Pakistan J. Zool., vol. 52(2), pp 659-667, 2020. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20190210070218

Detection and Phylogeny of Infectious Bursal Disease Virus (IBDV) during Field Outbreaks in Broilers

Umm-i-Habiba[1,*], Ayesha Maqbool[2], Muhammad Safdar[3], Nida Zia[1], Altaf Mehmood[4], Muhammad Usman[5], Mehwish Sharif[6], Amanullah Khan[7] and Sajid Umar[8]

[1]*Department of Biology, Virtual University, Lahore, Pakistan*
[2]*Department of molecular Biology, Virtual University of Pakistan*
[3]*Department of Breeding and Genetics, Cholistan University of Veterinary & Animal Sciences, Bahawalpur*
[4]*Punjab Livestock and Dairy Development Department, Chakwal, Pakistan*
[5]*Poultry Research Institute, Rawalpindi, Pakistan*
[6]*University of Veterinary and Animal Sciences, Lahore, Pakistan*
[7]*Department of Microbiology, Friedrich Loeffler Institute, Jena, Germany*
[8]*Department of Pathology, PMAS-Arid Agriculture University, Rawalpindi, Pakistan*

Article Information
Received 10 February 2019
Revised 22 March 2019
Accepted 30 March 2019
Available online27 January 2020

Authors' Contribution
UH conducted the research and collected data. AM, MS and SU supervised the study and designed the study. NZ, AM, MU, MS and AK helped in sample collection, interpretation of results and article writing.

Key words
Infectious bursal disease, RT-PCR, Phylogenetic, Commercial poultry, Pakistan.

ABSTRACT

Infectious bursal disease (IBD) is economically important disease causing great losses to poultry industry worldwide. Field outbreaks of IBD in 18 different poultry farms in the Chakwal district were confirmed by clinicopathologic examination and PCR. A total of 6 isolates of IBDV from these outbreaks were genetically characterized based on hyper-variable region of the VP2 gene. IBDV strains were grouped into two distinct clusters on the basis of nucleotide sequences of hyper-variable region of the VP2 gene. According to phylogenetic analysis, 5 IBDV strains showed characteristic amino acid signatures in the VP2 gene (*A*222, *I*242, *I*256, *I*294, *S299*) and classified as vvIBDV. Furthermore, the sequencing analysis of detected field strains revealed the high similarity and close clustering with vvIBDV strains isolated from neighboring countries, suggesting geographic and temporal relationships among these strains. Interestingly, one IBDV strain clustered togather with vaccinal IBDV strains and showed 99% sequence homology with attenuated vaccine strains. Our study revealed exclusive circulation of vvIBDV and these evidences emphasize the need of further detailed and more systemic approaches to study IBDV distribution for the implementation of effective control measures.

INTRODUCTION

Infectious bursal disease (IBD), continues to be a serious threat to the poultry industry worldwide. Despite the availability and application of IBDV vaccines, the emergence of new IBDV variants can threaten poultry health and production all over the world causing significant economic losses (Yilmaz *et al*., 2018). It is one of the most devastating viral diseases and targets immune cells thus lead to severe immunosuppression (Alkie and Rautenschlein, 2016). Chickens are the primary host species for IBDV, but the antibodies and the virus are also found in wild birds including guinea fowls, ducks, quails, pheasants and ostriches with no signs of infection (Lukert and Saif, 2003). It is caused by infectious bursal disease virus (IBDV), which was first discovered in Delaware in the United States of America (USA) in 1962. IBDV primarily infects immature B cells causing pathological changes in the bursa of Fabricius of chickens, resulting in immunosuppression which in turn facilitates secondary infections and poor immune response to pathogens and vaccination (OIE, 2016).

IBDV is a non-enveloped, icosahedral, bi-segmented (segment A and Segment B), double-stranded RNA (dsRNA) virus belonging to the genus Avibirnavirus within the family *Birnaviridae* (OIE, 2016; Lukert and Saif, 2003; Alkie and Rautenschlein, 2016). The larger segment A (3.2 kbp) of the bisegmented dsRNA genome of IBDV encodes a precursor poly-protein that is processed in three mature proteins; VP2 (outer capsid), VP3 (inner capsid), and VP4 (protease). The VP2 is the major structural protein that contains antigenic regions, that is, those responsible for

* Corresponding author: Umihabiba25@gmail.com
0030-9923/2020/0002-0659 $ 9.00/0

the production of neutralizing antibodies in chickens, and for serotype specificity. The middle third of the VP2 gene contains a variable region, where stretches of hydrophilic amino acids (aa) have been recognized as the molecular basis for antigenic variation (OIE, 2016) and is used for molecular characterization of IBDV. The segment B (2.8kbp) of the IBDV genome that is a double-stranded polymer encodes a protein, VPl. Additionally, genetic analysis of VP1 gene is also performed along with VP2 gene for better In addition to VP2 sequences, the analysis of the VP1 gene of segment B is useful in providing complementary genetic information for more precise characterization of IBDV (Lukert and Saif, 2003).

