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Sequence analysis and biological characterization of virulent avian avulavirus 1 isolated from asymptomatic migratory fowl

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Summary. – Continuous monitoring and surveillance of avian avulaviruses (AAvVs) in water/migratory fowl is imperative to ascertain the evolutionary dynamics of these viruses. Here, we report genomic and amino acid characteristics of two AAvVs strains isolated from asymptomatic waterfowl (*Anas carolinensis*). Sequence characteristics including the presence of virulent motif ($_{112}$RRQKR↓F$_{117}$) and biological assessment confirmed the virulent nature of study isolates. Phylogenetic analysis of complete fusion (*F*) and hemagglutinin-neuraminidase (*HN*), and hyper-variable region of *F* gene revealed clustering of both strains within genotype VII and sub-genotype VIIi. The inferred residue analysis of complete *F* and *HN* genes revealed a number of substitutions in functionally and structurally important motif/s compared to reference strains of each genotype (I-XI). This study concludes an evolutionary nature of avian avulavaris 1 (AAvV-1), ascertaining continuous surveillance of migratory fowl to better elucidate their infection, epidemiology and subsequent impacts on commercial and backyard poultry.

Keywords: virulent AAvV-1; migratory fowl; genetic characterization; evolutionary analysis; Pakistan

Introduction

Newcastle disease, caused by avian avulavirus 1 (AAvV-1), is a highly contagious viral disease of multiple avian species. The virus is an enveloped, mono-partite, negative sense single stranded RNA virus, classified into the genus *Orthoavulavirus* within the family *Paramyxoviridae* (Kuhn *et al.*, 2019). The presence of mono- or poly-basic amino acids in fusion (F) protein is considered a key determinant for its virulence and, based on pathogenicity, it is categorized as velogenic, mesogenic, lentogenic and avirulent (de Leeuw *et al.*, 2005). The phylogenetic analysis classified the AAvV-1 strains into two distinct classes; class-I has nine genotypes (I-IX), whereas, class-II has eleven genotypes (I-XI) (Dimitrov *et al.*, 2016). Within class-II, genotypes VI and VII can further be classified into eight (a-h) and eleven (a-k) subgenotypes, respectively (Esmaelizad *et al.*, 2017; Molini *et al.*, 2017; Aziz-ul-Rahman *et al.,* 2018).

Being a natural reservoir of infectious agents, water fowl has potential to shed virus into the environment and, therefore, could be a potential source of disease transmission to susceptible hosts such as chickens (Shabbir *et al.*, 2013). Virulent AAvVs have previously been reported from clinically healthy green-winged teal (Pearson and McCann, 1975); nevertheless, there is a paucity of data revealing genomic and residue characteristics of genes that are considered important for replication and neutralization. Also, biological assessments such as mean death time and infection dose 50

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Abbreviation: AAvVs = avian avulaviruses; AAvV-1 = avian avulavirus 1; HA = hemagglutination assay; MDT = mean death time; EID_{50} = embryo infective dose; ND = Newcastle disease; F = fusion; HN = hemagglutinin-neuraminidase; HI = hemagglutination inhibition; HR = hydrophobic heptad repeat region

(EID_{50}) have not been assessed so far and, as such, warrant necessary investigations. That being said, we determined genetic and biologic assessments of two virulent AAvVs isolated from clinically healthy waterfowl from Pakistan, a disease endemic country where substantial economic losses are associated with frequent ND outbreaks in commercial poultry setting.

Materials and Methods

Sample collection and characterization. Cloacal and oropharyngeal swabs (n = 119) were collected from asymptomatic green-winged teals and processed for isolation of virus by inoculating into 9-day-old embryonated chicken eggs following standard protocol (Stear, 2005). The harvested fluid was confirmed to be AAvV-1 by spot hemagglutination (HA) and hemagglutination inhibition (HI) assays using AAvV-1 specific antisera (Stear, 2005) followed by *F* gene-based PCR (Munir *et al.*, 2010). The confirmed isolates were stored at -80°C until further use**.** The pathogenicity of each isolate was assessed through egg infectious dose (EID_{50} ml^{-1}) using 9-day-old embryonated chicken eggs as described earlier (Reed and Muench, 1938). Mean death time (MDT) was also determined following previously described procedure (Stear, 2005). All essential protocols were approved by the Ethical Review Committee for Use of Laboratory Animals (ERCULA) of University of Veterinary and Animal Sciences, Lahore (Permit Number: ORIC/DR-70).

