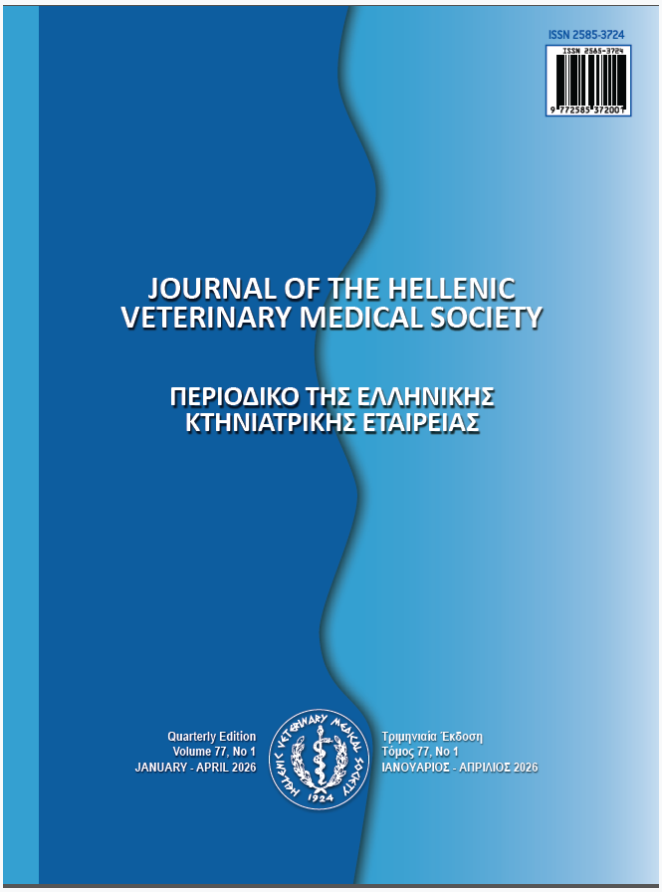
Journal of the Hellenic Veterinary Medical Society

Vol 77, No 1 (2026)



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doi: 10.12681/jhvms.37686

To cite this article:

Ahmad, M., Aiman , U., Sarfraz , M., & Bhutta, Z. (2026). Molecular and Phylogenetic Characterization of Infectious Bursal Disease Virus Re-emerging in Backyard Poultry in Pakistan: IBD Virus Re-emergence in Pakistan. *Journal of the Hellenic Veterinary Medical Society*, *77*(1), 10073–10082. https://doi.org/10.12681/jhvms.37686

J HELLENIC VET MED SOC 2026, 77 (1): 10073-10082
ΠΕΚΕ 2026, 77 (1): 10073-10082

Research article
Ερευνητικό άρθρο

Molecular and Phylogenetic Characterization of Infectious Bursal Disease Virus Re-emerging in Backyard Poultry in Pakistan

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ABSTRACT: In recent periods, infectious bursal disease (IBD) has re-emerged with virulent strains, resulting in significant losses to the economy of Pakistan. IBD is caused by a virus of the genus Avibirna, leading to severe immune suppression and other secondary infections. A total of 110 broiler chickens with a history of IBD infection were selected from poultry diagnostic laboratories in Faisalabad, Multan, Arif Wala, Lahore, and from private farms in Gojra and Bahawalnagar in the Punjab region of Pakistan. After the necropsy, the samples were processed for histopathology, and only selected positive samples from histopathology were processed for molecular detection and phylogenetic analysis. According to our findings, the bursa of Fabricius during the necropsy was edematous, hemorrhagic, and enlarged in size, along with hemorrhages on the pectoral muscles and the gizzard and proventriculus junction. The histopathological examinations of 20 samples revealed that the virus severely affected the bursa as there were atrophic, necrotic changes, increased interfollicular spaces, and replacement of fibrous cells. Then, 13 samples were confirmed through reverse transcription polymerase chain reaction (RT-PCR) by amplifying the hypervariable region of the VP2 (HVR-VP2). The samples were sequenced to analyze the nucleotide sequence of the IBD virus (IBDV), referring to ‘‘UVAS-01’’ as a predominant, virulent strain from the samples obtained from the backyard poultry. The obtained data from the sequence were analyzed with the reported strains of Pakistan and other parts of the world. The similarity index for ‘‘UVAS-1’’ was 98-100% with the reported very virulent infectious bursal disease virus (vvIBDV) strains in Pakistan, while the identity index was 92-100% with the very virulent strains prevalent in other regions of the globe. Using phylogenetic trees, the first vvIBDV strain was identified in the southeastern part of Punjab, Pakistan, in backyard poultry. Further work is required to study the reassortment in local strains for the emergence of vvIBD in indigenous breed farming.

Keyword: Backyard poultry; bursa of Fabricius; histopathology; RT-PCR; Sequencing; VP2

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Date of submission: **5-5-2024**
Date of acceptance: **6-12-2025**

INTRODUCTION

Backyard poultry is one of the major sources of income for rural areas in developing countries. Before the start of the commercial poultry business, domestic poultry was the only source of chicken meat and eggs; despite the more significant contribution to the economy, its role in the spread of poultry diseases is undefined, and there are no proper strategies and procedures to control and prevent the spread of infectious diseases (Ahmed *et al*., 2021).

