 major wildlife sanctuary for aquatic and terrestrial habitat under the provision of Punjab

97 Wildlife Act, 1974. Precisely, comprised of 0.327 Mha, it is located in the provinces of Punjab

98 and Khyber Pakhtun Khwa (KPK), Pakistan. Major part of sanctuary lies in the Punjab
99 province (district Mianwali) while a small proportion lies in Tehsil Lakki Marwat of Dera
100 Ismail Khan District in KPK province. The barrage is considered as a wetland of international
101 importance that accommodates a large variety of migratory and indigenous birds each year (2).
102 Cloacal and oropharyngeal swabs were collected from clinically healthy teals and transferred
103 to laboratory as described by Halverson et al. (21). Each sample was prepared and processed
104 for the isolation of virus using 9-day-old embryonated chicken eggs following standard
105 protocol (52). Harvested fluid was confirmed as AAvV 1 by *F* gene-based PCR (49, 66)
106 followed by spot hemagglutination (HA) assays (52). The AAvV 1 confirmed isolates were
107 stored at -80°C until used.

108 **Biological characterization of isolates**

109 Hemagglutination inhibition (HI) test with antisera against AAvV 1 was performed for
110 both isolates according to standard protocol (52). The pathogenicity of each isolate was
111 assessed through egg infectious dose 50 (EID_{50} ml^{-1}) by inoculating into 9-11-day old
112 embryonated chicken eggs as described by Reed and Muench (43). Intra-cerebral pathogenicity
113 index (ICPI) tests in one-day-old chicken and the mean death time (MDT) in 9-day-old
114 embryonated chicken eggs were determined following previously described procedures (52).

115 **Complete genome sequencing of isolates**

116 Extraction of viral RNA from harvested allantoic fluid was performed using QIAamp
117 Viral RNA extraction Mini Kit as per manufacturer's instructions (Qiagen, Valencia city, CA,
118 USA). The whole genome was amplified by one-step Reverse Transcriptase Polymerase Chain
119 Reaction (RT-PCR) using our previously reported primers and protocols (35). The amplified
120 PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System as
121 recommended by manufacturer (Promega, Co., Madison, WI, USA). Amplicons were

122 sequenced in both directions with primers used for amplification through ABI PRISM Genetic
123 Analyzer 3130x1 version (Applied Biosystems, Foster City, CA, USA).

124 **Phylogenetic and amino acid residue analysis**

125 Complete nucleotide sequence of each isolate was assembled using Geneious® version
126 8.1.6 (16). The obtained sequences were compared to GenBank database using BLAST tool at
127 NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Assembled sequence of each isolate was
128 aligned with strains representing different genotypes (GenBank) using ClustalW methods in
129 BioEdit® version 5.0.6 (20) for subsequent phylogeny, prediction of deduced amino acid
130 substitution sites for the *F* and *HN* genes, and for nucleotide and amino acid similarity indices.
131 To determine sub-genotype, the hypervariable region of the *F* gene of both isolates was
132 analysed in comparison with previously reported AAvVs around the globe
133 (http://www.ncbi.nlm.nih.gov/) using distance-based neighbour-joining method (1000
134 replication bootstrap values)in MEGA® version 6.0 software (54). Nucleotide identity among
135 whole genome and individual protein was determined using ORF of selected strains of all
136 genotypes (I-XVIII); Pairwise Sequence Comparisons (PASC) analysis was performed using
137 MEGA software. The complete F and HN amino acid sequences were submitted to I-TASSER®
138 (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) for 3-dimensional structural analysis of
139 each protein (63). Substitutions of amino acid in the *F* and *HN* proteins were annotated through
140 PyMol® software (https://www.pymol.org/).

