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Detection and Phylogenetic Analysis of Infectious Bursal Disease Virus Based on both Genome Segments A and B Isolated from Backyard Poultry Punjab, Pakistan

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ABSTRACT

In recent years, the re-emergence of virulent strains of infectious bursal disease virus (IBDV) has resulted in substantial economic losses in Pakistan despite mass and intense vaccination regimens. Infectious bursal disease (IBD) is one of the most important immunosuppressive diseases of the poultry and has been a constraint on sustainable food security around the globe, including Pakistan. This disease damages the bursa of Fabricius (BF), which causes immunosuppression in birds. A total of 50 tissue samples from backyard chicken flocks presenting suspected symptoms were collected during February 2020 and March 2021 for genetic characterization, followed by phylogentic analysis. A total of 4 isolates were sequenced based on partial VP1 and hyper-variable region of the VP2 genes simultaneously. According to phylogenetic analysis, the study isolates genotype A3B3 were identified as predominant strains in country backyard poultry. Concerning the identity matrix analysis of VP1 representative part, study isolates shared (87-88% nt; 95-96% aa) identity with the vvIBDV and (97-98% nt; 98-99% aa) with non-vvIBDV. While VP2 revealed (99-100% nt; 99-100% aa) identity with previously reported Pakistan vvIBDV strains and (91-95% nt; 91-95% aa) with non vvIBDV. Amino acid alignment analysis of VP1 revealed that current IBDVs have three characteristic aa residues of vvIBDV (287-A, 508-K, and 511-S) and four characteristic aa residues of non-vvIBDV (146-E, 147-G, 242-D and 390-L). While VP2 gene sequence alignment revealed eight characteristic aa residues of vvIBDV (222-A, 242-I, 253-Q, 256-I, 279-D, 284-A, 299-S, and 330-S and a distinct aa 384-I). Based on phylogeny, this is the first identification of IBDV segment reassortants having segment A of A3 (very virulent) and segment B of B3 (early Australian-like) genogroups reported in Pakistan backyard poultry. Further study is required to determine the pathogenicity of the IBDV reassortant and development of new policies for IBDV intervention in the country.

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SFW conducted the research. AA supervised the study and AA, MRK and KA designed the study. SFW wrote the manuscript. BZ helped in interpretation of results and article writing.

Key words
Infectious bursal disease, Backyard poultry, Phylogenetic analysis, Pakistan

INTRODUCTION

Before 1960s backyard poultry production was the sole poultry meat source in the country, with flock size of about 20-50 birds of different breeds and ages. It was a low input business without veterinary care facilities (Ahmed *et al*., 2021). Infectious bursal disease (IBD) is a highly contagious and immunosuppressive disease of young chickens that causes considerable economic losses to the poultry industry globally (Qin and Zheng, 2017; Hussain *et al*., 2020; Maqbool *et al*., 2020). The disease is caused by the infectious bursal disease virus (IBDV), a non-enveloped dsRNA virus, which belongs to the genus Avibirnavirus of family Birnaviridae with two distinct serotypes (Müller *et al*., 2003). Serotype I is pathogenic and induces pathological lesions in chickens, whereas serotype II is non-pathogenic, primarily isolated from turkeys. Pathogenic IBDV is classified into four subtypes, i.e., attenuated, classical virulent, antigenic variants, and very virulent IBDV (Hussain *et al*., 2020). IBDV targets immature B lymphocytes (Zahid *et al*., 2018) in the lymphoid organ (bursa of Fabricius), leading to age-

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dependent immunosuppression exposing birds to other opportunistic pathogens. Very virulent IBDV (vvIBDV) has been reported in different countries with more than 70% chicken mortality, posing a severe threat to the poultry industry (Dey *et al*., 2019).