Hemagglutination-inhibition test. In order to assess an *in vitro* genomic and residue divergence, the cross-HI assay was performed with studied and vaccine (LaSota) strains using their specific antisera. The titre was expressed as a reciprocal of the highest dilution of antisera that caused a complete inhibition of agglutination activity of the virus. The antigenic relatedness of studied and vaccine strains was expressed in R-value as described previously (Archetti and Horsfall, 1950).

RNA isolation. Viral RNA was extracted using QIAamp viral RNA extraction mini kit as per manufacturer's instructions (Qiagen, USA). Genome was amplified by one-step reverse transcriptase PCR (RT-PCR) using previously reported primers and protocols (Munir *et al.*, 2010). The products were purified using the Wizard® SV gel and PCR clean-up system (Promega, Co., USA) and were sequenced in both directions with the same primers through ABI PRISM Genetic Analyzer 3130x1 version (Applied Biosystems, USA).

Multiple sequence alignment. The obtained sequence of each isolate was aligned with strains representing different genotypes using ClustalW methods in BioEdit® version 5.0.6 (Hall, 1999). To determine sub-genotype, the hyper variable region of *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 neighbor-joining (1000 replication bootstrap values) method in MEGA® version 6.0 software (Tamura *et al.*, 2013). To know the residue diversity for cleavage site pattern in the fusion protein at position 112–117, a multiple sequence alignment was generated through WebLogo 3.1 (http://weblogo.threeplusone.com/create.cgi). A pairwise nucleotide identity matrix among *F* and *HN* gene of aforementioned isolates from all genotypes (I-XI) was generated using Sequence Demarcation Tool (SDT) version 1.2 in muscle mode (Muhire *et al*., 2014). 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. Substitutions of these proteins were compared using PyMol® software (https://www.pymol.org/). The complete *F* and *HN* nucleotide sequences of both isolates were submitted to GenBank and available under Acc. No. MH891651-54.

Results

Hemagglutination of both isolates showed a high titre (log_2 9) and inhibition of hemagglutination with AAvV-1 specific antisera. The mean embryo infective dose for isolates was found to be $10^{-6.51}$ and $10^{-6.53}$ whereas; mean death time ranged 49.2 to 50 hrs. After conducting cross-HI assay using specific viruses and their antisera, the calculated R-values between studied isolates and viruses of genotype VII was 0.75 and 0.79**,** showing an increased antigenic similarity as compared to vaccine strains that showed a noticeable antigenic variation between vaccine strain (LaSota) and these prevailing strains (R-value = 0.26 and 0.19).

The phylogenetic analysis of complete *F* and *HN* gene sequences 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 (Fig. 1a,b). Phylogenetic analysis of hyper-variable region of *F* gene grouped both isolates within sub-genotype VIIi revealing a close relationship to isolate reported previously from backyard poultry and wild birds in different regions of Pakistan (Fig. 1c).