There are two serotypes of IBDV and designated I and II (Jackwood and Sommer-Wagner, 2007; Lukert and Saif, 2003). Serotype II is not considered pathogenic because it does not cause disease in poultry however serotype I causes infection in poultry and consists of pathogenic strains of IBDVs (OIE, 2016). According to their pathogenicity, serotype I viruses are classified as avirulent, classical virulent strains (cIBDV), antigenic variant strains (vaIBDV), and very virulent strains (vvIBDV) (Jackwood and Sommer-Wagner, 2007; Lukert and Saif, 2003; OIE, 2016). Until the 1980s, IBDV strains were characterized low virulence, causing less than 2% specific mortality and satisfactorily controlled by vaccination. Despite vigorous vaccination strategies, it has been difficult to control IBD. Since its first isolation in 1957 the IBDV has been continuously mutating, and the wIBDV strains were isolated from many countries of Europe, Asia, and Middle East (Jackwood and Sommer-Wagner, 2007; Michel and Jackwood, 2017; Alfonso-Morales *et al*., 2013; Hernández *et al*., 2013; Mohamed *et al*., 2014; Norouzian *et al*., 2017; Vera *et al*., 2017).

A number of useful methods have been developed for the diagnosis of IBDV infections. However, most of them are laborious and time-consuming. Polymerase chain reaction (PCR) assays have been regarded cost effective, rapid and reliable for the diagnosis of poultry pathogens. This method is not only rapid, but also more sensitive and specific than other diagnostic tests. Utilization of PCR for the detection and phylogentic analysis of IBDV can help in to produce highly specific vaccines and in turn provide rapid diagnosis. The VP2 gene of IBDV is highly variable among strains and carries main protective epitopes and determinants of virulence. Therefore, this gene is mainly targeted in PCR and phylogenetic assays to study evolution within IBDVs (Michel and Jackwood, 2017; Hernández *et al*., 2013).

In Pakistan, a vvIBDV outbreak were first recorded in 1980 from Punjab, causing heavy mortality (Khan *et al*., 1998) and since then continues to be a serious problem all over country (Shabbir *et al*., 2016; Zahoor *et al*., 2011; Lone *et al*., 2009). Severe outbreaks of IBD with more than 60% mortality rate has been reported in commercial broiler flocks, despite the use of live attenuated vaccines (mild and intermediate plus) in the field. Due to lack of organized laboratory diagnostic set up and facilities in Pakistan, poultry diseases are usually diagnosed on the basis of necropsy and clinical findings. These practices in the field often leads to misdiagnosis of serious poultry pathogens thus creating hindrance in the control of contagious pathogens. Therefore, exact data about distribution and genotyping is not available for IBDVs strains. The IBDV continuous to be a serious problem in Pakistan despite vaccination in commercial poultry (Shabbir *et al*., 2016; Zahoor *et al*., 2011). Therefore, there is an urgent need to develop a more stable and efficacious vaccine for IBD control in Pakistan. The variations in the genome of pathogens due to mutations often result in misdiagnosis and inefficient control measures at poultry farms. Therefore, it is important to investigate the distribution pattern of IBDV in different regions and study mutations in its genome to develop better vaccines and risk based prevention measures against IBD infections. No data are available regarding IBDV distribution and phylogeny from commercial broiler flocks of district Chakwal, Pakistan. To the best of our knowledge, this is the first attempt on IBDV genotyping and phylogeny from this region. The main goal of the study was to detect and genotype IBDV strains circulating on poultry farms, and to add a new piece of knowledge about its characterization for the better implementation of control measures in Pakistan.

MATERIALS AND METHODS

Collection of samples

The current study plan was approved by animal welfare, ethics and research committee of Virtual University of Lahore, Pakistan. Outbreaks of severe IBD in commercial broiler flocks at different locations (Dbab Kalan, Chakral, Minwal) of Chakwal district during the period from March 2018 to October 2018 were investigated. Necropsy of dead birds was performed to collect Bursae tissue samples and to record gross lesions. For each flock, 1 or 2 pools of 10 bursae tissue were taken in small Ependorf tubes for molecular diagnosis.

RNA extraction and reverse transcription

Total RNA was extracted from bursa of Fabricius tissues using the RNeasy RNA extraction kit following the manufacturers protocols (Qiagen, USA). After that, RNA was quantified using a NanoDrop spectrophotometer (NanoDrop 1000c, Thermo Scientific, Waltham, USA). Reverse transcription and generation of cDNA was performed by using High Capacity cDNA reverse

transcription kit (Applied Biosystems) as described by the manufacturer.

Detection of IBDV using conventional PCR

A set of VP2 gene based primers 743-F: 5′-GCCCAGAGTCTACACCAT-3′ and 743-R: 5′-CCCGGATTATGTCTTTGA-3′ for the detection of IBDV was synthesized as described previously (Jackwood and Sommer, 2005). Conventional PCR was conducted to amplify VP2 gene (743 bp) using an automatic DNA thermal cycler. For this purpose, our optimized PCR reaction consisted of a total volume of 50 µl reaction mixture containing 3 µl (10 µM) of each forward and reverse primer, 25 µl Maxima Hot Start PCR Master Mix (Thermo Scientific), 15 µl nuclease-free water, 1 µl $MgCl_2$ and 3 µl of cDNA. Amplification was carried out in a thermal cycler (Biorad, Chromo-4) by following the protocol described elsewhere (Jackwood and Sommer, 2005). Briefly, 5 min at 95°C for initial denaturation followed by 30 cycles of denaturation (94°C for 60 sec), annealing (60°C for 60 sec) and extension (72°C for 60 sec) and a final extension at 72°C for 10 min. Positive and negative controls were also considered for each PCR reaction. Nuclease free water was used for negative controls in place of DNA template for all PCR reactions. A 100 bp DNA ladder was used to analyze PCR amplicons on 1.5% agarose gel.