IBDV has a bi-segmented genome (segment A and B) of approximately 6.0 kb. Segment A is 3.2 kb long, with two overlapping open reading frames (ORF) encoding four proteins. ORF-1 encodes longer precursor polyprotein, cleaved by autoproteolysis and yields capsid protein VP2, nucleoprotein VP3 and protease VP4. In comparison, ORF-2 encodes only a nonstructural VP5 protein, a significant determinant of viral virulence and host protective antigen (Luque *et al*., 2009). The VP2 protein is the primary protective antigen containing specific epitopes responsible for inducing neutralizing antibody responses (Reddy *et al*., 2017). Amino acid substitutions in the VP2 hypervariable region can lead to variations in antibody recognition, antigenicity, virulence, and tissue tropism (Mato *et al*., 2020). Segment B is 2.8kb long, and it contains only one ORF which encodes RNA-dependent RNA polymerase VP1 protein, involved in virus replication and evolution (Ye *et al*., 2018). Both VP1 and VP2 proteins have been used to study pathogenicity, virulence, phylogenetic relationship and molecular characterizations (Fan *et al*., 2019).

IBDV may undergo segment reassortment due to the genome's bi-segmented structure. It has been proposed that vvIBDV arose through segment reassortment between segment A of circulating IBDV strains and segment B from an unknown IBDV strain (Hon *et al*., 2006). Natural reassortant IBDV strains have been reported frequently in recent years from various parts of the world, including reassortment between serotypes 1 and 2 (Soubies *et al*., 2017; Stoute *et al*., 2019) and reassortment between different genotypes of serotype 1 IBDVs (Mato *et al*., 2020).

In 1980 a vvIBDV outbreak was first recorded with high mortality in Punjab Pakistan (Khan *et al*., 1988) and since then continues to pose a threat by various strains all over the country (Lone *et al*., 2009; Zahoor *et al*., 2011). Despite extensive vaccination programs, IBD still prevails at the poultry farms posing significant economic losses in Pakistan, which emphasizes the characterization of field circulating IBDV strains to understand the causes of vaccination failure, hence incorporating local viruses in vaccine development (Shafqat *et al*., 2017; Sajid *et al*., 2021). Traditionally, nucleotide sequencing of the segment A coded VP2 hypervariable region is used for genotypic characterization of IBDV strains. Both segments should be characterized to detect genetic variations, influencing strain pathogenicity (Gao *et al*., 2018). Both genome segments contribute to the virulence of IBDV and segment reassortment plays a role in virus evolution, which is not possible to be evaluated by targeting VP2 gene only. Since VP1 and VP2 proteins are involved in virus evolution, there is still insufficient information to predict the actual dynamics of IBDV in Pakistan. Recently, some authors have characterized the chicken isolated IBDV VP1 and VP2 genes (Hussain *et al*., 2019, 2020), but the literature on backyard poultry is silent. Therefore, this study was designed for IBDV genotyping and phylogenetic analysis covering both segments from indigenous breed of backyard poultry. The study's primary objective was to detect and genotype IBDV strains circulating in country backyard poultry and add a new piece of knowledge about its characterization to better implement control measures.

MATERIALS AND METHODS

Ethical approval

The present work was approved by the ethical review committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan (Permit Number: DR/944).

Study area

The current study was conducted at the Department of Pathology, University of Veterinary and Animal Sciences Lahore, Pakistan.

Clinical samples

A total number of 50 tissue samples (bursa of Fabricius) were collected from backyard chicken flocks for genome extraction from February 2020 to March 2021. The dead bird subjected to virus isolation had typical hemorrhagic lesions on skeletal muscles and mucosal surface of the bursa of Fabricius. The bursal tissues were ground to make homogenate in phosphate buffered saline (pH 7.2) containing penicillin and streptomycin. The homogenate was finally centrifuged at 5 000×g for 5 min at 4°C. Lastly, the upper aquous layer was collected for further detection.