141 **Recombination Analysis**

142 To estimate the inter- and intra-class recombination of under-study isolates, complete
143 genome sequence of representative strains of each genotype (GenBank database) was subjected
144 to SimPlot Version 3.5.1 (42), GARD (48) and RDP version 4.70 (28). Distance-based
145 similarity method in SimPlot allowed percentage identity of query sequence to a panel of
146 reference sequences. BootScan analysis, maximum χ^2 method (implementing GARD online,

147 http://www.datamonkey.org/GARD) and RDP were used to assess likelihood of a locus for
148 recombination events and putative breakpoint within genotype of both isolates. Utilizing
149 several recombination detection methods into single suite of tool, the RDP package is
150 considered a fast, simple and sensitive method for identification of putative recombination
151 breakpoints (29).

152 **Accession Numbers**

153 Complete nucleotide sequences of both isolates were submitted to GenBank database
154 and are available under accession numbers MF437286 (*Anas carolinensis*-I-UVAS-Pak-2015)
155 and MF437287 (*Anas carolinensis*-II-UVAS-Pak-2015).

156 **Experimental challenge and transmission in chickens**

157 A total of 20 clinical healthy chickens were used in the present study to assess the patho-typing
158 of isolates based on the clinical presentation of infection, gross lesions and histopathology. All
159 chickens were screened negative for avian influenza virus (AIV) and AAvV 1-specific HI
160 antibodies in blood, and antigen in naso-oral and cloacal swabs samples using ELISA and RT-
161 PCR, respectively. These 27-day-old chickens (n=20) were randomly divided into three groups
162 [Group 1 had 10 birds (challenged chickens), Group 2 had 5 birds (contact chickens) and Group
163 3 had 5 birds (mock or negative control chickens)]. The challenge group of chickens was
164 inoculated with 0.1 mL $10^{-6.51}$ EID_{50} of *Anas carolinensis*-I-UVAS-Pak-2015 isolate bilaterally
165 *via* intranasal route. The control group was inoculated with 0.2 mL phosphate-buffered saline
166 (PBS). After 24 hrs of infection, the contact chickens were kept together with virus-challenged
167 chickens for the assessment of possible horizontal transmission of virus. All chickens were
168 housed in separate negative pressure isolators and were provided food and water *ad libitum*.
169 All chickens were clinically monitored every day for clinical presentation of the ND. With the
170 onset of infection evidenced by observation of clinical signs, infected chicken was sacrificed
171 and tissue samples (lung, liver, spleen, brain, bursa and small intestine) were collected in 10%

172 neutral buffered formalin for fixation and hematoxylin and eosin staining for subsequent
173 histopathological observations.

174 **RESULTS**

175 **Biological assessment of the two AAvV 1 isolates**

176 Alongside influenza surveillance, genome corresponding to avian avulavirus was
177 detected in 19 independently harvested allantoic fluid (8.76%) of teals. Since isolates were
178 obtained during one-time surveillance programme and were detected in the same flock, we
179 processed only two of randomly selected isolates for further biological and molecular
180 characterization. Hemagglutination assay on both isolates showed a high titre ($log_2 9$/50ul) and
181 revealed a high positive inhibition of haemagglutination (HI titre) with AAvV 1-specific
182 antisera. The ICPI value for both isolates was found to be 1.5. The mean embryo infective
183 doses (EID_{50}) for isolates were $10^{-6.51}$ and $10^{-6.53}$, respectively. A general description of each
184 isolate along with various studied parameters is provided in Table 1.

185 **Phylogenetic and evolutionary analysis**

186 Full-length genome sequences of both isolates showed similar length (15,192 nts),
187 followed the "rule of six" and genes in an order of 3′-*NP*-*P*-*M*-*F*-*HN*-*L*-5′ (Table 2).
188 Phylogenetic analysis of whole genome (Fig. 1A), *HN* (Fig. 1B) and *F* genes (Fig. 1C),
189 clustered both isolates within genotype VII, closely related to previously reported isolates from
190 layer chicken (KX791185-87) and duck (KU845252) in Pakistan and vaccinated commercial
191 broiler chicken (HQ697254) from Indonesia. Phylogenetic analysis of hyper-variable region
192 grouped both isolates within sub-genotype VIIi with a close relationship to isolates reported
193 previously from backyard poultry and wild birds in different regions of Pakistan (Fig. 1D).
194 Nucleotide divergence indices of study isolates revealed a minimum difference (1%) for
195 genotype VII. Based on coding regions of both isolates, *HN* gene showed a low nucleotide
196 percentage divergence (0.7%) with genotype VII followed by *L* gene (0.7%), *NP* gene (0.6-

197 0.9%), *F* gene (1.1%), *M* gene (1.3%) and *P* gene (1.4%). Added to this, percentage nucleotide
198 divergence to vaccine strains were observed to be 16.1% and 19.3% for Mukteswer and LaSota,
199 respectively (Table 2).

