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

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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;

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

INTRODUCTION

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

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

chickens (49). Virulent AAvVs have previously been reported from clinically healthy green-winged teal (39, 44); nevertheless, there is a paucity of higher resolution characterization of genome, evolutionary dynamics and occurrence of potential recombination events across the length of viral genome. While aquatic bird-origin AAvV strains are reported, the characterization is mainly attributed to partial sequencing of hypervariable region of *F* gene (24, 25, 27). Additionally, biological assessments of AAvVs isolated from teal have not been assessed before. Therefore, owing to proven roles in virus transmission and potential threats to commercial vulnerable poultry, it is imperative to investigate the evolutionary and infectious potential of these viruses from waterfowl. In this study, we determined genetic and biologic assessments of two AAvVs isolated from clinically healthy green-winged teal from Pakistan.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals by National Institutes of Health and Animal Research Council (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf). The swab sampling, embryonated eggs inoculation and other essential protocols were approved by the Ethical Review Committee for the Use of Laboratory Animals (ERCULA) of University of Veterinary and Animal Sciences, Lahore vide permit number ORIC/DR-70 dated March 15, 2015.

Sample collection and virus isolation

During 2015-2016, a total of 217 Green-winged teal (*Anas carolinensis*) were captured with mist-net during an avian influenza surveillance program at Chashma Barrage, Pakistan (60). 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), 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 (2). Cloacal and oropharyngeal swabs were collected from clinically healthy teals and transferred to laboratory as described by Halverson et al. (21). Each sample was prepared and processed for the isolation of virus using 9-day-old embryonated chicken eggs following standard protocol (52). Harvested fluid was confirmed as AAvV 1 by *F* gene-based PCR (49, 66) followed by spot hemagglutination (HA) assays (52). The AAvV 1 confirmed isolates were stored at -80^{o}C until used.

Biological characterization of isolates

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

Complete genome sequencing of isolates

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

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

Phylogenetic and amino acid residue analysis

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

Recombination Analysis

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

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

Accession Numbers

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

Experimental challenge and transmission in chickens

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

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

RESULTS

Biological assessment of the two AAvV 1 isolates

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

Phylogenetic and evolutionary analysis

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

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

Residue analysis of F and HN proteins

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

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

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

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

Recombination Analysis

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

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

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

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

DISCUSSION

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

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

and Indonesian strains (HQ697254) originated from poultry, proposes an ancestral link between these isolates (62). Together, it highlights circulation of virulent nature of circulating sub-genotype VIIi in multiple avian hosts (3, 48) in disease endemic countries including Pakistan. The AAvVs of genotype VII are known to be genetically diverse and are associated with recurrent poultry outbreaks, mainly in the Middle East, Asia (4, 33, 48), Africa and South America (4, 50). For instance, the intercontinental spread of recently panzootic sub-genotype VIIi demonstrates global significance and economic importance of these viruses (4).

Maximum homology between isolates reported here and characterized previously from chicken origin highlights susceptibility of multiple avian species as has been documented earlier (25). Nucleotide divergence from vaccine strains raises concerns on vaccine efficacy against field circulating AAvV 1 strains of different genotypes (53). While challenge-protection studies are warranted, potential divergence in residues may predict concerns on efficacy of vaccine being used to protect susceptible population (10). Also, genetic divergence among different genotypes (Table 2) highlights continuous evolutionary nature of APMVs in different geographic location across the globe (10, 36, 61). Under-study isolates were considered velogenic with the presence of typical cleavage site in the F protein which is a key molecular determinant of virulence (40). The predicted residue analysis revealed several conserved neutralising sites (31) that are considered significant for emergence of escape variants. The presence of six glycosylation sites indicated high virulent nature of isolates as compared to low virulent AAvVs (38). Whereas, eleven or twelve conserved cysteine residues in AAvV 1 strains (46) may plays a vital role to maintain connection between F1 and F2 subunit (30). Substitutions in different influential regions of F protein can enhance the virulence of a virus (38). For instance, variations in signal peptide and fusion peptide motifs may hinder viral envelope-cell membrane fusion activity of the F protein (26, 47, 59). In fact, the signal peptide 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

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

CONCLUSIONS

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

CONFLICT OF INTEREST

All authors declared no conflict of interest for this study.

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FIGURE LEGENDS:

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

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

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

Fig. 4. Structural features of head, neck, and stalk regions of AAvVs 1 F (**A**) and HN (**B**) proteins. Substitutions compared to LaSota strain are highlighted.

Fig. 5. Microscopic examination of histopathological changes at different resolution in different tissue collected from chickens infected with the Anas carolinensis-I-UVAS-Pak-2015 isolate. Arrows indicate histological and pathological lesions in liver at 40x (**A**), lung at 10x (**B**), bursa at 10x (**C**), spleen at 40x (**D**), small intestine at 40x (**E**) and brain at 40x (**F**).

572 **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

573 **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
574 hours represented the mesogenic and lentogenic NDVs, respectively), **b**: Cleavage site pattern, **CS**: Cloacal Swabs, **OS**: Oropharyngeal Swabs, **vvNDV**:
575 Viscerotropic velogenic Newcastle disease virus, **nt**: Nucleotide
576

577 **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

578 **a**: Including stop codon, **b**: exclusive of stop codon, *****Vaccine strain LaSota and Mukteswer (**Note:** Both study isolates have 15,192 genome lengths, 122-
579 15001 is coding region and remaining is non-coding region including, Leader, 3′UTR, Intragenic regions, 5′UTR and Trailer)