Table I.- Infectious bursal disease virus (IBDV) reference strains used in the present study.

Genogroup	Previous classification	Reference strains (GenBank accession number)
1	Classical	228E (AF457104)
		D78 (AF499929)
		F52-70 (AY321953)
		Lukert (AY918948)
		STC (D00499)
2	Antigeneic variants	AL-2 (JF736011)
		DelE (AF133904)
		T1 (AF281238)
3	vvIBD	Henan (KT884486)
		HK46 (AF092943)
		OKYM (AF092943)
		UK661 (NC_004178)
4	dIBD	dIBDV/UY/2014/2202 (KT336459)
		MG4 (JN982252)
		TY2 (LC136880)
3	Variant/classical recombinant	Mexico04M101 (DQ916210)
6	ITA	ITA-02 (JN852986)
7	Australian	V877-W (HM071991)

Sequencing

Purification of PCR amplicons was performed following the manufacturers protocols (NucleoSpin®Gel and PCR Clean-up kit, Macherey-Nagel, Düren, Germany). Sequencing was performed by a commercial company using the PCR primers as sequencing primers.

Statistical analysis and phylogeny

Bio Edit with Clustal W aligning methods was used for editing and alignment of raw nucleaotide sequences. After alignment of sequences in BioEdit software, MEGA5 software was used for phylogeny and evolutionary analysis of VP2 gene of IBDV. Neighbor-joining method was adopted for construction of phylogenetic tree of VP2 gene based sequences. The reference sequence set for IBDV classification (Michel and Jackwood, 2017) was used to reconstruct the topology of the VP2 gene sequences generated in this study (Table I).

RESULTS

Clinical observations

The clinical signs observed in the infected flocks were depression, ruffled feathers, lethargy, poor feed intake, poor growth, and white watery diarrhea. The feathers around the cloaca were stained with feces. Mortality rates in clinically affected broiler flocks ranged from 35% to 45%. At necropsy, various gross changes were seen in bursa of Fabricius in broiler flocks detected vvIBDV. Atrophy was remarkable in some of them. They were firm and almost receded to one third of their original sizes. There were distinct edema, hemorrhages and enlargement in some bursae of Fabricius. In addition, petechial and stripe-like hemorrhages were seen on the pectoral and leg muscles of some IBDV infected birds (data not shown).

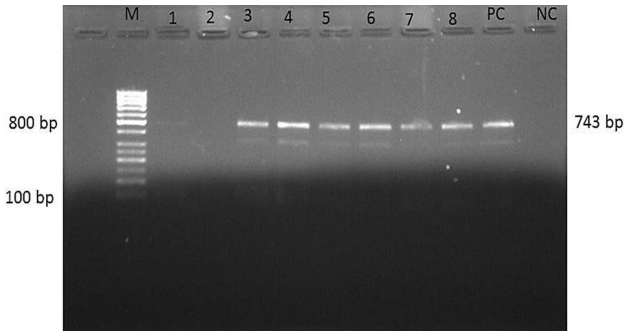

Fig. 1. Amplification product of VP2 gene from IBDV. L, DNA marker; Lanes 3 to 8, positive samples; PC, positive control; NC, negative control.

Overall detection of partial IBDV VP2 gene in broiler flocks by RT-PCR

All diseased flocks were detected in positive for IBDV by RT-PCR targeting the hypervariable region of the VP2 gene. The VP2 gene is often used to study the taxonomy and antigenic properties of IBDV. We used previsiouly reported primers to amplify the hypervariable region of VP2 gene. The designed primers amplified a DNA fragment of the expected size (743 bp) from clinical samples from infected flocks (Fig. 1). All positive controls resulted in the expected fragments and no fragment was observed in the negative control. IBDV positive samples were also analysed for the presence of Newcastle disease virus as part of routine diagnostic work by using conventional PCR to rule out any possible co-infection. All IBDV positive samples were tested negative for Newcastle disease virus. However, none of the samples were analysed for the co-infection of avian influenza, infectious bronchitis virus and fowl adenovirus. Therefore, presence of these viruses in positive samples cannot be ruled out.

Phylogenetic analysis of the partial IBDV VP2 gene sequences

The specificity of the amplified fragment was confirmed by DNA sequencing and BLAST analysis. Following submission to NCBI nucleotide blast tool, only sequences from IBDV were returned with significant similarity scores. Positive samples were successfully sequenced and subjected for phylogeny by using MEGA 5 programme. The five sequences were deposited in the GenBank database under accession numbers (MK139655, MK139656, MK139657, MK139658, MK139659). According to phylogenetic analysis, 6 IBDV strains were found to