Molecular identification

The virus genome was extracted according to the protocol as described by Qi *et al*. (2015). Viral RNA was extracted from the bursa homogenate using QIAamp Viral RNA Kit (Qiagen, Germany) as directed by the manufacturer. Viral cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) (Zahid *et al*., 2016). The selected DNA fragment was amplified through PCR in a 25µL reaction mixture, containing 12.5µL master mix (PrimeSTAR Max DNA polymerase, Catalog Number: R045A), 1µL DNA template, 1µL each forward and reverse primers and 9.5µL

PCR grade water. Previously reported *VP1* gene-based primers; F(5'-AGGAGAAGCCCAATGCGT-3') and R(5'-GTCATCAATGGACCTCTC-3') with amplicon size 1255bp (Hussain *et al*., 2020); *VP2* gene-based primers; F(5'-AGCCAACATCAACGACAAA-3') and R(5'-CAAGACGGTCCCTCTCACT-3') with an estimated amplicon size of 782bp were employed (Ali *et al*., 2019). The PCR was performed under the following conditions: preheating (95℃ for 5 min), with subsequent 35 cycles of denaturation (95℃ for 30 s), annealing (56℃ for 30 s) and extension (72℃ for 1 min) followed by a final extension at 72℃ for 10 min. The RT-PCR products were checked by 1% agarose gel, stained with GelRed™ Nucleic Acid Gel Stain in gel electrophoresis and visualized in a gel documentation system (Bio-Rad Laboratories, United States). Amplified PCR product was purified using GeneJET PCR Purification Kit (Thermo Fischer Scientific, USA). The purified samples were sent to the Comate Bioscience Co., Ltd., China for Sanger sequencing.

Statistical and phylogenetic analysis

The obtained oligonucleotide sequences were properly trimmed with Chromas Software (version 2.6.6). Multiple sequence alignments were performed on BioEdit Software (version 7.2.5) using the ClustalW (version 1.8) (Thompson *et al*., 1997). The evolutionary analysis was conducted on MEGA Software (version 6) inferred through the Neighbor-Joining method with the maximum likelihood method. The reliability of the tree was tested by performing 1000 bootstrap replicates (Tamura *et al*., 2013). The pairwise nucleotide (nt) and amino acid (aa) distance matrix were calculated with UGENE software.

RESULTS AND DISCUSSION

In the present study, the results of RT-PCR revealed that the positive rate of IBDV samples was 32% (16/50). Afterwards, four positive samples were selected randomly to sequence the partial *VP1* and *VP2* gene fragments and the nucleotide sequences were determined and submitted to NCBI GenBank database (Table I). The phylogenetic analysis revealed that serotype I was subgrouped into vvIBDV, classical, attenuated, and variant IBDV. All the vvIBDV isolates, including reference strain (NC_004178), were placed in a single clade with a bootstrap value of 100. In contrast, classical, attenuated, and variant IBDV were grouped into another monophyletic clade. In the evolutionary tree based on the 89 nucleotide sequences of *VP1* retrieved from GenBank, serotype I strains revealed distinct branches and current study isolates with previously reported Pakistan strains were branched out of the vvIBDV branch and formed a cluster with HLJ0504-like strains, including HLJ0504 and Harbin-1 (Fig. 1). Based on the 72 nucleotide sequences of *VP2*, the phylogenetic tree was distinctly divided into two major branches, vvIBDV and non-vvIBDv. Present study isolates belonged to vvIBDV branch with already reported isolates (Pakistan reference strains) and formed one subgroup with a bootstrap value of 100 (Fig. 2). Segment A-coded *VP2* gene phylogenetic analysis exhibited that our isolate had a close relationship with other Pakistan and Indian isolates. Currently identified isolates acquired both segments (A and B) from different genetic relatives and were further confirmed by sequence alignment and phylogeny. *VP2* gene (segment A) of current isolates was clustered into vvIBDV branch while *VP1* gene (segment B) branched out of vvIBDV and formed a unique branch with strain like HLJ0504 previously reported in China (Hussain *et al*., 2019; Qi *et al*., 2011), Algeria (Nwagbo *et al*., 2016) and Pakistan (Hussain *et al*., 2019). Strains like HLJ0504 and Harbin-1 with unique segment (segment A originated from vvIBDV) and segment B acquired from a unique ancestor. So the current study reported unique segment-reassortant IBDV from backyard poultry. The results agree with (Hussain *et al*., 2019, 2020), who reported Pakistan originated reassortant strains from commercial poultry. Concerning the identity matrix analysis of VP1 representative part, study isolates shared (87-88% nt; 95-96% aa) homology with the vvIBDV and (97-98% nt; 98-99% aa) with non-vvIBDV. VP1 of isolates have comparatively higher identity of (97-98% nt; 98-99% aa) with previously reported HLJ0504-like strains which are the unique reassortant retrieving segment A from vvIBDV and B from unique ancestor (He *et al*., 2014; Qi *et al*., 2011). Concerning the identity matrix analysis of VP2 representative part, present study isolates revealed 99-100% nt and 99-100% aa identity with previously reported Pakistan vvIBDV strains and 91-95% nt and 91-95% aa homology with non vvIBDV.