The predicted residue analysis of F protein revealed a typical proteolytic cleavage motif RRQKR↓F for residues between 112–117 aa with high residue conservness of arginine (R) at 113 aa following glutamine (Q) and arginine (R) at 114 and 116 aa position, respectively (Fig. 2a). 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}), several neutralizing sites (D^{72}, E^{74}, A^{75}, K^{78}, A^{79}, and L^{343}) across a stretch of residues between 156–171 aa, and twelve cysteine residues were found conserved. However, two substitutions (V121I and I125V) in fusion peptide (117–142 aa), one substitution each in hydrophobic heptad repeat regions as HRa region (S171A; 143–185 aa), HRb region (Y272N; 268-29 aa) and HRc region (R494K; 471–500 aa) and three substitutions (A506V, I512L and G520V) in major trans-membrane domain (501–521

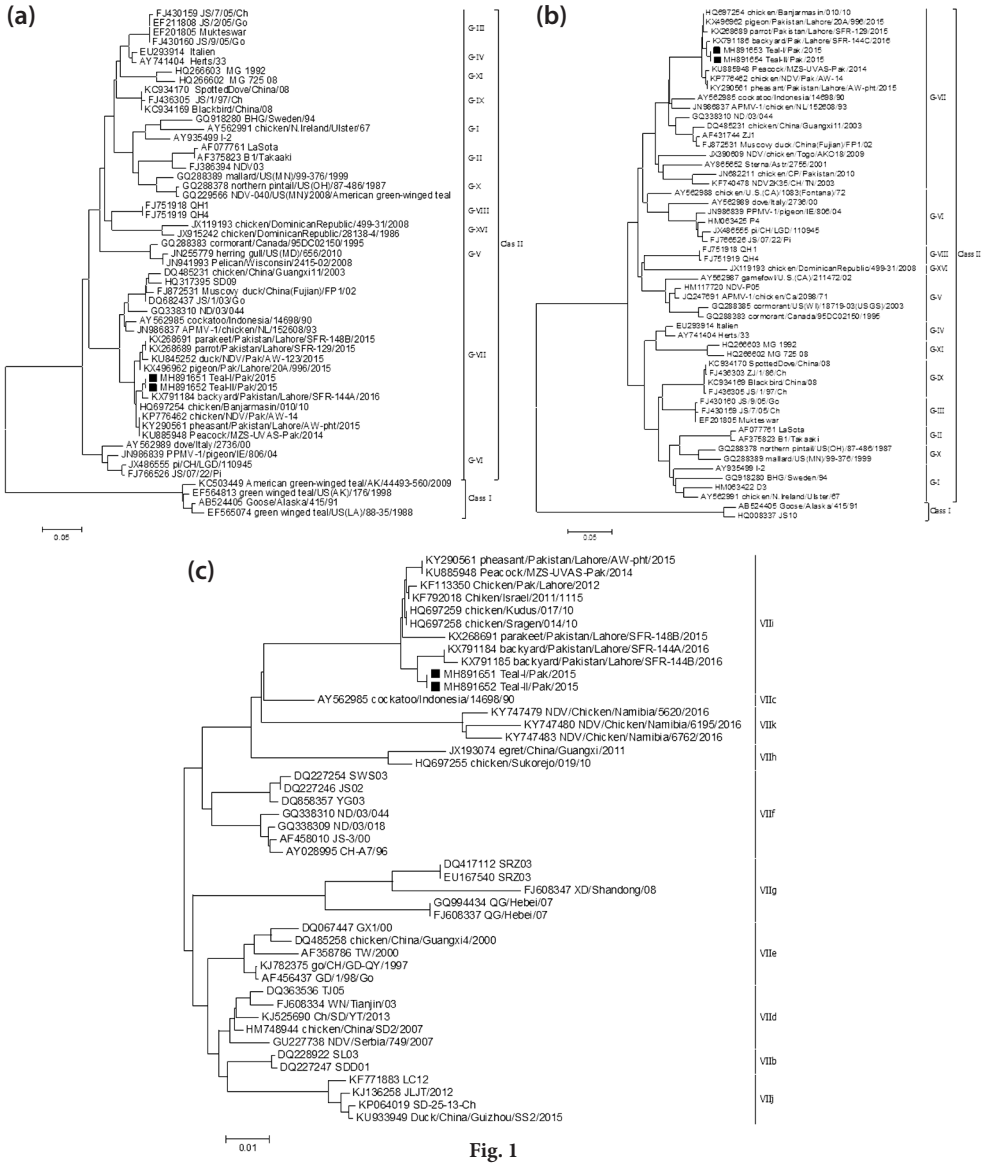

Fig. 1

The phylogenetic analysis of the studied and previously characterized AAvV-1 strains