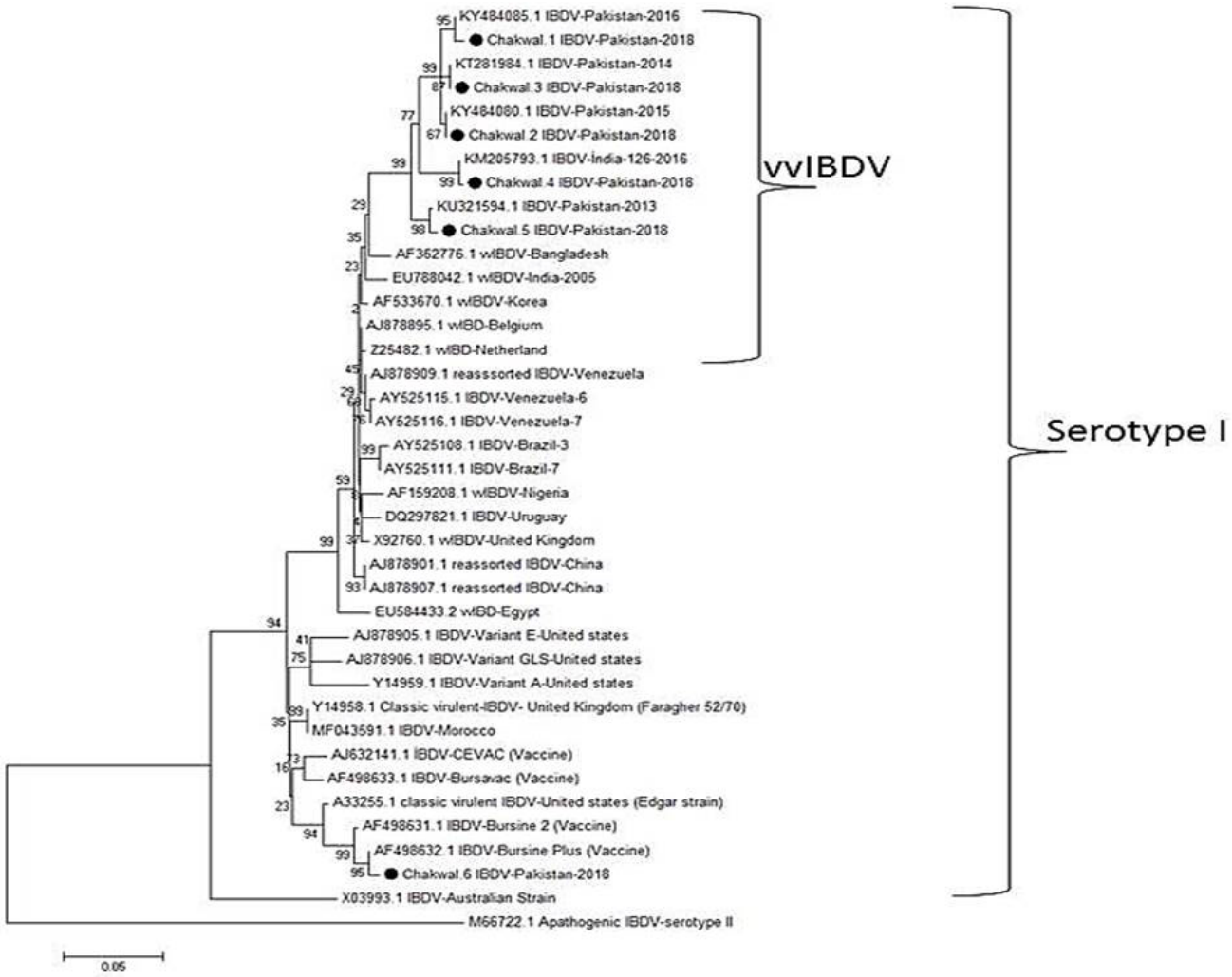

Fig. 2. Phylogenetic tree based on the VP2 gene sequences of IBDV. IBDV detected in present study have been represented with black circles.

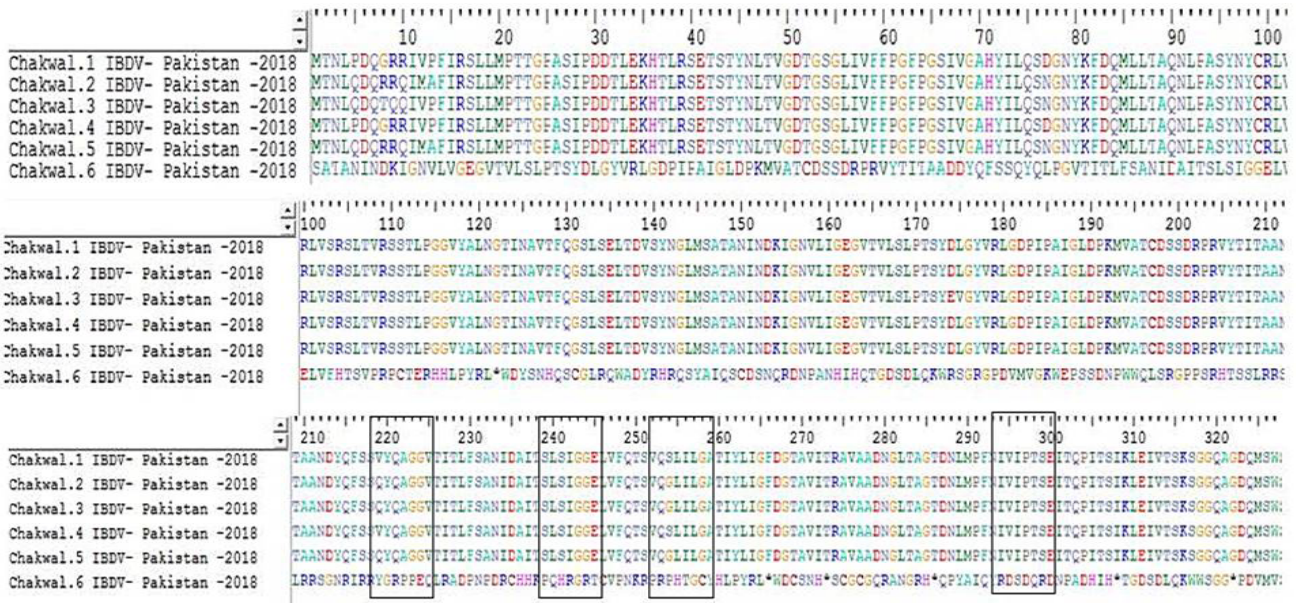

Fig. 3. VP2 protein based analysis showing amino acid pattern of IBDV. The amino acid alanine at the position 222 and other amino acids *I*242, *I*256, *I*294 and *S*299 is characteristic for vvIBDV.

