Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(1): 1036-1040

Received: 06-11-2018
Accepted: 10-12-2018

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Molecular detection of very virulent strains of infectious bursal disease virus from commercial broiler chickens

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Abstract
The present study was conducted to characterize the infectious bursal disease (IBD) virus by phylogenetic analysis from IBD-affected broiler flocks in Haryana, India. Phylogenetic analysis revealed very virulent, virulent strains and classical strains of IBDVs of this study. All the IBDV strains of this study had 96.7-99.9% homology among themselves. The similarity with other already reported Indian strains was between 92.3-97.4% while it was 89.5-93.2% with IBDV strains from abroad. The deduced amino acids sequence analysis of VP2 gene revealed that 18 of the 20 IBDVs were of very virulent nature due to amino acid substitution at positions 242I, 253Q, 256I, 272I, 279D, 284A, 294I, 299S and 330S, specific to very virulent IBDV strains. The remaining two field strains showed substitutions at positions 272T, 279N, 284A, 294I, 299S and 330S which are characteristics of intermediate plus variant strain of IBD viruses. The substitution of amino acid at position 300 (glutamic acid to alanine) in a field strain HR/2960/15, at 359 (threionine to lysine) in a field strain HR/3123/16 and at 384 (isoleucine to valine) in field strains HR/0955/15, HR/2960/15 and HR/2940/15 was observed. Hence, it was concluded that there is a circulation of very virulent, virulent, classical and intermediate plus variant strains of IBDVs in broiler chickens in Haryana, India.

Keywords: Infectious bursal disease, reverse transcription polymerase chain reaction, RNA

1. Introduction
Infectious bursal disease (IBD), caused by infectious bursal disease virus (IBDV), is a highly contagious and immunosuppressive disease in young chicken causes significant losses to the poultry industry. It was first discovered by Cosgrove in 1962 [4] from an outbreak in Gumboro area of Southern Delaware, so it was called Gumboro disease. Infectious bursal disease virus (IBDV) belongs to family *Birnaviridae* of the genus *Avibirnavirus*. The virus is double stranded RNA, bi-segmented (segments A and B), non-enveloped and icosahedral (Dobos *et al*., 1979; Murphy *et al*., 1995, Nwagbo *et al.*, 2016) [5, 19, 20]. Two serotypes: serotype-1 and serotype- 2 have been discovered on the basis of virus neutralization test. Serotype-1 IBDVs vary in virulence and pathogenicity, and cause disease and immunosuppression in chickens. On the basis of heterogeneous antigenecity and sequence analysis, serotype-1 viruses are classified as attenuated, classical virulent, intermediate virulent, very virulent (vv) and antigenic variant strains (Kataria *et al.*, 2001) [10]. Viruses of serotype-2 were isolated from turkeys and are non-pathogenic to both turkeys and chickens. Serotype-2 viruses produce neither disease nor immunity against pathogenic strains of serotype-1 (Muller *et al*., 2003; Cortey *et al.*, 2012; Vera *et al.*, 2015) [18, 3, 26].
In India the IBD was reported for the first time by Mohanty *et al*. (1971) [15] and it remained in its classical form until 1990's. The vvIBDVs are capable of establishing themselves even in the presence of maternal antibodies that are normally protective against virulent IBDVs (Jackwood and Sommer, 2010) [7]. In an earlier study, Mor *et al*. (2013) [16] collected bursal samples from 30 IBD-affected commercial broiler flocks during 2008-2009 and reported the presence of IBDVs in 28 flocks by reverse transcription-polymerase chain reaction (RT-PCR). It is important that the viruses prevalent in the region are characterized regularly to better understand the genetic variability in them. Hence, the present study was undertaken to characterize the IBDV strains of India and abroad.

2. Material and Methods

A) Sample collection

Bursal samples were collected aseptically from 85 chicken flocks suspected to be suffering from IBD during the period from 2012- 2016 from different parts of Haryana, India. In almost all the IBD-affected flocks, the symptoms of dullness, depression, anorexia, ruffled feathers and yellowish white or greenish yellow diarrhoea were observed. The post-mortem lesions included oedematous and swollen bursa, presence of gelatinous exudates around bursa with haemorrhages and haemorrhages on thigh muscles were recorded. The collected samples were stored immediately at -20^{0}C until further processed for total RNA isolation. Reverse transcription-polymerase chain (RT-PCR) was carried out to amplify the hypervariable region of VP2 gene of IBDV using one-step RT-PCR Kit (Qiagen, Germany). A primer pair, VP2 forward 5′-GGCCCAGAGTCTACACCATAAC-3′ and reverse 5′-CCGGATTATGTCTTTGAAGCC-3′ was used which generated PCR product of size 743 bp (Preeti, 2016) [23].