The neighbor-joining method with 1000 bootstraps was used for analysis of evolutionary relationship between study isolates (marked with black square) and representative isolates from worldwide using MEGA 6 software. The phylogeny analysis was conducted based on complete *F* gene (**a**), *HN* gene (**b**), hyper-variable region of *F* gene (**c**).

aa) were observed (Table 1). The *HN* gene comprised of a single open reading frame (ORF) encoding 571 amino acids. A total of four glycosylation sites (^{119}N-N-S^{121}, ^{433}N-K-T^{435}, ^{481}N-H-T^{483} and ^{538}N-K-T^{540}) and 13 cysteine residues were found conserved in both isolates. However, four substitutions (M33T, I34V, M35V/I, I36T) in trans-membrane domain (25–45 aa), two (S77N, I81V) in HRa region (74–88 aa) and one (N569D) at site 2 were observed (Table 1). Cumulatively, nucleotide similarity matrix revealed a maximum identity (99%) among study isolates and strain from genotype VII. Moreover, percentage of nucleotide similarities to vaccine strains were observed to be 80.7 and 83.9% for LaSota and Mukteswer, respectively (Fig. 2b).

Since both isolates shared a high percentage of nucleotide similarity (99.9%), three-dimensional protein structures were simulated for F and HN proteins of Teal-I/Pak/2015 isolate alone. Compared to vaccine strain (LaSota; AF077761), significant substitutions of residues in signal peptide region and cleavage site of F protein were noted. These were two (N145K, S176A) in hydrophobic heptad repeat region (HRa), four in the major trans-membrane domain and two in cytoplasmic tail. Similarly, for HN protein, the trans-membrane