The infectious bursal disease (IBD), also known as Gumboro disease, is an immunosuppressive and highly contagious disease of young broiler chickens that causes heavy economic losses to the industry (Qin and Zheng, 2017; Hussain *et al.*, 2020; Maqbool *et al.*, 2020). IBD is caused by the infectious bursal disease virus (IBDV), a double-stranded RNA, non-enveloped virus that belongs to the family *Birnaviridae*, and the genus *Avibirnavirus* (Müller *et al*., 2003). IBDV is classified into two different serotypes based on its pathogenicity; serotype 1 is pathogenic and causes severe illness in chickens, while serotype 2 is non-pathogenic and only causes infections in turkeys. *Avibirnavirus* has further classification into four subtypes based on its antigenicity. They're regarded as attenuated, classical, antigenic, and virulent viruses (Hussain *et al.*, 2020).

The target of Gumboro or IBD is the immature B lymphocytes in the bursa of Fabricius, which leads to the immunosuppression of birds dependent on age and exposes the birds to other secondary infections (Zahid, 2018). The very virulent IBDV (vvIBDV) has been investigated with 70 % mortality of young birds in different parts of the globe, indicating a severe threat to commercial poultry businesses (Dey *et al.*, 2019).

The genome of the IBD virus contains two segments, A and B, of dsRNA (Müller *et al*., 1979). Segment A (3.4 kb) of the *Avibirnavirus* genetic makeup consists of two partially overlapping open reading frames (ORFs) (Hudson *et al.*, 1986). Polyprotein is encoded by the major ORF, which produces VP2, VP3, and VP4 through autoproteolysis. VP3 and VP2 are 2 structural proteins, while VP4 is only a virus protease. The other minor ORF of the A segment encodes VP5. At the same time, there is only one ORF in segment B that is playing a role in encoding the VP1 RNA-dependent RNA polymerase (Ye *et al.*, 2018; Drissi Touzani *et al.*, 2019).

The capsid of the IBDV consists mainly of VP2 proteins and has been considered a significant antigen that evokes serotype-neutralizing antibodies (Abs), proving to be a major objective for the genotyping and molecular diagnosis of IBDV. We identified the Hypervariable region (HVR) between amino acids 206 and 350 within VP2 by performing sequence analysis of various pathotypes of IBDV. This VP2-HVR is considered to have a particular immunodominant epitope essential for giving rise to host protective Abs against the IBDV (Jackwood and Saif, 1987; Bayliss *et al.*, 1990; Vakharia *et al.*, 1994).

Substitutions of amino acids in HVR-VP2 can produce antigenicity, antibody recognition, tissue tropism, and virulence variations (Mató *et al.*, 2020). IBDV can undergo segment reassortment because the structure of the genome is bi-segmented. It has been investigated that the very virulent IBDV evolved by reassortment of segments between the A-segment of prevalent strains of IBDV and segment B of an unknown IBDV strain (Hon *et al.*, 2006). In the last few years, from different regions of the world, naturally reassortment strains of the IBDV have been produced after the assortment of serotypes 1 and 2 (Soubies *et al.*, 2017; Stoute *et al.*, 2019) and the reassortment among various genotypes of serotype one IBDV (Mató *et al.*, 2020; Umm-i-Habiba *et al.*, 2020).

In a recent study, four isolates from the backyard poultry of the Chakwal district of Punjab, Pakistan, were sequenced based on HVR-VP2 and partial VP1 together. A new genotype, the A3B3 strain, was identified through phylogenetic analysis, and VP2 shows a 99-100 % similarity index with the already present very virulent IBDV. VP2 amino acids alignment analysis of the current strain reveals that this new strain contains 8 Amino acid residues of already present vvIBDV strains, but the studies in the other parts of Pakistan have not been done (Waheed *et al.*, 2023). Therefore, our study was designed to detect IBD clinic-pathologically, genotyping, and phylogenetic analysis covering the HVR VP2 of segment A isolated from the birds of backyard poultry farming. The main objective of our study was to detect the IBDV strain genotypically prevalent in the backyard poultry industry of Pakistan and to enhance the understanding and knowledge of controlling this devastating disease.

MATERIALS AND METHODS

Sample collection and processing

A total of 110 tissue samples from broiler chickens aged between 3 to 5 weeks, with IBD disease

history with no vaccination record, were collected from different diagnostic of various cities, including 55 samples from poultry diagnostic laboratories in Faisalabad (15), Multan (10), Arif Wala (12), and Lahore (13) towns of Pakistan, while 55 samples from two private farms located in Gojra (35) and Bahawalnagar (20) cities of province Punjab Pakistan. Ethics approval was not required for this study because the sampling was performed on the dead birds available at the disease diagnostic laboratories and broiler farms at the request of farm owners. Dead birds were transferred to the University Diagnostic lab, University of Agriculture, Faisalabad, for the necropsy with vaccination records and diagnosis during normal working days. We were able to collect samples under the supervision of the Veterinarian. All the gross pathological lesions were recorded, and different tissues were collected for Histopathological and molecular-level investigation. The Bursa of Fabricius was our major organ of interest, as particular attention was given to the gross lesion and size of the bursa. We preserved the organ in 10% buffered formalin for histopathological examination, while half the samples were stored at -20 °C for genetic analysis. Tissues were cut, and sections of 4 µm thickness were obtained from the blocks of paraffin-embedded tissue before being taken out of formalin. These tissue sections were stained as described in the protocol for histopathological examination.