cluster into two distinct clade (Fig. 2). One cluster containing 5 strains (MK139655, MK139656, MK139657, MK139658, MK139659) belonged to the vvIBDV and were related to strains reported from China, Iran, India, and Pakistan. One RT PCR positive samples (16.6%) formed cluster with vaccinal IBDV strains (Fig. 2). It is most likely that these strains might be vaccine strain since this vaccine used in the field where the samples were collected. Amino acid pattern in VP2 protein of IBDV strains showed presence of the amino acid alanine at the position 222 and other amino acids *I*242, *I*256, *I*294 and *S*299 that is characteristic for vvIBDV (Fig. 3).

DISCUSSION

Pakistan has one of the largest poultry farming operation in the world. İntensive commercial farming and improper control measures has led to rapid rise in viral infections. Thus, epidemiologic research is very important for monitoring disease outbreaks and developing vaccines. IBD has become endemic and major immunosuppressive disease in poultry in Pakistan since its emergence in early 1980s. İt is an economically important disease, causing huge losses to poultry farmers in Pakistan. Live and killed vaccines produced around the world are imported into Pakistan because there are no regulations for the importation of poultry vaccines; therefore, mild, intermediate, and hot strains are available in the local market. Virulent IBDV strains have been detected in different countries with an economic impact on poultry production worldwide (Alkie and Rautenschlein, 2016; Jackwood and Sommer-Wagner, 2007; Alfonso-Morales *et al*., 2013; Dolz *et al*., 2005; Shabbir *et al*., 2016). Poor protection due to a low titer of maternally derived antibodies is of a concern in controlling IBDV in the field as well as poor sanitation and hygiene (OIE, 2016; Lukert and Saif, 2003). The vaccines used and vaccination strategy applied are also important factor in prevention and control of IBDV infection. In order to apply good preventive and control strategies, field IBDV strains as well as vaccine strains need to be monitored. There is no comprehensive study on current IBDV strains circulating in broilers at present in Chakwal, Pakistan. The main goal of the present study was to detect and characterize the genetics of IBDV involved in recent outbreaks in broiler flocks in Chakwal district of Pakistan.

The clinical features of the vvIBDV outbreaks observed in present study were similar to those reported previously in Asia, Europe, Africa, and United States (Alkie and Rautenschlein, 2016; Shabbir *et al*., 2012; Ikuta *et al*., 2001; Nwagbo *et al*., 2016; Eterradossi *et al*., 1999; Hoque *et al*., 2001). Mortality rates in clinically affected broiler flocks ranged from 35% to 45%. The predominant necropsy findings included atrophy, enlargement, edema, hemorrhages and congestions in the bursa of Fabricius and petechial and ecchymosis hemorrhages in the pectoral and leg muscles which are consistent with previous findings reported from the field studies (Khenenou *et al*., 2017). Field outbreaks of IBD in 18 different poultry farms in

the Chakwal district were confirmed by clinicopathologic examination and reverse transcriptase PCR. A total of 6 isolates of IBDV from these outbreaks were characterized by analyzing the pattern of nucleotides in the hypervariable region of the VP2 gene. IBDV strains were grouped into two distinct clusters on the basis of nucleotide sequences, deduced amino acid sequences, and genetic analysis of the VP2 gene. Five (83.3%) of them were found to be similar to vvIBDV VP2 gene reported to GenBank previously. These strains were similar to strains reported from other countries. Only one strain was clustered with classical IBDV vaccine strains. However, presence of other IBDV strains cannot be excluded in other regions of the country. In a previous study performed in Pakistan, classical and variant IBDVs were found by RT-PCR method (Shabbir *et al*., 2016). According to phylogenetic analysis, 5 IBDV strains showed characteristic amino acid signatures in the VP2 gene (*A*222, *I*242, *I*256, *I*294, *S299*) for vvIBDV and classified as vvIBDV. They showed 97%–99% identity at the nucleotide level. The amino acids A222, I256, and I294 are considered a main characteristic feature attributed to the vvIBDV strain, and the amino acids I242, I256, I294, and S299 are known to be highly conserved in vvIBDV strains (Shabbir *et al*., 2016; Hoque *et al*., 2001; Rajkhowa *et al*., 2018). The results indicated exclusive presence of field isolates with similar nucleotide patterns as documented in Pakistan previously (Lone *et al*., 2009). Furthermore, the sequencing analysis of detected field strains revealed the high similarity and close clustering with vvIBDV strains isolated from India, Pakistan, and China, suggesting geographic and temporal relationships among these strains. Interestingly, one IBDV strain showed 99% sequence homology with attenuated vaccine strains in the VP2 gene and clustered together suggesting possible role of attenuated vaccines in the outbreaks of IBD.