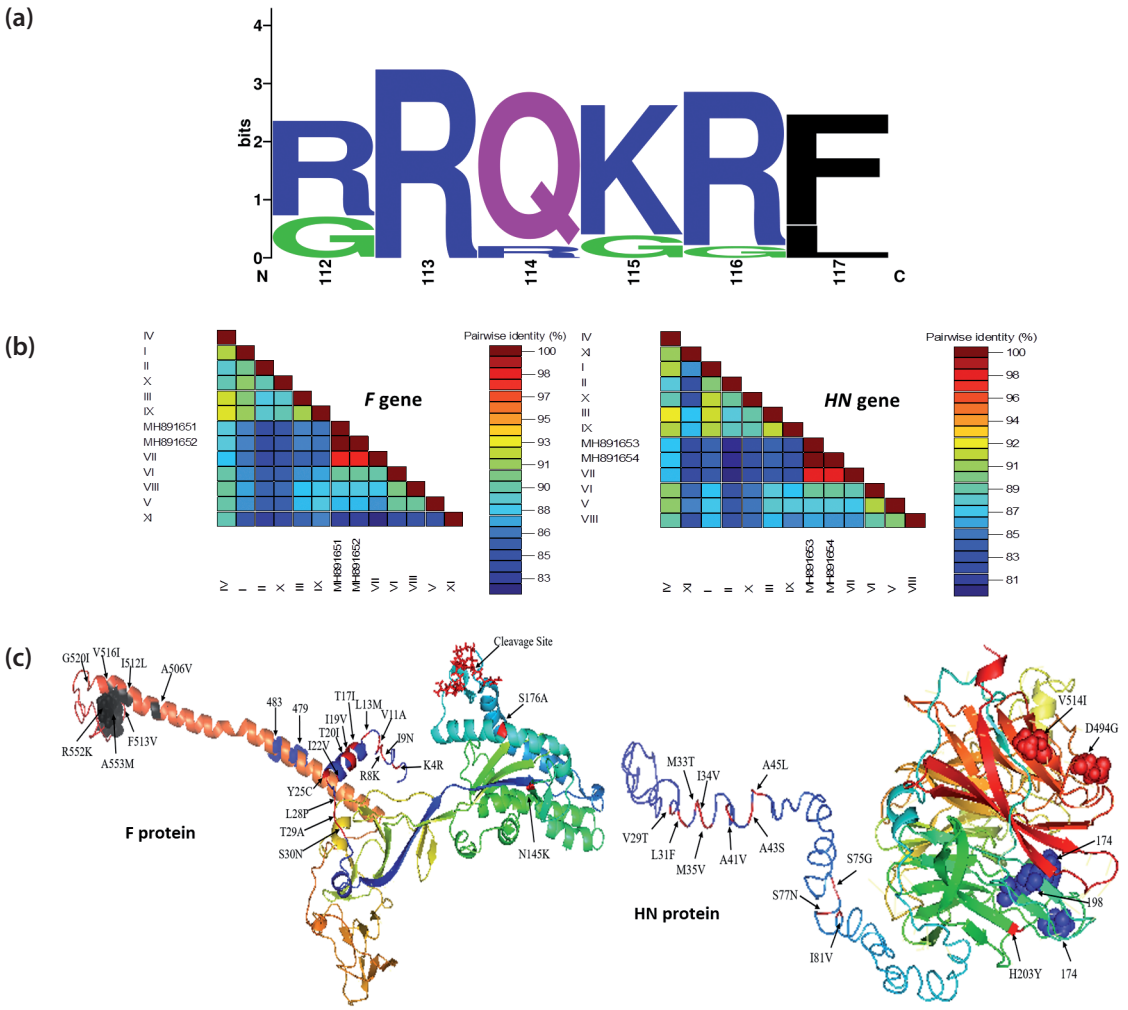

Fig. 2

Genome-based comparison of F and HN protein of the studied and previously characterized AAvV-1 strains

The graphical presentation of residue conservness at cleavage site (112–117) (**a**); the nucleotide homology of studied isolates with strains of genotypes (I-XI) including vaccine strain (LaSota; AF077761, Mukhteswer; EF201805) (**b**); three-dimensional presentation of structural regions of AAvV-1-F and -HN proteins compared to LaSota strain (**c**).

domain contained eight residue substitutions, three in HRa region, and one each for antigenic site 2, 12 and 23 (Fig. 3c).

Discussion

The study presents biologic and genetic assessment of two AAvV-1 strains isolated from healthy green-winged teal together highlighting their significance in epizootology of ND, particularly in disease endemic countries such as Pakistan. Based upon mean infectivity and mean death time, both isolates were found velogenic in nature. The viruses are considered velogenic with a MDT of up to 60 h, mesogenic if it is 61–90 h and lentogenic if it is >90 h (Stear, 2005). The phylogeny analysis clustered both isolates with strains originating from different avian species indicating continuous circulation of genotype VII and sub-genotype VIIi viruses in multiple avian hosts in Pakistan (Shabbir *et al.*, 2013). The strains of sub-genotype VIIi are known to be genetically diverse and are associated with recurrent poultry outbreaks, mainly in the Middle East and Asia (Aldous *et al.*, 2003; Shabbir *et al.*, 2013) along with intercontinental spread demonstrating their global significance (Aldous *et al.*, 2003).

Maximum homology between under-study isolates and chicken originated isolate highlights susceptibility of multiple avian species (Aldous *et al.*, 2003). Whereas, genetic divergence from vaccine strains raises concerns about vaccine efficacy against field circulating AAvV-1 strains. This is important because predicted residue analysis revealed some conserved neutralizing sites that are considered significant

Table 1. The inferred amino acid of complete *F* and *HN* gene of studied isolates was compared with AAvVs strain from different genotypes (I-XI) within Class II