Phylogenetic analysis

RT-PCR analysis was conducted for molecular detection of IBDV and phylogenetic characterization of the hypervariable VP2 gene.

RNA Extraction

Thirteen samples with obvious pathological lesions were selected after necropsy and histopathology to identify the strain. RNA extraction was done from the samples of the bursa by using TRIzol-LS as performed by (Wizol™ Reagent, Cat. No: W76100 by Korean WizBio Solutions) (Hussain *et al.*, 2019).

Complementary DNA

After primer optimization for the PCR reaction, cDNA was synthesized by the M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA, Cat. No: 28025013), according to the standard procedure after minor modifications.

The synthesis of complementary deoxyribonucleic acid was done by mixing extracted RNA 10 µL (1ng/Ml), Random Hexamer primer 1 µL, and 1 µL dNTP(10mMol) and incubating at 65 °C for five minutes. After incubation, 5X Buffer solution 4 µL in quantity, along with 2 µL DTT and one µL of RNase Ribolock Inhibitor, was added into the mixture. The solution mixture was warmed for two minutes at 37 ^{0}C after adding 1 µL of M-MLV reverse transcriptase. After mixing, the solution was incubated at 25 °C for 10 minutes, the second incubation at 370 °C for 50 minutes, and the last heating for 15 minutes at 70 °C.

Polymerase Chain Reaction (PCR)

VP2 gene-based primer: F(5'-CCTCAGCTTACCCACATC-3') with nucleotide position 628-645 and R(5'-CCTTCCCCAATTGCATGG-3') 540-1557 with an amplicon product size of 929 bp were used (Hussain et al., 2019).

After the final mixture with the sample cDNA, the samples were loaded into a thermocycler (Thermo Scientific, USA) and subjected to the set PCR conditions, including an initial denaturation at 95 °C for 5 minutes, followed by annealing at 56 °C for 30 seconds. The initial extension was set at 70 °C for 1 minute and 30 seconds. For subsequent cycles, denaturation was performed at 95 °C for 30 seconds, and a final extension at 70 °C for 10 minutes was included.

To confirm the PCR products, the process of Gel Electrophoresis was performed after forming a 1.5 % agarose gel and loading the PCR product samples along with a 2 kb ladder in the gel with ethidium bromide into the gel in the electrophoresis equipment, which possesses positive and negative anodes. Conditions with 360 Volts with 180 A current for 18 minutes, separated the amplicons of DNA, and their size was measured according to the ladder, and identification of the concerned gene was made. Gel was analyzed under a UV trans illuminator and gel documentation system (Bio-Rad, USA), and different sizes were investigated by comparing the DNA size of samples with the size of the ladder.

Amplification and sequencing of the HVR VP2 region

Based on the PCR results and severity of histopathological lesions, including the destruction of the architecture of bursal tissue, vacuolation, cyst formation, and interfollicular gaps, 13 samples were selected among the positive. Those PCR products previously analyzed were purified using a gel purification kit (Biobasic, USA). The Sanger dideoxy sequencing protocol was applied with the help BiqDye Termina-

tor Sequencing Kits Method. PCR products without purification were delivered to a 3100 DNA analyzer (Applied Biosystem, Foster City, C.A., USA), and similar primers were used for the sequencing reaction, which was used for PCR. For assembling and editing of sequences for direct sequence in two directions, the SeqMan Pro software (DNAStar; Lasergene 7.1.0, Madison, USA) was applied.

The hypervariable region was confirmed by the application of BLAST http://www.ncbi.nlm.nih.gov/blast (NCBI), the National Center for Biotechnology and Information (Ali Khan *et al.*, 2019). In the end, the final sequence in the investigation was submitted to the available GenBank, and a specific Accession Number MW075221.1 was allocated.

Phylogenetic analysis of the VP2 region

The software BioEdit, Lasergene DNASTAR, and MegAlign were used for the analysis of Nucleotide sequences for phylogenetic analysis. We used the hypervariable region of the VP2 gene for phylogenetic investigation (Burland, 1999; Tamura *et al.*, 2013).

The evolutionary and phylogenetic analyses were carried out on software called Sanger dideoxy sequencing Technology by applying the maximum likelihood method with one thousand bootstrap replications (Tamura *et al*., 2013) (Version: MW075221.1). We applied the ClustalW process with the MegAlignTM program for the amino acids. While analyzing the predicted sequence of amino acids, we used the ProteanTM program of Lasergene (DNASTAR Inc) as utilized by (Raja *et al*., 2018) (Version UJE38523 1).

Histopathology

25 out of 110 tissue samples of the Bursa of Fabricius were processed for histopathology based on structural integrity and obvious gross pathological lesions to seek the actual pathological picture at the tissue level. Tissue was cut for sectioning, and then sections were stained with hematoxylin and eosin (Pereira). After cutting the sample tissue, washing, dehydration, clearing, embedding of tissue, section cutting, mounting of tissue, and examination under a light microscope were performed, respectively.