200 **Residue analysis of F and HN proteins**

201 The predicted residue analysis of F protein revealed a typical proteolytic cleavage
202 motif of RRQKR↓F for residues between positions 112-117. Several neutralising sites (D^{72},
203 E^{74}, A^{75}, K^{78}, A^{79}, and L^{343}) across a stretch of residues between 156-171 aa, six glycosylation
204 sites (^{85}N-R-T^{87}, ^{191}N-N-T^{193}, ^{366}N-T-S^{368}, ^{447}N-I-S^{449}, ^{471}N-N-S^{473} and ^{541}N-N-T^{543}) and
205 twelve cysteine residues were conserved in the putative F protein. However, a single
206 substitution in signal peptide (1-31 aa, Y20C) and two substitutions in fusion peptide (117-142
207 aa, V121I, I125V) were noticed. Hydrophobic heptad repeats (HR) regions showed one
208 substitution, each in HRa region (143-185 aa, S171A), HRb region (268-29 aa, Y272N) and
209 HRc region (471-500 aa, R494K). Three substitutions were found in major trans-membrane
210 domain (501-521 aa, V506A, L512I and V521G). Substitutions at 52, 107 and 445 residue
211 position were exclusive in non-conserved regions of studied isolates (Fig. 2).

212 The HN gene comprised of a single ORF encoding 571 amino acids. A total of 13-
213 cysteine residues and four glycosylation sites (^{119}N-N-S^{121}, ^{433}N-K-T^{435}, ^{481}N-H-T^{483} and ^{538}N-
214 K-T^{540}) were observed in both isolates. However, four substitutions (M33T, I34V, M35V/I,
215 I36T) in transmembrane domain (25-45 aa), two (S77N, I81V) in HRa region (74-88 aa) and
216 one (N569D) at site 2 were observed. No significant substitution was found in site 23, P1, site
217 1 and 14, P2, P3, HRc domain and site 2 and 12. Five substitutions at 58, 218, 308, 387 and
218 431were exclusive in studied isolates (Fig. 3).

219 Because both isolates shared a high percentage of nucleotide similarity (99.9%), three-
220 dimensional protein structures were simulated for F and HN proteins of MF437286 isolate
221 alone. Compared to vaccine strain (LaSota; AF077761), significant substitutions in signal

222 peptide region and cleavage site of F protein were identified. These include N145K and S176A
223 in hydrophobic heptad repeat region a (HRa), four in the major trans-membrane domain and
224 two in cytoplasmic tail (Fig. 4A). Similarly, for the HN protein, the trans-membrane domain
225 contained eight residue substitutions, three in HRa region, and one each for antigenic site 2, 12
226 and 23 (Fig. 4B).

227 **Recombination Analysis**

228 Complete sequences of both isolates were also compared for possible recombination
229 events. SimPlot showed similarities at non-coding intergenic regions among selected strains
230 from Class I and II. Utilizing different approaches for detection of putative recombination
231 events or breakpoints integrated in SimPlot and RDP software, we found a lack of potential
232 recombination event for study isolates.