The vvIBDVs were also reported in neighboring countries to Pakistan. Rajkhowa *et al*. (2018) characterized IBDV on the basis of highly variable parts of the VP2 gene of IBDV from field outbreaks in North east region of İndia and revealed two genetically diverse strains of very virulent IBDV (vvIBDV) and one intermediate strain circulating in the North east region of İndia. Phylogenetic analyses of the hypervariable region of the VP2 gene of IBDV in 10 pooled bursa Fabricius samples collected from broiler farms in Iraq have demonstrated that 5 of these viruses were vvIBDV (Amin *et al*., 2014). In Iran, a total of 10 vvIBDVs were analyzed for the diversity of the VP2 gene. Amongst these viruses, 97.6-100% similarities were found indicating the presence of a common ancestor virus circulating in Iran. However, they were partially different from previous Iranian and neighboring countries' isolates (96.2-97.3% similarity to Shiraz isolate and 95.7-96.7% to Iraq) (Norouzian *et al*., 2017). A recently published study indicate that vvIBDV strains similar to those circulating in various countries in the Middle East are present and undergoing evolution in chickens from Turkish broiler flocks (Yilmaz *et al*., 2018). In Egypt, hypervariable region of theVP2 gene in seven strains were sequenced and homology was found to be around 95.6-99.1%. On the basis of amino acid patterns, these viruses were considered as vvIBDV (242I, 256I, 294I, 299S) (Mohamed *et al*., 2014). Phylogenetic analysis revealed three different clusters of IBDV strains. These isolates showed close association with vvIBDV strains of European and Asian countries (Japanese and Hong Kong) indicating different ancestors of the isolates (Mohamed *et al*., 2014). Similarly, Li *et al*. (2015) analyzed genomic pattern of segment A and B and reported a close association and similar clustering for IBDV Strain (SH99) with previously reported strains of IBDV in China. On the other hand, extensive evolution and changes were shown by recently isolated Chinese vvIBDV (HLJ0504, HeB10 and HuN11) strains in both segments and formed a divergent genetic lineage from previously reported Chinese vvIBDV.

Previous reports from different geographical regions indicated that phylogenetic analyses of the hypervariable region of VP2 gene sequences of vvIBDV strains clustered in one major phylogenetic lineage (Cortey *et al*., 2012; Silva *et al*., 2013). However, recent reports from China on vvIBDV showed distinct clusters and genetic lineages based on nucleotide pattern of VP2 gene. Despite similarity in antigenic characteristics with previous isolates of vvIBDV, the recent isolates of vvIBDVs have several changes in their genome and exhibited enhanced pathogenicity than to the European vvIBDV (Li *et al*., 2015; Alkie and Rautenschlein, 2016). Similar findings have been reported in the USA since numerous VP2 gene sequences of IBDV field isolates were different from any known IBDV VP2 gene sequence (Jackwood *et al*., 2009). All of these reports indicate that evolution of the VP2 gene is continuous and diverse vvIBDVs are circulating in the fields posing a risk to poultry health. IBDV Sequence analysis from field outbreaks in USA has revealed that their amino acid sequences in the hypervariable region of the VP2 gene were similar to the vvIBDV strains UK 661, OKYM and Harbin (Jackwood *et al*., 2009; Jackwood and Sommer-Wagner, 2007). Similarly, a study in Europe revealed higher similarity and close association of European IBDV strains to USA variant viruses (Zahoor *et al*., 2011). In Italy, researchers detected four vvIBDV strains (DV86), one classical strain (HPR2) along with eight vaccine strains at the same time from field samples indicating circulation of

divergent IBDV strain in İtalian poultry flocks (Lupini *et al*., 2016). In Slovenia, phylogenetic analyses of VP2 gene has indicated that recent vvIBDV isolates were closely related to those from outbreaks reported in the 1990s (Rojs *et al*., 2008). Alfonso-Morales *et al*. (2013) retrieved IBDV sequences Genbank of different geographical locations and studied nucleotide pattern in the VP2 gene from IBDV strains. Phylogenetic analysis revealed a tendency of close association and clustering among IBDV Phylogenetic analysis revealed that viruses analyzed from individual countries tend to cluster in the same phylogeny. According to spatial analysis, IBDV strains with high virulence characters were appeared in 1981 in Iran for the first time and then simultaneously spread to other European (Belgium, 1987), African (Egypt, 1990), Asian (China and Japan, 1993) and USA (Cuba, 1995 and Brazil, 2000) (Alfonso-Morales *et al*., 2013).

In Pakistan, chickens are routinely vaccinated with various IBDV vaccines, representing heterogeneous genotypes, including recent introduction of virulent (intermediate plus) vaccine strains to control vvIBDV in poultry. The presence of these recently introduced vaccine a strain was only detected in one flock as part of this study. The co-persistence of vvIBDV and vaccine strains in poultry flocks, which could favor the emergence of new IBDV variants and escape mutants. The vvIBDV strains identified in the present study displayed an evolutionary rate within the range expected for RNA viruses. Further studies are warranted to understand if the observed evolution is attributable to vaccine-induced selection or to a stochastic genetic drift. Moreover, study of the relevant role of recombination events in the genesis of the vvIBDV genetic variability would also helpful to understand the mechanism of evolution among circulating strains. The spread of the vvIBDV strains in broiler farms in Chakwal, Pakistan might be associated with improper vaccination strategy, biosecurity, trade between neighbor countries and backyard poultry farming. Moreover, field exposure of young chicks with other immunosuppressive pathogens might decreases immunity resulting in the entry of field strain of vvIBDV. Pohjola *et al*. (2017) reported antibodies to IBDV in non-vaccinated backyard chicken in Finland. Similar investigation should also be done in backyard chicken in Pakistan to elucidate the epidemiological picture of IBDV. The vvIBDVs' similar to those circulating in various countries in the Asia and Middle East were identified in chickens from Chakwal broiler flocks. Pathologic changes were mainly detected in the bursa of Fabricius of infected chickens. The results of phylogenetic analysis indicate that there is exclusive presence of vvIBDV strains at Chakwal broiler flocks. A comprehensive epidemilogical surveys for the detection and characcterization of IBDV strains involved in recent outbreaks throughout the country using molecular tools will improve the understandings of the global epidemiology of the infection and help in better control of the disease.