RESULTS

Clinical outcomes

This study was conducted on rural poultry from the regions of Punjab, including Multan, Faisalabad, Lahore, Arif Wala, and Bahawalnagar of Pakistan. A private farm located in the district of Gojra, Punjab, Pakistan, has had a history where we could observe some clinical signs in a few broiler chickens. Clinical findings included dizziness, lethargy, solid vent, loose whitish dropping, and prostration. The hens started showing clinical outcomes at the age of twenty-five days. The outbreak period was between 3 to 6 weeks of age. The percentage mortality was 25 % approximately.

Gross lesions

Upon necropsy, we found the following lesions **(Table 1)** in the bursa with length (17–20 mm) and width (12-15 mm), hemorrhagic, swollen, edematous, atrophied with dimension (length: 5-7 mm, width: 4-6 mm), and yellowish coloration **(Figure 1a, c, and e)** in the hens including. Hemorrhages were also observed on pectoral and thigh muscles **(Figure 1f)**, deposition of urates in the kidneys **(Figure 1d)**, liver congestion, and marginal hepatomegaly with dimensions' length (55-60 mm) and width (35-40 mm) **(Figure 1b)**. Additionally, nonspecific lesions, including hemorrhages on the junction of the proventriculus and gizzard. There was evidence of an edematous thymus.

Histopathological Findings

The results of the histopathology investigation **(Fig. 4)** indicated inflammatory cell infiltration, including macrophages, lymphocytic necrosis, connective tissue hyperplasia, and vacuole formation in the parenchyma of the Bursa of Fabricius **(Fig. 4 and Table 2)**. We also observed atrophic follicles, some haemorrhages, and congestion in the bursa tissues.

Representative images of the histopathology of the bursa of Fabricius, indicating the **(A)** Intrafollicular gap, Hemorrhagic Dissemination; **(B)** Vacuolar Degeneration in the parenchyma cells and infiltration of inflammatory cells, including macrophages, heterophils, and lymphocytes, and connective tissue hyperplasia are shown.; **(C)** Loss of demarcation among the bursal defined structures and random increase in the intra-follicular spaces; **(D)** Cavity formation, Fibrotic Wall, and Exudate ; **(E)** Loss of bursal architecture by the necrotic cells destruction and depletion of B-cells and epithelial lining of bursal lobes and there is loss of demarcation among the bursal defined structures; **(F)** Fibrous tissues, Inter Follicular Spaces, and Atrophy of follicles.

Intrafollicular gap: IFG, Hemorrhagic Dissemination: HD, *Vacuolar Degeneration (VD)*, *Inflammatory Cells (ICS)*, *Cavity formation: CF, Fibrotic*

Table 1. Macroscopic lesions of various organs infected with IBDV collected from the cities of the province of Punjab, Pakistan.

Organ	Gross Lesions	Total	Percentage
Bursa of Fabricius	Edema, Enlarged, Thickened	30/50	60
	Hemorrhages	8/50	15
	Atrophy	7/50	15
	Necrotising material in the lumen	5/50	10
Kidney	Pale and Swollen	36/50	72
Spleen	Relatively enlarged	40/50	80
Liver	Congested and Enlarged	36/50	75
Thymus	Atrophied	0/50	-
Thigh and Pectoral Muscles	Petechial hemorrhages on the thigh only	33/50	66
	On both muscles	18/50	36
Gizzard and Proventriculus Junction	Hemorrhages present	16/20	80
	Not present	4/20	20