233 **Potential of wild bird origin AAvV 1 to cause infection in experimental chicken**

234 The experimentally challenged and contact chickens showed 100% mortality within 6th day of
235 post-infection (DPI), highlighting the infectious potential of reported AAvV 1. Wherein,
236 challenged chickens showed clinical signs from 2nd DPI with death of three chickens. The
237 clinical infection was aggravated and peaked at 3rd DPI with death of four chickens. After three
238 days, minor clinical signs were observed in one contact chicken. At the end of 4th DPI, the
239 remaining challenged chickens also succumbed. After five days, severe clinical presentation of
240 ND was observed in three contact chickens and all contact birds died at the end of sixth day.
241 The clinical signs in both isolated infected chickens were observed since 2nd DPI, consisting of
242 anorexia, depression, green-white diarrhea with foamy presence, nasal and ocular discharge,
243 open mouth breathing, sneezing and coughing. However, no neurological signs were observed
244 in challenged chickens. The chickens in the control group remained healthy during the entire
245 experimental duration. On the post-mortem examination of infected chickens, hemorrhages in
246 lungs and liver, enlarged liver, congested kidneys, mottled spleen, pinpoint hemorrhages in

247 proventricular glands and edamatous bursa were observed. The histopathological observations
248 were consistent with aforementioned gross lesions including degeneration in hepatocytes,
249 venous congestion and infiltration of inflammatory cells in portal card of liver (Fig. 5A). Also,
250 congestion, hemorrhages with mononuclear inflammatory cells infiltration in sub-mucosa of
251 lung (Fig. 5B), damaged basal membrane and degeneration in follicles of bursa (Fig. 5C),
252 infiltration of inflammatory cells and congestion in spleen (Fig. 5D), presence of dead/necrotic
253 tissue mass, dropout of epithelium and inflammatory cells infiltration in small intestine (Fig.
254 5E) and mild congestion in brain (Fig. 5F) were observed. Whereas, all collected tissues from
255 chickens of control group had no apparent histological or pathological changes.

256 **DISCUSSION**

257 We presented the first biological, genetic and evolutionary analysis of two AAvV 1 strains
258 isolated from asymptomatic green-winged teal from Pakistan. These findings highlight the
259 potential roles of waterfowl in the epizootology of ND, especially in countries where the
260 disease remains endemic. Based upon mean infectivity and mean death time, both of the under-
261 study isolates were found virulent (53, 57). The AAvVs are considered velogenic with a MDT
262 of up to 60 hrs, mesogenic if it is 61- 90 hrs and lentogenic if it is > 90 hrs (52). Both isolates
263 showed 1.5 ICPI value, which is a typical biological characteristic of virulent AAvV 1 strains.
264 Whereas, virus showing ICPI lower than 0.7 was considered to be a low virulent strain (52). In
265 accordance to the pathogenicity assessment and horizontal transmission, the clinical and
266 histopathological observation indicates velogenic nature of virus and categorized them as
267 velogenic viscerotropic AAvV 1 (39). These clinico-pathological observations of studied
268 isolates were similar to previously characterized virulent strains of genotypes VII (39).

269 Phylogenetic clustering of under-study isolates with viruses from different host-origin showed
270 an evidence of continuous circulation of genotype VII in Pakistan along with potential inter-
271 species transmission (34, 35, 48, 57). A strong phylogenetic relationship between these isolates

272 and Indonesian strains (HQ697254) originated from poultry, proposes an ancestral link
273 between these isolates (62). Together, it highlights circulation of virulent nature of circulating
274 sub-genotype VIIi in multiple avian hosts (3, 48) in disease endemic countries including
275 Pakistan. The AAvVs of genotype VII are known to be genetically diverse and are associated
276 with recurrent poultry outbreaks, mainly in the Middle East, Asia (4, 33, 48), Africa and South
277 America (4, 50). For instance, the intercontinental spread of recently panzootic sub-genotype
278 VIIi demonstrates global significance and economic importance of these viruses (4).
279 Maximum homology between isolates reported here and characterized previously from chicken
280 origin highlights susceptibility of multiple avian species as has been documented earlier (25).
281 Nucleotide divergence from vaccine strains raises concerns on vaccine efficacy against field
282 circulating AAvV 1 strains of different genotypes (53). While challenge-protection studies are
283 warranted, potential divergence in residues may predict concerns on efficacy of vaccine being
284 used to protect susceptible population (10). Also, genetic divergence among different
285 genotypes (Table 2) highlights continuous evolutionary nature of APMVs in different
286 geographic location across the globe (10, 36, 61). Under-study isolates were considered
287 velogenic with the presence of typical cleavage site in the F protein which is a key molecular
288 determinant of virulence (40). The predicted residue analysis revealed several conserved
289 neutralising sites (31) that are considered significant for emergence of escape variants. The
290 presence of six glycosylation sites indicated high virulent nature of isolates as compared to low
291 virulent AAvVs (38). Whereas, eleven or twelve conserved cysteine residues in AAvV 1 strains
292 (46) may plays a vital role to maintain connection between F1 and F2 subunit (30).
293 Substitutions in different influential regions of F protein can enhance the virulence of a virus
294 (38). For instance, variations in signal peptide and fusion peptide motifs may hinder viral
295 envelope-cell membrane fusion activity of the F protein (26, 47, 59). In fact, the signal peptide
296 of F protein is the most hypervariable region among different sub–genotypes of genotype VII