Table I. Viral strains isolated from backyard chicken (age 3-5 weeks) during Apil and May 2020 and included in current study.

Gene	Strain	GenBank accession No.
Partial VP1	PAK-SFW	MW541059
	PAK-SFW2	OL690424
	PAK-SFW3	OL690425
	PAK-SFW4	OL690426
Partial VP2	PAK-SFW	MW452665
	PAK-SFW2	OL690421
	PAK-SFW3	OL690422
	PAK-SFW4	OL690423

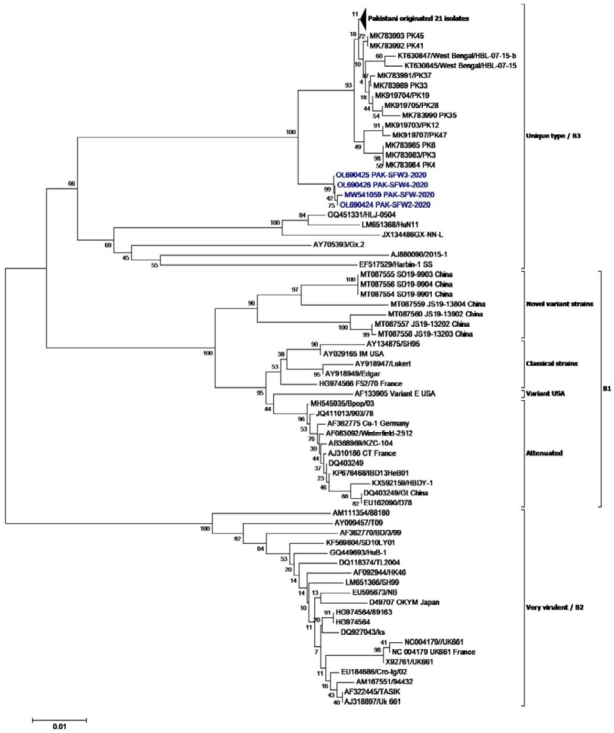

Fig. 1. Evolutionary tree constructed from segment B-coded *VP1* gene sequences reported in different geographical regions of the world with present study isolates highlighted with blue color