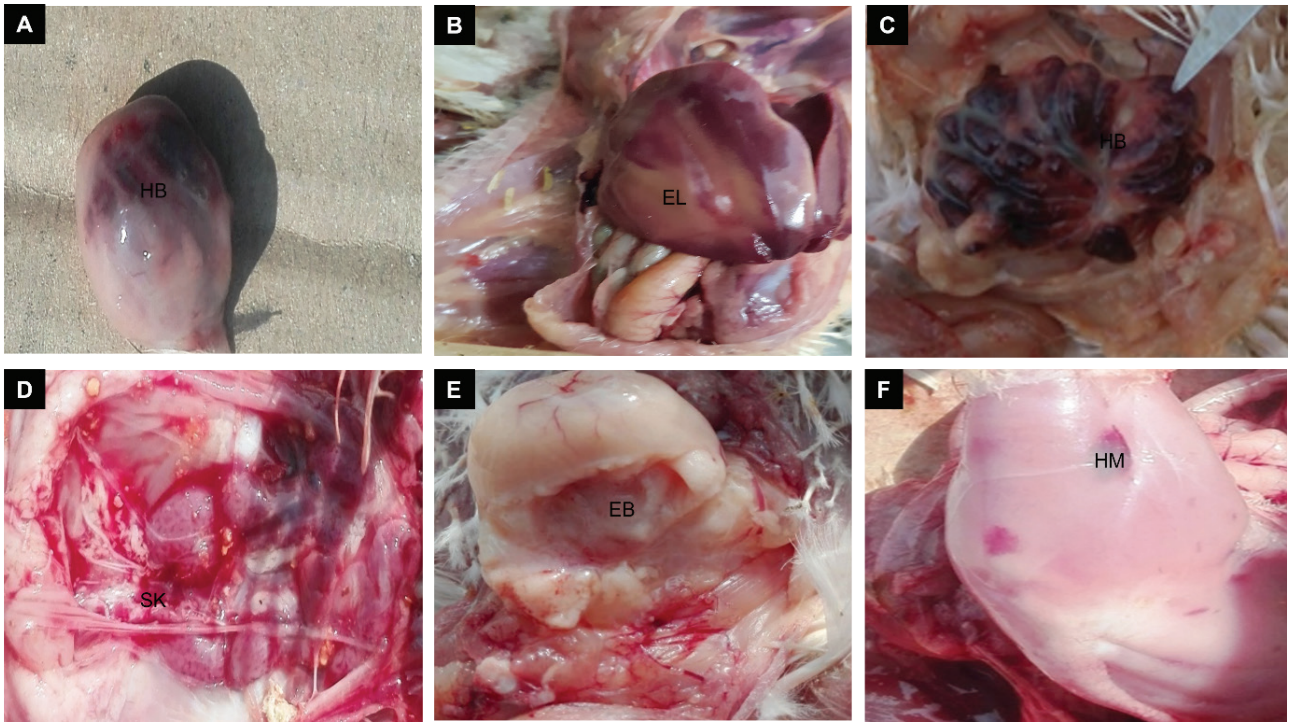

Figure 1. Gross lesion in broiler chicken infected with IBD: Representative images indicating the gross lesions on **(A)** bursa of Fabricius were observed with hemorrhagic streaks (0.3–0.4 mm). **(B)** Liver changed colour from pale to yellow with enlargement and haemorrhages; **(C)** haemorrhages inside the bursa. **(D)** urate deposition and swollen kidneys **(E)** enlarged bursa in size with length (35–50 mm) and width (20-25 mm) as compared to normal length (17-20 mm) and width (10-15 mm). **(F)** hemorrhages in the thigh muscles. *HB: Hemorrhagic Bursa; EL: Enlarged Liver; SK: Swollen Kidneys; HM: Hemorrhagic Muscles*.

Table 2. Bursa of Fabricius histopathological lesions and their scoring

Organ	Lesions	Total
Bursa of Fabricius	Normal bursa with no gaps in bursal follicles	1/25
	Edema and hemorrhages in the parenchyma of the bursa	7/25
	Necrotic and fibrotic changes in the bursa and interfollicular space development	4/25
	Cyst formation in the bursal follicles is lined with the fibrotic wall and inflammatory exudates	5/25
	Sloughing of the interfollicular epithelium and vacuolar degeneration of cells of the bursa follicle	2/25
	Atrophic changes in the follicles of the bursa	3/25
	The bursal architecture was destroyed completely	3/25

Wall: FW, Exudate: E, Necrotic Cell: NC, Loss of Demarcation: LD, Fibrous Tissue: FT, Inter Follicular Spaces: IFS, Atrophy: AT.

Molecular Detection

PCR analysis confirmed the presence of the IBDV through the visualization of amplicon bands at 929 bp for the VP2 gene fragment **(Figure 2)**. It was evident that the negative control had no band on the gel, while a sample of the bursa of Fabricius, given the names B1-B11 bands were matched the positive control, while B12 and B13 showed no bands. Control positive has a 929 base pair length, according to the ladder.

Phylogenetic Analysis

The phylogenetic study was conducted to detect of viral strain, and the VP2 sequence of the IBD virus was submitted to GeneBank. This nucleotide sequence was compared to the existing strains to find a new strain. The phylogenetic tree was established based on the bootstrap value. While 70 to 100 was considered a significant value. The values discovered closer to 98-100 were considered highly significant, while those near 70 were given non-significant status. Our investigation confirmed the value of 100 and hence considered as a highly significant value and confirmed it as 'UVAS-1' **(Fig. 2),** which showed variation compared to the other prevalent strains in Pakistan. The phylogenetic tree results were formulated by applying the Kimura 2 pattern.

Development of Similarity Index

We compared our newly discovered strains and strains prevalent in Pakistan and different countries. We concluded that the'UVAS-1' strain is approximately 98-100% similar in the index compared to other strains within Pakistan. Therefore, the virus is highly conserved in Pakistan. Comparing the 'UVAS-1' strain we discovered with strains found in other countries, the similarities are insignificant, ranging from 92 to 97.9% with strains from China, the U.S.A., the UK., and Germany, to 70 to 77% with the strain isolated in Canada. These indices indicate that the IBDV continuously changes, especially in different countries.