297 (37). A lack of conserve residue pattern was also observed in hydrophobic heptad-repeat
298 regions and major transmembrane domains. These domains are usually considered essential for
299 efficient virus fusion; however potential substitutions may affect protein integrity and
300 subsequent functional changes (7).
301 Genomic characterization of HN protein revealed a specific ORF length (571aa) in a pattern
302 similar to those observed for virulent AAvVs (52, 56). Avirulent strains usually carry an ORF
303 encoding 577 residues where insertion of six amino acids at the C-terminus can act as motif for
304 inhibition of HN activity (65). The 13-cysteine residues and four glycosylation sites were found
305 highly conserved (31). However, inter-genotype comparison revealed few substitutions in
306 trans-membrane domain, HRa region and at site 2. Such substitutions, particularly in a
307 structural motif in stalk domain (HRa region) are considered responsible for mediating protein-
308 protein interactions, and are proposed to be responsible for increased virulence (65). Key
309 receptors, antigenic sites responsible for NA activity and sialic acid binding sites including P1,
310 P2 and P3 were also conserved among studied isolates (9, 18). Compared to vaccine strain
311 (LaSota isolate), few substitutions were observed in antigenically important regions of the
312 protein (11). Noteworthy, substitutions in signal peptide, hydrophobic heptad repeat region,
313 major transmembrane domain and cytoplasmic tail may result in escape mutants owing to the
314 involvement in structural transition of protein from metastable to stable form (11). These are
315 in accordance with a previous investigation, where conformational variations in linear epitopes
316 of HN protein may influence the binding sites for monoclonal antibodies (12) subsequent to
317 escape mutant. Moreover, substitutions in each site 2, 12 and 23 may affect NA activity,
318 receptor binding ability and cell fusion of protein suggestive to cause infection (53).
319 While analysing of putative recombination events among investigated isolates, we observed
320 potent inter- and intra-genotypes resemblances in coding and non-coding regions (19, 58).
321 SimPlot revealed maximum similarity for genotype VII whereas a maximum divergence was

322 observed for vaccine isolates in accordance to previous study (45). Due to lack of significance
323 for non-coding regions, we analysed only coding regions of studied and other representative
324 isolates originating from multiple avian species for subsequent identification of breakpoints.
325 GARD analysis revealed putative recombination events at two positions; one breakpoint at
326 3811 nt (p = 0.046, AIC score = 76.68) and the other one at 7789 nt (p = 0.0016, AIC score =
327 4.19). However, these outcomes were not consistent with other tools for detection of
328 recombination event such as RDP and, therefore could not be considered as recombinant. The
329 findings are in consistent with observations made previously by Diel et al. (14) who concluded
330 absolute lack of significance of such recombination events. Though occurrence of natural
331 recombination may help in emergence of a novel/new mutant virus (22, 41, 45, 57, 64, 66), an
332 absent or rare occurrence of recombination events has been documented for negative-sense
333 RNA viruses (23). Taken together, the influence of natural occurrence of recombination in
334 AAvV 1 evolution is not well documented and remain largely debatable (1, 51).