Statement of conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

Alfonso-Morales, A., Martínez-Pérez, O., Dolz, R., Valle, R., Perera, C. L., Bertran, K., Frías, M.T., Majó, N., Ganges, L. and Pérez, L.J., 2013. Spatiotemporal phylogenetic analysis and molecular characterisation of infectious bursal disease viruses based on the VP2 hyper-variable region. *PLoS One*, **8**: e65999. https://doi.org/10.1371/journal.pone.0065999

Alkie, T.N. and Rautenschlein, S., 2016. Infectious bursal disease virus in poultry: Current status and future prospects. *Vet. Med*., **7**: 9–18. https://doi.org/10.2147/VMRR.S68905

Amin, O.G.M. and Jackwood, D.J., 2014. Identification and molecular analysis of infectious bursal disease in broiler farms in the Kurdistan Regional Government of Iraq. *Trop. Anim. Hlth. Prod*., **46**: 1297–1301. https://doi.org/10.1007/s11250-014-0643-0

Cortey, M., Bertran, K., Toskano, J., Majó, N. and Dolz, R., 2012. Phylogeographic distribution of very virulent infectious bursal disease virus isolates in the Iberian Peninsula. *Avian Pathol*., **41**: 277–284. https://doi.org/10.1080/03079457.2012.682562

Dolz, R., Majó, N., Ordóñez, G. and Porta, R., 2005. Viral genotyping of infectious bursal disease viruses isolated from the 2002 acute outbreak in Spain and comparison with previous isolates. *Avian Dis*., **49**: 332–339. https://doi.org/10.1637/7299-110204R1.1

Eterradossi, N., Arnauld, C., Tekaia, F., Toquin, D., Le Coq, H., Rivallan, G., Guittet, M., Domenech, J., van den Berg, T.P. and Skinner, M.A., 1999. Antigenic and genetic relationships between European very virulent infectious bursal disease viruses and an early West African isolate. *Avian Pathol*., **28**: 36–46. https://doi.org/10.1080/03079459995028

Hernández, M., Tomás, G., Hernández, D., Villegas, P., Banda, A., Maya, L., Panzera, Y. and Pérez, R., 2011. Novel multiplex RT-PCR/RFLP diagnostic test to differentiate low- from high-pathogenic strains and to detect reassortant infectious bursal disease virus. *Avian Dis*., **55**: 368-374. https://doi.

org/10.1637/9672-013111-Reg.1

Hoque, M. M., Omar, A. R., Chong, L. K., Hair-Bejo, M. and Aini, I., 2001. Pathogenicity of SspI-positive infectious bursal disease virus and molecular characterization of the VP2 hypervariable region. *Avian Pathol*., **30**: 369–380. https://doi.org/10.1080/03079450120066377

Ikuta, N., El-Attrache, J., Villegas, P., García, E.M., Lunge, V.R., Fonseca, A.S., Oliveira, C. and Marques, E.K., 2001. Molecular characterization of Brazilian infectious bursal disease viruses. *Avian Dis*., **45**: 297–306. https://doi.org/10.2307/1592968

Jackwood, D.J. and Sommer, S.E., 2005. Molecular studies on suspect very virulent infectious bursal disease virus genomic RNA samples. *Avian Dis*., **49**: 246–251. https://doi.org/10.1637/7294-102604R

Jackwood, D.J. and Sommer-Wagner, S., 2007. Genetic characteristics of infectious bursal disease viruses from four continents. *Virology*, **365**: 369–375. https://doi.org/10.1016/j.virol.2007.03.046

Jackwood, D.J., Sommer-Wagner, S.E., Stoute, A.S.T., Woolcock, P.R., Crossley, B.M., Hietala, S.K. and Charlton, B.R., 2009. Characteristics of a very virulent infectious bursal disease virus from California. *Avian Dis*., **53**: 592–600. https://doi.org/10.1637/8957-061109-Reg.1

Khan, K.N.M., Shah, S.A. and Afzal, M., 1998. Observations on Gumboro disease (infectious bursal disease) in Pakistan. *Rev. Sci. Tech. Off. Int. Epiz*. **7**: 625–629. https://doi.org/10.20506/rst.7.3.366

Khenenou, T., Bougherara, M., Melizi, M. and Lamraoui, R., 2017. Histomorphological study of the bursae of fabricius of broiler chickens during Gumboro disease in Algeria Area. *Glob. Vet*., **18**: 132-136.