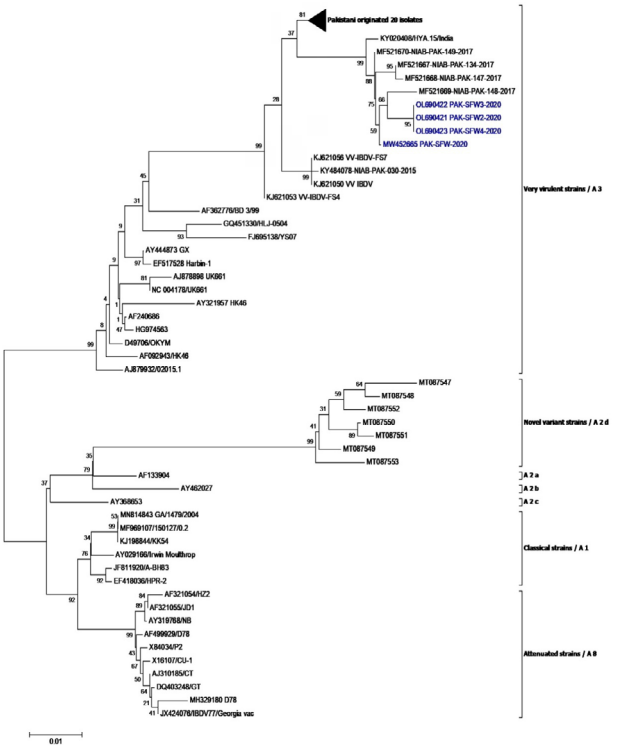

Fig. 2. Evolutionary tree constructed from segment A-coded *VP2* gene sequences reported in different geographical regions of the world with present study isolates highlighted with blue color.

In VP1 representative part sequence alignment, Pakistan IBDVs has three characteristic aa residues of vvIBDV (287-A, 508-K, and 511-S) and four characteristic aa residues of non-vvIBDV (146-E, 147-G, 242-D and 390-L) (Fig. 3). There are three domains in VP1 of IBDV; N-terminal (1-167aa), the polymerase (168-658aa), and C terminal domain (659-878aa). Pakistan IBDV isolates revealed recombinant characters in the N-terminal domain and the central polymerase domain between very virulent and attenuated strains. Self guanylylation site of RNA dependent RNA polymerase responsible for Protein priming of stains found in the N terminal domain (1–67 aa). Additionally, sequence alignment of VP2 revealed eight characteristic aa residues of vvIBDV (222-A, 242-I, 253-Q, 256-I, 279-D, 284-A, 299-S, and 330-S and a distinct aa 384-I) (Fig. 4) also presented in Pakistan IBDVs previously reported by (Hussain *et al*., 2020). Likewise the reassortants are also prevailing in the neighboring countries including India and China, it is thought-provoking to discover the relation among them.

As far as genotype is concerned current isolates belong to A3B3 genotype (Table II) according to the new classification scheme proposed by Wang *et al*. (2021) and

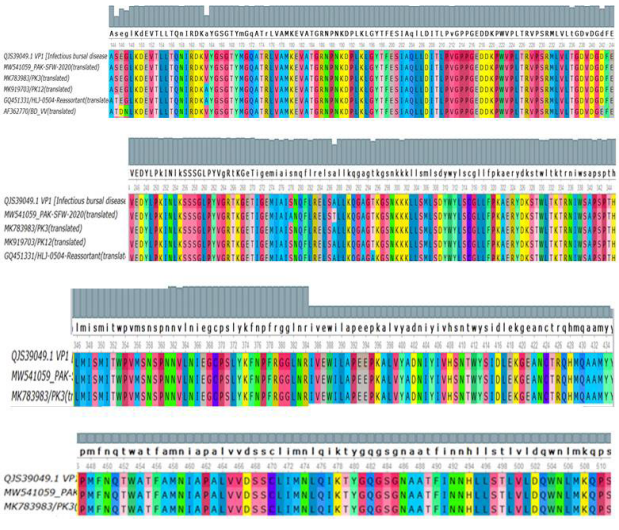

Fig. 3. Mean pairwise amino acid identity of present IBDV isolate with previous strains *VP1* gene based sequence alignment revealed presence of characteristic amino acids of non vvIBDV at position 146-E, 147-G, 242-D, 390-L, 508-k, 511-S. characteristic amino acids of very virulent IBDV at position 287-A.