335 **CONCLUSIONS**

336 We sequenced and characterized two virulent AAvV 1 strains isolated from clinically
337 healthy teal. Biological characterization confirmed the F protein cleavage-based virulence of
338 both isolates, and phylogenetically clustered them within sub-genotype VIIi in genotype VII.
339 Both showed significant residue substitutions at different sites that are considered important
340 for virulence and pathogenicity. The finding of current study highlights the potential
341 transmission of wild bird origin AAvV 1 in commercial poultry. Therefore continuous
342 monitoring and surveillance of asymptomatic natural reservoirs particularly in disease endemic
343 regions across the globe are warranted.

344 **CONFLICT OF INTEREST**

345 All authors declared no conflict of interest for this study.

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551 **FIGURE LEGENDS:**

552 **Fig. 1. The phylogenetic analysis of the studied and previously characterized AAvV 1
553 strains.** The neighbour-joining method with 1000 bootstraps was used for analysis of
554 evolutionary relationship between study isolates (marked with black circles) and representative
555 isolates using MEGA 6 software. (**A**) The phylogenetic analysis was conducted based on the
556 whole genome, (**B**) *HN* gene (**C**) *F* gene and (**D**) hypervariable region of the *F* gene.

557 **Fig. 2.** For observation of substitutions, the inferred amino acid of complete *F* gene of studied
558 isolates was compared with AAvV strains from different genotypes (I-XVIII) including
559 vaccine strains within Class II. Conserved regions are highlighted with red colour, and
560 structurally and functionally importance residues were labelled.

561 **Fig. 3.** The inferred amino acids of complete *HN* gene of studied isolates were compared with
562 AAvV strains from different genotypes (I- XVIII) including the vaccine strains within Class
563 II. Conserved regions are highlighted with red colour. Structurally and functionally importance
564 residues were labelled.

565 **Fig. 4.** Structural features of head, neck, and stalk regions of AAvVs 1 F (**A**) and HN (**B**)
566 proteins. Substitutions compared to LaSota strain are highlighted.
567 **Fig. 5.** Microscopic examination of histopathological changes at different resolution in
568 different tissue collected from chickens infected with the Anas carolinensis-I-UVAS-Pak-2015
569 isolate. Arrows indicate histological and pathological lesions in liver at 40x (**A**), lung at 10x
570 (**B**), bursa at 10x (**C**), spleen at 40x (**D**), small intestine at 40x (**E**) and brain at 40x (**F**).
571

Table 1 Epidemiological, genetic and biological characteristics of reported isolates

Isolate	Location	Year	Bird Species	Bird sex	Sample Type	Pathotype	HA titre	HI	EID_{50} ml^{-1}	ICPI	MDT(h)[a]	CSP[b]	Genotype	Genome Length	GenBank
Anas carolinensis-I-UVAS-Pak-2015	Pakistan	2015	Green-winged teal	Male	CS, OS	vvNDV	1:512	+	$10^{-6.51}$	1.5	49.2	$_{112}$RRQKR↓F$_{117}$	VIIi	15,192 nt	MF437286
Anas carolinensis-II-UVAS-Pak-2015	Pakistan	2015	Green-winged teal	Female	CS, OS	vvNDV	1:512	+	$10^{-6.53}$	1.5	50	$_{112}$RRQKR↓F$_{117}$	VIIi	15,192 nt	MF437287

a: Mean Death Time in hours (**Note:** According OIE standard < 60 hours of MDT indicated the velogenic NDV whereas MDT between 60-90 and > than 90 hours represented the mesogenic and lentogenic NDVs, respectively), **b**: Cleavage site pattern, **CS**: Cloacal Swabs, **OS**: Oropharyngeal Swabs, **vvNDV**: Viscerotropic velogenic Newcastle disease virus, **nt**: Nucleotide

Table 2 Comparative estimation of evolutionary distances among different genotypes of Class II and studied isolates.