Similarity percentage among the discovered strains

Our investigation found that all virus strains were quite similar, although we have collected the samples from different regions of Punjab, Pakistan. All the discovered strains of the virus had 100 % similarity, and no genetic variation was evident. It can

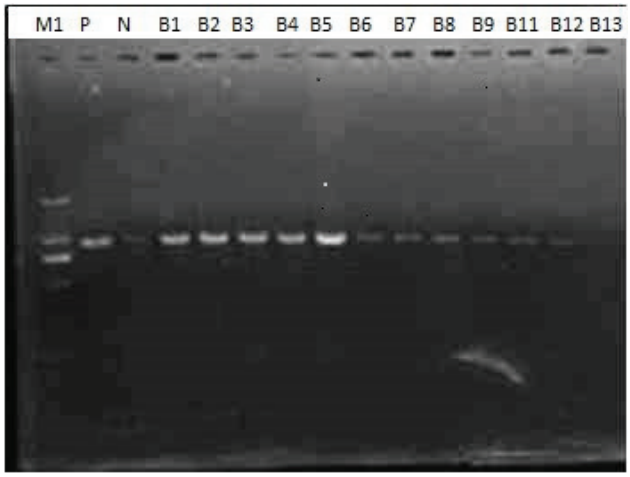

Figure 2. PCR amplicons band at base pair length of 929 for the VP2 gene fragment of IBDV. M1=DL2000, B1- B13 = Number of samples, P=Positive control, N =Negative control

be confirmed that the virus is highly conserved in Pakistan's regions. At the same time, there is no evidence of significant similarities with strains from other countries **(Fig. 3)**.

DISCUSSION

IBD is considered the second most common disease in the poultry industry in Pakistan after Newcastle disease. IBDV has an immunosuppression role due to the compromised humoral and cellular immunity after the B cell destruction, which leads to a reduced feed conversion ratio, causing huge economic losses to the poultry industry. The only solution to this problem is to control this pathogen by vaccination. However, there are reports that vaccination failure can occur due to the genetic variability of this virus to form new strains, resulting in outbreaks (Sajid *et al.*, 2021). The possible reason could be the amino acid changes (mutation), which possibly can alter the immune response of the chicken. The only way to protect the broiler birds is to vaccinate frequently and keep the hens of the same ages, but on the other side of the story, it is also problematic that frequent vaccination at various intervals of broiler chicken's growth can be the probable cause of mutation in IBDV. The quality, quantity, and method of vaccination can also be questionable. As Pak-Asian avian isolates are the hallmark of genetic variation, the

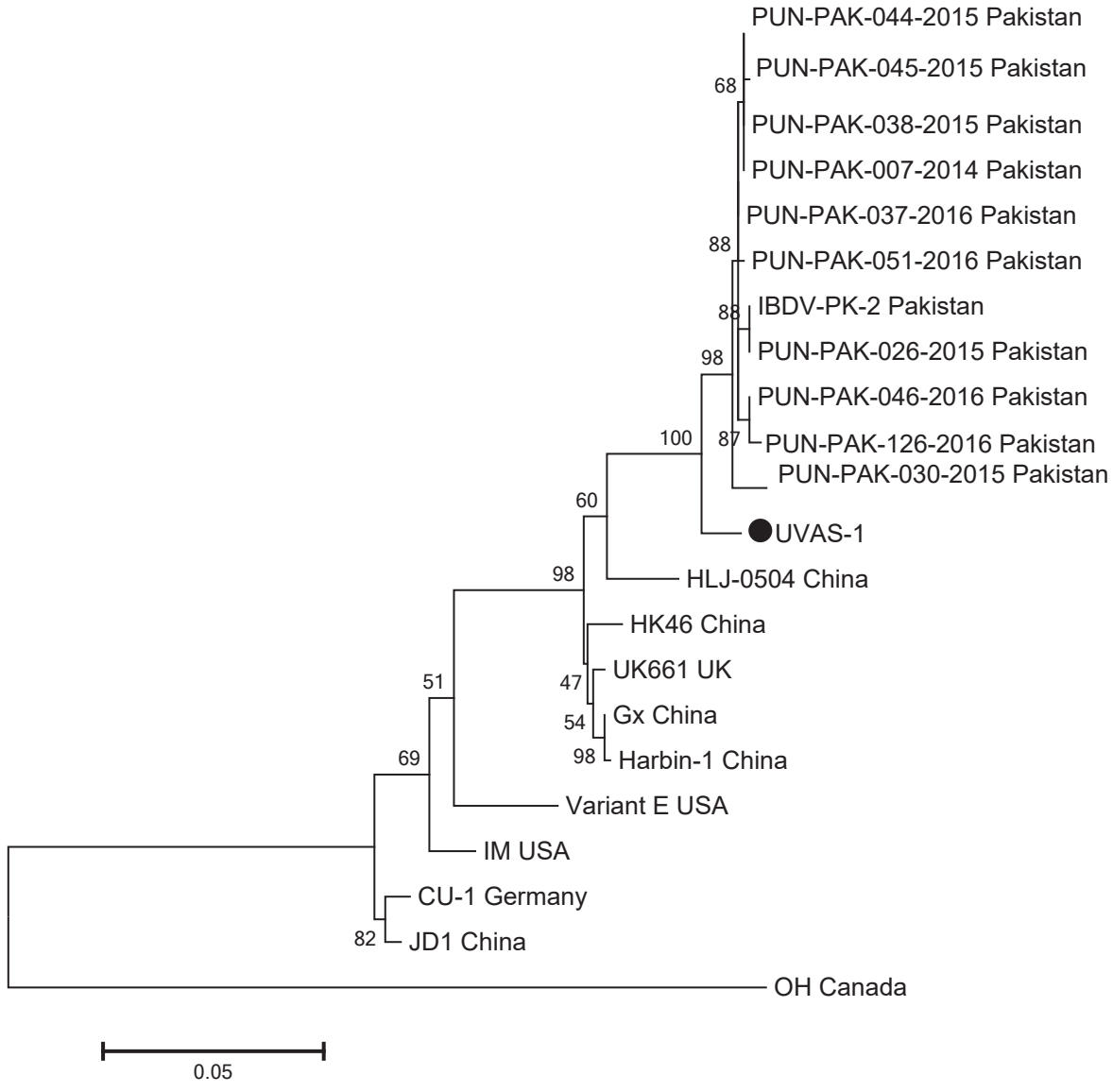

Figure 3. Phylogenetic tree of the UVAS-1 analyzed with other reported vvIBDV strains of Pakistan and other countries, with the samples collected.