Genotype	Isolates	Fusion protein					Hemagglutinin-neuraminidase protein			
		Fusion peptide (117-142 aa)	HRa (143-185 aa)	HRb (268-299 aa)	HRc (471-521 aa)	Trans-membrane domain (501-521 aa)	Trans-membrane domain (25-45 aa)	HRa (74-88 aa)	Site 2 (569 aa)
VII	Teal-I/Pak/2015	FIGAVIGSIALGV	QANQNAANILRLKESIAATNEA	LITGYPILYDSQTQLLG	NNSISNALDKLTESN	LITYIAITVIS	FRIAVLLLMIM	LSSSQDVI	N
		ATAAQITAAAALI	VHEVTDGLSQLSVAVGKMQQF	IQVNLPSVGNLNNMR	SKLDKVNVRLTSTSA	IFFGVISLGL	ILAISAAALA	DRIYKQV	
VII	Teal-II/Pak/2015	.....	.....	.....	.....	.....	.....	.....	N
I	AY562991	...I..V.....	.....A.....	...N.....	.....K.....	...V...L.....V.	.....TVVT.....	...N..V.....	D
II	AF077761	...I..V.....	.....A.....	...N.....	.....K.....	...V...L.....V.	.....TVVT.....	...N..V.....	D
III	EF201805	...I..V.....	.....A.....	...N.....	.....	...V...L.....V.	.....TVVT.....	...N..V.....	D
IV	EU293914	...I..V.....	.....A.....	...N.....	.....K.....	...V...L.....V.	.....TVIT.....	...N..V.....	D
V	HM117720	...I..V.....	.....A.....	...N.....	.....K.....	...V...L.....V.	.....TVVT.....	...N..V.....	D
VI	AJ880277	...I..V.....	.....A.....	...N.....	.....K.....	...V...L.....V.	.....TVVT.....	...N..V.....	D
VII	JX854452	... ...V.....	.....A.....	.....	.....	.....	.....	...N..V.....	D
VIII	FJ751919	...I..V.....	.....A.....	...N.....	.....K.....	...V...L.....V.	.....TVVT.....	...N..V.....	D
IX	HQ317394	...I..V.....	.....A.....	...N.....	.....K.....	...V...L.....V.	.....TVVT.....	...N..V.....	D
X	GQ288391	...I..V.....	.....A.....	...N.....	.....K.....	...V...L.....V.	.....TVVT.....	...N..V.....	D
XI	HQ266602	...I..V.....	.....A.....	...N.....	.....K.....	...V...L.....V.	.....TVIT.....	...N..V.....	D

for emergence of escape variants. Though, it needs challenge-protection studies, potential divergence in residues along with outcome of cross-HI assay may predict concern on efficacy of vaccine being used to protect susceptible population. The under-study isolates were considered velogenic with the presence of typical cleavage site (Peeters *et al.*, 1999), six glycosylation sites in F protein (Panda *et al.*, 2004) and a specific ORF length (571aa) of HN protein (Stear, 2005).

The intra-genotype comparison for HN protein revealed a few substitutions in trans-membrane domain, the HRa region and at site 2. Such substitutions, particularly in a structural motif in HRa region are considered responsible for mediating protein-protein interactions and could be responsible for increase in virulence (Yuan *et al.*, 2012). Compared to vaccine strain (LaSota strain), a few substitutions were observed in significant antigenic regions of HN protein. For instance, substitutions in signal peptide, hydrophobic heptad repeat region, major trans-membrane domain and cytoplasmic tail may result in escape mutants because of the involvement in structural transition of protein from metastable to stable form (Chen *et al.*, 2001). These observations are consistent with previous investigation where conformational variations in linear epitopes of *HN* gene may influence the binding sites for monoclonal antibodies resulting in escape mutant (Cho *et al.*, 2008). Moreover, substitutions in each of the site 2, 12 and 23, may affect NA activity, receptor binding ability and cell fusion of protein (Sun *et al.*, 2015). Taken together, the current study highlights an evolutionary nature of AAvV-1 and, therefore ascertains continuous monitoring and surveillance of asymptomatic natural reservoirs particularly in disease endemic regions across the globe.

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