Region Compared	Nucleotide Length (bp)[a]	Amino Acid Length[b]	Genotype-I (AY562991)		Genotype-II (AF077761)*		Genotype-III (EF201805)*		Genotype-IV (EU293914)		Genotype-V (HM117720)		Genotype-VI (AJ880277)		Genotype-VII (KX791185)		Genotype-VIII (FJ751919)		Genotype-IX (HQ317334)		Genotype-X (GQ288391)		Genotype-XI (HQ266602)	
			MF437286	**MF437287**	**MF437286**	**MF437287**	**MF437286**	**MF437287**	**MF437286**	**MF437287**	**MF437286**	**MF437287**	**MF437286**	**MF437287**	**MF437286**	**MF437287**	**MF437286**	**MF437287**	**MF437286**	**MF437287**	**MF437286**	**MF437287**	**MF437286**	**MF437287**
			nt/aa	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**	**nt/aa**
Whole Genome	15,192	5064	16.5	16.5	19.3	19.3	16.1	16.1	13.9	14	13.9	13.9	11.8	11.8	1	1	14	14	16.3	16.3	18	18	19.4	19.5
NP	122-1591	489	13.4/24.5	13.5/24.8	17.2/31.6	17.5/32.2	14.4/26.4	14.7/27	11.4/20.8	11.5/21.1	12.2/22.3	12.4/22.6	11.8/21.6	11.8/21.6	0.9/11.1	0.6/11.4	12.6/23	12.8/23.3	13.5/24.8	13.5/24.8	15/27.9	15.3/28.1	16.1/29.7	16.3/29.9
P	1893-3080	395	17.3/32.3	17.3/32.3	18.6/34.7	18.6/34.7	16.9/31.5	16.9/31.5	14.5/27	14.5/27	14.7/27.3	14.7/27.3	13.2/24.6	13.2/24.6	1.4/25.4	1.4/25.4	15.9/29.7	15.9/29.7	17.8/33.1	17.8/33.1	19.7/36.6	19.7/36.6	20.4/37.9	20.4/37.9
M	3296-4390	364	17/30.9	17/30.9	19.1/34.4	19.1/34.4	15.5/27.9	15.5/27.9	13.9/25.1	13.9/25.1	18.5/33.4	18.5/33.4	9.7/17.4	9.7/17.4	1.3/16.1	1.3/16.1	12.4/22.3	12.4/22.3	16.1/29	16.1/29	17.5/31.8	17.5/31.8	18.1/32.5	18.1/32.5
F	4550-6211	553	13.9/24.9	13.9/24.9	17.2/30.7	17.2/30.7	14.1/25.1	14.1/25.1	11.7/20.9	11.7/20.9	13.1/23.5	13.1/23.5	10.3/18.5	10.3/18.5	1.1/33	1.1/33	12.2/21.8	12.2/21.8	14.4/25.7	14.4/25.7	15.8/28.2	15.8/28.2	18.3/32.9	18.3/32.9
HN	6418-8133	571	16/28.7	16.1/28.8	20.2/36.3	20.3/36.4	16.2/28.9	16.2/29	13.7 24.5	13.8/24.6	12.6/22.5	12.7/22.6	11/19.7	11.1/19.8	0.7/33	0.7/33	14.5/25.9	14.5/26	17.3/31.1	17.4/31.2	17.9/32.1	18/32.3	18.6/33.4	18.7/33.5
L	8387-15001	2,204	14/24.9	14/24.9	15.8/28.1	15.8/28.2	13.1/23.2	13.1/23.2	11.7/20.8	11.7/20.8	11.1/19.6	11.1/19.7	9.3/16.5	9.3/16.5	0.7/5.1	0.7/5.1	11.5/20.4	11.5/20.4	13.3/23.6	13.3/23.6	15.2/26.9	15.2/27	17.2/30.6	17.2/30.6

a: Including stop codon, **b**: exclusive of stop codon, *****Vaccine strain LaSota and Mukteswer (**Note:** Both study isolates have 15,192 genome lengths, 122-15001 is coding region and remaining is non-coding region including, Leader, 3′UTR, Intragenic regions, 5′UTR and Trailer)