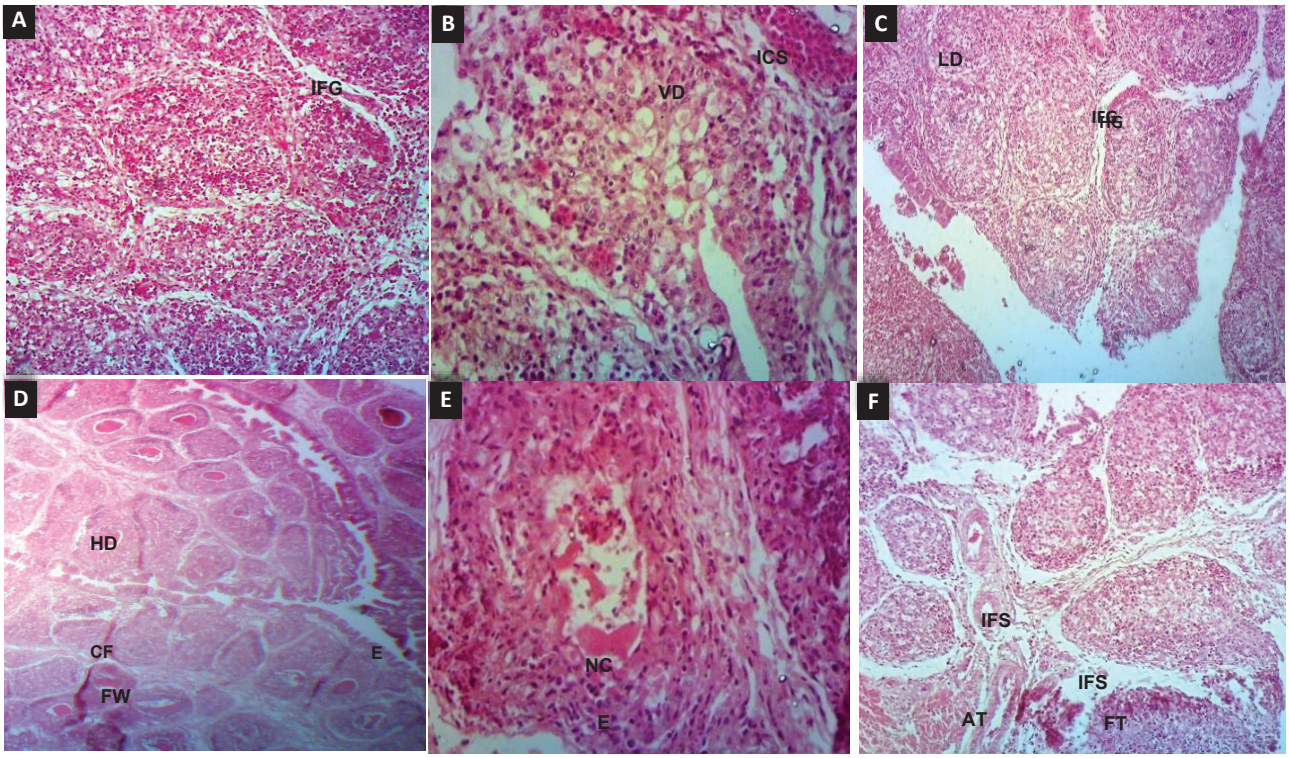

Figure 4. Histopathological lesions of the infected chicken.

location of amino acids was conserved in the same genotype (Khan *et al.*, 2017).

Our study of the clinical signs and symptoms, along with the postmortem lesions, was similar to the study done in the Chakwal district of Pakistan. Our investigation of clinical signs and symptoms also agreed with work done in Asia, Africa, Europe, and the United States. The mortality rate was almost 25% in the affected area, according to our investigation.

The pathology of the bursa induced by our newly discovered strain was severe, with enlarged and hemorrhagic bursa in all necropsy birds, and these symptoms were more evident in those birds that were in the second or third day of infection, and only at 3 weeks of age. Our findings are similar to the lesions reported by. In our investigation, we found that a few birds had a regressed and atrophied bursa, as those birds were from that age group that were facing infection for one week (3-7 days), which is quite similar to what has been investigated by.

There were hemorrhages on the thigh, pectoral muscles, junction of proventriculus and gizzard, and swollen kidney, along with mucoid material observed in a few birds during necropsy, which agrees with the research done by. There was a reduction of the bursa of Fabricius, along with atrophy in a few groups of birds where the infection was at an early stage, which again resembles the study done by which indicates that the strain was a vvIBDV. In some birds, it was observed that their liver was relatively enlarged and congested, with bladder gallbladder full of bile. Deposition of urates in the kidneys was also noted, along with bulging of the kidneys from their sockets. Similarly, the hemorrhages are found on the gizzard and proventriculus junction, thigh, and pectoral muscles. All these findings also agree with a study done by.