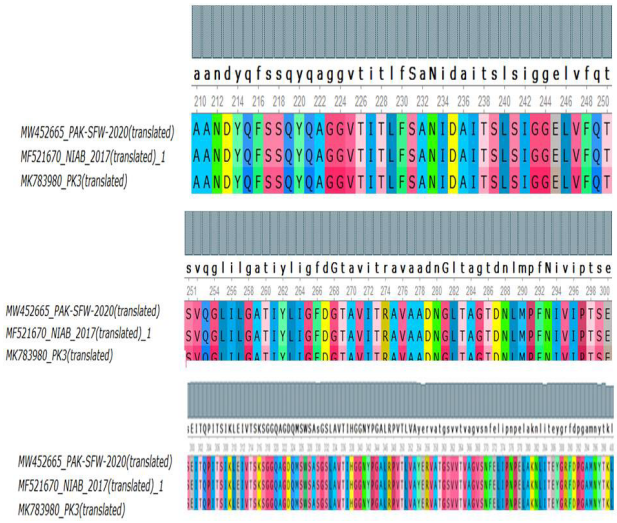

Fig. 4. Mean pairwise amino acid identity of present IBDV isolate with already reported strains. *VP2* gene based sequence alignment revealed presence of characteristic amino acids of vvIBDV at position 222-A, 242-I, 253-Q, 256-I, 279-D, 284-A, 299-S, 330-S and (384-I a distinct AA).

binomial scheme for genotype characterization suggested by Islam *et al*. (2021). In Serotype 1, segment A has genogroups classical (A1), US antigenic variant (A2), very virulent (A3), early European and recent South American distinct IBDV (A4), atypical or recombinant strains (A5), atypical Italian (A6), early Australian (A7) and Australian variant (A8). While segment B has five distinct genogroups; classical (B1), very virulent (B2), early Australian (B3), Polish and Tanzanian (B4), and Nigerian (B5). Segment A of the current four isolates clustered with vvIBDV genogroup A3, additionally, segment B of current isolates clustered with early Australian like IBDVs genogroup B3. The findings suggests that current isolates are segment reassortants with very virulent segment A and early Australian like segment B. Isolates with the same genotype A3B3 were also detected in Bangladesh in 2016. Different genotypes have appeared due to reassortment between two segments of previously circulating or newly evovled genotypes. The characterization of IBDV strains based on both genome segments information will help for a better understanding of IBDV evolution and molecular epidemiology.

CONCLUSION

The naturally occurring reassortant strains of IBDV genotype A3B3, harboring segment A from a very virulent strain and segment B from one unique ancestor, were identified through molecular characterization. To our knowledge, this is the first report on characterization of segment B coding VP1 and segment A-coding VP2 genes in Pakistan backyard poultry. The current work gives insight into a better knowledge of the nature and evolution of IBDV, which will assist in disease prevention and control programs for emerging reassortant strains.

Table II. Classification of IBDV isolates by phenotype and genogroups.

(Traditional classification) phenotype	Genotype	Reference strain	Accession No.	
			A	B
Classic strains	A1B1	F52/70 France	HG974565	HG974566
		IM USA	AY029166	AY029166
Variant strains	A2aB1	Variant E USA	AF133904	AF133905
	A2bB1	9109 USA	AY462027	AY459321
	A2cB1	GLS	AY368653	AY368654
Novel variant strains	A2dB1	SHG19 China	MH879045	MH879092
		SHG120 China	MH879063	MH879110
		SHG350 China	MH879081	MH879129
		SD19-9901 China	MT087547	MT087554
		SD19-9903 China	MT087548	MT087555
		SD19-9904 China	MT087549	MT087556
		JS19-13202 China	MT087550	MT087557
		JS19-13203 China	MT087551	MT087558
		JS19-13804 China	MT087552	MT087559
		JS19-13902 China	MT087553	MT087560
Very virulent strains	A3B2	UK661 France	NC_004178	NC_004179
		OKYM Japan	D49706	D49707
		89163 France	HG974563	HG974564
Attenuated strains	A8B1	Cu-1 Germany	X16107	AF362775
		CT France	AJ310185	AJ310186
		Gt China	DQ403248	DQ403249
Reassortant strains	A3B3	Harbin-1	EF517528	EF517529
		Gx	AY444873	AY705393
		PAK-SFW	MW452665	MW541059
		PAK-SFW2	OL690421	OL690424
		PAK-SFW3	OL690422	OL690425
		PAK-SFW4	OL690423	OL690426

Statement of conflict of interest

The authors have declared no conflict of interest.

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