Li, K., Courtillon, C., Guionie, O., Allée, C., Amelot, M., Qi, X., Gao, Y., Wang, X. and Eterradossi, N., 2015. Genetic, antigenic and pathogenic characterization of four infectious bursal disease virus isolates from China suggests continued evolution of very virulent viruses. *Infect. Genet. Evol*., **30**: 120–127. https://doi.org/10.1016/j.meegid.2014.12.016

Lone, N.A., Rehmani, S.F., Kazmi, S.U., Muzaffar, R., Khan, T.A., Khan, A., Khan, S.A. and Ahmed, A., 2009. Molecular characterization of Pakistani field isolates of infectious bursal disease virus. *Avian Dis*., **53**: 306–309. https://doi.org/10.1637/8325-042108-Reg.1

Lukert, P. and Saif, Y., 2003. Infectious bursal disease. In: *Diseases of poultry* (eds. Y. Saif, J. Glison, A. Fadly, L. McDougald, D. Swayne), 11th edn. Iowa State University Press, Amsterdam, pp. 161–179.

Lupini, C., Giovanardi, D., Pesente, P., Bonci, M., Felice, V., Rossi, G., Morandini, E., Cecchinato, M. and Catelli, E., 2016. A molecular epidemiology study based on VP2 gene sequences reveals that a new genotype of infectious bursal disease virus is dominantly prevalent in Italy. *Avian Pathol*., **45**: 458–464. https://doi.org/10.1080/03079457.2016.1165792

Michel, L.O. and Jackwood, D.J., 2017. Classification of infectious bursal disease virus into genogroups. *Arch. Virol*., **162**: 3661–3670. https://doi.org/10.1007/s00705-017-3500-4

Mohamed, M.A., Elzanaty, K.E.S., Bakhit, B.M. and Safwat, M.M., 2014. Genetic characterization of infectious bursal disease viruses associated with Gumboro outbreaks in commercial broilers from Asyut Province, Egypt. *ISRN Vet. Sci*., **2014**: 916412. https://doi.org/10.1155/2014/916412

Norouzian, H., Farjanikish, G. and Hosseini, H., 2017. Genetic and pathologic characteristics of infectious bursal disease viruses isolated from broiler chickens in Iran during 2014-2015. *Acta Virol*., **61**: 191–196. https://doi.org/10.4149/av_2017_02_09

Nwagbo, I.O., Shittu, I., Nwosuh, C.I., Ezeifeka, G.O., Odibo, F.J.C., Michel, L.O., Jackwood, D.J., 2016. Molecular characterization of field infectious bursal disease virus isolates from Nigeria. *Vet. World*, **9**: 1420–1428. https://doi.org/10.14202/vetworld.2016.1420-1428

OIE, 2016. Infectious bursal disease (Gumboro disease). Ch. 2.3.12. *OIE manual of diagnostic tests and vaccines for terrestrial animals*. OIE, Paris, pp. 1-21.

Pohjola, L., Tammiranta, N., Ek-Kommonen, C., Soveri, T., Hänninen, M.L., Fredriksson-Ahomaa, M. and Huovilainen, A., 2017. A survey for selected avian viral pathogens in backyard chicken farms in Finland. *Avian Pathol*., **46**: 166–172. https://doi.org/10.1080/03079457.2016.1232804

Rajkhowa, T.K., Vanlalruati, C. and Arya, R.S., 2018b. Genetic characterization of infectious bursal disease viruses from field outbreaks of the North East Region of India. *Avian Dis*., **62**: 218–225. https://doi.org/10.1637/11810-021118-Reg.1

Rojs, O.Z., Krapez, U., Slavec, B., Mankoc, S., Juriric-Cizerl, R. and Barlic-Maganja, D., 2008. Molecular characterisation of infectious bursal disease viruses isolated in recent acute outbreaks in Slovenia. *Acta Vet. Hung*., **56**: 255–264. https://doi.org/10.1556/AVet.56.2008.2.13

Shabbir, M.Z., Ali, M., Abbas, M., Chaudhry, U.N.,

Zia-Ur-Rehman and Munir, M., 2016. Molecular characterization of infectious bursal disease viruses from Pakistan. *Arch. Virol*., **161**: 2001–2006. https://doi.org/10.1007/s00705-016-2869-9

Silva, F.M.F., Vidigal, P.M.P., Myrrha, L.W., Fietto, J.L.R., Silva, A. and Almeida, M., R. 2013. Tracking the molecular epidemiology of Brazilian infectious bursal disease virus (IBDV) isolates. *Infect. Genet. Evol*., **13**: 18–26. https://doi.org/10.1016/j.meegid.2012.09.005

Vera, F., Craig, M.I., Olivera, V., Rojas, F., König, G., Pereda, A. and Vagnozzi, A., 2015. Molecular characterization of infectious bursal disease virus (IBDV) isolated in Argentina indicates a regional lineage. *Arch. Virol*., **160**: 1909–1921. https://doi.org/10.1007/s00705-015-2449-4

Yilmaz, A., Turan, N., Bayraktar, E., Gurel, A., Cizmecigil, U.Y., Aydin, O., Bamac, O.E., Cecchinato, M., Franzo, G., Tali, H.E., Cakan, B., Savic, V., Richt, J.A. and Yilmaz, H., 2018. Phylogeny and evolution of infectious bursal disease virus circulating in Turkish broiler flocks. *Poult. Sci*., **98**: 1976–1984. https://doi.org/10.3382/ps/pey551

Zahoor, M., Abubakar, M., Naim, S. and Arshed, Q., 2011. Molecular typing of field isolates from two outbreaks of infectious bursal disease virus from Pakistan. *Vet. World*, **4**: 297-300. https://doi.org/10.5455/vetworld.4.297