There were severe macroscopic as well as microscopic lesions in the samples of bursa collected from different areas of Bangladesh, which shows similarity with the work done by (Hasan *et al.*, 2010). (Taghavian *et al*., 2013) had conducted a study showing the development of interfollicular as well as interlobular spaces due to alteration in the parenchyma of the bursa. There was a fibrotic replacement and shrinkage of lobes of the bursa, which resulted in a reduction in the size of the bursa agreed with our investigation in current research.

There was vacuole formation in the parenchyma of the bursa along with infiltration of other cells into the bursa follicles in some groups of birds. Increased interfollicular spaces and depletion of lymphocytes

in our studies show that the IBDV strain is a very virulent strain, and similar lesions were observed by.

In our research, we found out that there were continuous changes in the size of the bursa at the start of the infection, the size increases and at the end of the infection it reduces, following the atrophy of the bursa and these alterations in the bursa occurs due to the exudate formation and infiltration of different kind of cells including macrophages, heterophils and lymphocytes. There was the same pattern of cyst inside the bursa, providing a clear idea of vvIBDV, and these results matched with investigations done by.

Our results from some groups of birds indicate that there was necrosis, atrophy, and infiltration of inflammatory cells in the medulla and cortex of the bursa. The necrosis of lymphocytes, fibrin deposition along with thickening of the interfollicular space, followed by hyperemia, oedema, and reticuloendothelial cell hyperplasia, gives evidence that our new strain UVAS 01 is a very virulent infectious bursal disease, and our findings are matching with a study conducted in the area of Faisalabad, Pakistan.

Field samples of different farms and diagnostic laboratories in different districts of Pakistan, including Lahore, Faisalabad, Multan, Bahawalnagar, Gojra, and Arifwala, were confirmed by pathological examination and RT-PCR. A total of 12 isolates of IBD virus from different outbreaks were characterized by performing the analysis of nucleotide sequence in the HVR of gene VP2. Twelve strains of Very Virulent IBDV gene VP2, as submitted previously to GenBank, were found to be like the existing strains. All the strains were similar to the strains reported in other countries, as well as in Pakistan. Only one strain was found to be very virulent IBDV. However, the presence of the other prevalent vvIBD virus strains cannot be excluded in other provinces of Pakistan. In previous research done in Pakistan, classical and variant IBD viruses were found by reverse Transcriptase PCR procedure (Shabbir *et al.*, 2016). As per phylogenetic analysis, five infectious bursal disease virus strains represented characteristics of amino acid (A.A) signatures and sequences in gene VP2 (*I*256, *I*242, *I*294, *S*299, *A2222*) for very vIBDV and hence classified as vvIBDV. They presented 97-99% similarity at the nucleotide level. The A.A *I*256, *A*222, and *I*294 have specific features towards the vvIBD virus strain, while Amino acids *I*256, *I*294, *I*242, and *S*299 are considered highly considered portions of very Virulent Infectious BDV (Hoque *et al.*, 2001; Shabbir *et al.*, 2016). previously documented in Pakistan, field isolates are present exclusively with similar nucleotide sequences (Lone *et al.*, 2009). Moreover, the phylogenetic analysis of diagnosed strains of vvIBDV is closely identical to those detected from China, India, and Pakistan, suggesting that there are some close temporal and geographic relationships between the strains.

The vvIBD viruses were found in border-sharing countries with Pakistan, where IBDV was characterized based on the HVR of the VP2 of infectious bursal disease virus from different outbreaks in India's North East region and pointed out two genetically different strains of Gumboro disease virus (Rajkhowa *et al.*, 2018). A study was conducted in Iraq where ten pooled samples of the bursa of Fabricius from various broiler farms were analyzed for the HVR of gene VP2 of infectious bursal disease virus, and they demonstrated five different viruses were of vvIBD (Amin and Jackwood, 2014).

We made a comparison between the newly discovered ''UVAS-01'' with previously discovered strains in Pakistan and other states. Our strain has a 98 % identity index with only 2 % dissimilarity, while compared with Pakistan, the newly discovered strain has 92 per cent per cent similarity and only 8 per cent differences from already prevalent strains discovered in other parts of the world (Hoque *et al*., 2001; Hamoud *et al*., 2006; Islam *et al*., 2012).

CONCLUSION

The resurgence of vvIBDV-caused IBD in Pakistan has resulted in substantial economic losses. Backyard poultry are particularly vulnerable due to the lack of proper surveillance. Our study confirms that IBDV is constantly mutating in the environment, and strict surveillance through advanced diagnostic techniques should be implemented on a priority basis to reduce the loss of the poultry industry in Pakistan. For the inhibition of the virus, gene VP2 is a much better focus point as compared to gene VPA because its capsid contains a unique component, neutralizing antibodies can recognize the proteins of this virus, which is very helpful in future studies about IBD infections.

Funding source

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of Interest

The authors declare no conflict of interest.

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