B) Purification of PCR products and Sequencing: Sequencing and Phylogenetic analysis

The PCR products of 20 IBDV positive samples were purified using QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol. The purified PCR products were got sequenced using forward and reverse primers at DNA Sequencing facility, Department of Animal Biotechnology, LUVAS, Hisar, using ABI3130XL (Applied Biosystem, USA) sequencer. These purified PCR products were subjected to sequence analysis of hypervariable region of VP2 gene. Using 'BioEdit' programme, the nucleotide sequence data was aligned and analysed for nucleotide position (704-1249 of KJ547674). The nucleotide sequences were translated to amino acid sequences position (239-400 based on amino acid sequence of KJ547674) were compared with 29 previously reported IBDV strains from different countries using MEGA 6.0. The evolutionary tree was drawn (Fig.1) using the Neighbor-Joining method and tree robustness was estimated using 1000 replicates (Tamura *et al*. 2013) [25].

C) Sequence data to GenBank

The VP2 gene sequence data of this study used for phylogenetic analysis were submitted to the GenBank database with the following accession numbers: MF978250(HR/2178/15), MF978251(HR/2391/15), MF97825(HR/2377/15), MF978253(HR/2210/15), MF978254(HR/2230/15), MF978255 HR/2335/15), MF978256(HR/2409/15), MF978257(HR/2273/15), MF978258(HR/2275/15), MF978259(HR/2410/15), MF978260(HR/2932/15), MF978261 (HR/2939/15), MF978262(HR/2940/15), MF978263(HR/2956/15), MF978264 (HR/2957/15), MF978265(HR/2960/15), MF978266(HR/0955/15), MF978267 (HR/2969/15), MF978268(HR/3123/16) and MF978269(HR/3245/16).

3. Results and Discussion

The Department provides disease investigation services to the livestock and poultry farmers in the state. The aim of the present study was to detect and characterize the IBDV strains from broiler flocks affected with IBD. A band of 743 bp for VP2 gene was observed in 83 (97.64%) of the 85 samples from IBD-affected flocks by gel electrophoresis. The specificity of RT-PCR was further confirmed by the absence of amplification in the negative control.

Phylogenetic analysis based on partial nucleotide sequences of 20 IBDV strains of this study with 29 previously reported IBDV strains from different countries revealed that 17 of the 20 IBDV strains of this study clustered with vvIBDVs from India and abroad (Fig. 1). Of these 17 vvIBDVs, nine strains (HR/2335/15, HR/2957/15, HR/2391/15, HR/2377/15, HR/2409/15, HR/3245/16, HR/2178/15, HR/2230/15 and HR/2275/15) were close to a virulent strain from India i.e. VRDC-IBDV-NZ/India. Three strains namely (HR/2210/15, HR/2273/15 and HR/2969/15) were close to another virulent strain from India i.e. VV-IBDV-FS16/India. Two IBDV strains of this study namely (HR/2939/15 and HR/2956/15) were close to the virulent strain VRDC-IBDV-EZ/India. Three strains of this study (HR/2410/15, HR/2932/15 and HR/3123/16) were close to a virulent strain of Pakistan (PK-1) and India (Barielly, India). Two strains namely HR/2940/15 and HR/0955/15 of this study clustered with NKL-14/India, again a virulent strain from India. One of the IBDV strain i.e. HR/2960/15 clustered with classical strains of IBDV i.e. 52/70 UK, AR-C7/IBDV/Argentina, D78/USA and GLS/USA strains.

The previously reported vvIBDV strains from Haryana (HR/9/03/India, HR/1/03/India and HR/5/03/India) though were in the same cluster but were distantly placed from IBDVs of this study. The IBDVs showed further divergence. Hence, phylogenetic analysis revealed that out of 20 IBDVs of this study, 17 were of very virulent nature. Two field strains (HR/2940/15 and HR/0955/15) clustered with a virulent strain of India and the remaining one field strain was classical virulent type. Phylogenetic analysis done by Vera *et al.* (2015) [26] revealed that out of 51 field strains, 42 formed a unique cluster, seven strains were typical classical strains in which one of them was a vaccine strain and two belonged to the very virulent strains. Owolodun *et al.* (2015) [21] reported four IBDV isolates from two outbreaks of Northern Nigeria as very virulent type.

The deduced amino acid sequence analysis of hypervariable region of VP2 gene showed the presence of amino acids as 242I, 253Q, 256I, 272I, 279D, 284A, 294I, 299S and 330S in 18 IBDVs of this study; the presence of these amino acids at the positions is suggestive of very virulent nature of field strains (Tables 1,2; Fig. 2). Similar findings of amino acid pattern for very virulent strains of IBD virus were observed by many workers (Jackwood *et al.,* 2008; Islam *et al.,* 2012; Xu *et al.,* 2015; Morla *et al.,* 2016; Patel *et al.,* 2016, Shehata *et al.,* 2017, Awandkar *et al.,* 2018) [8, 6, 27, 17, 22, 24, 2].

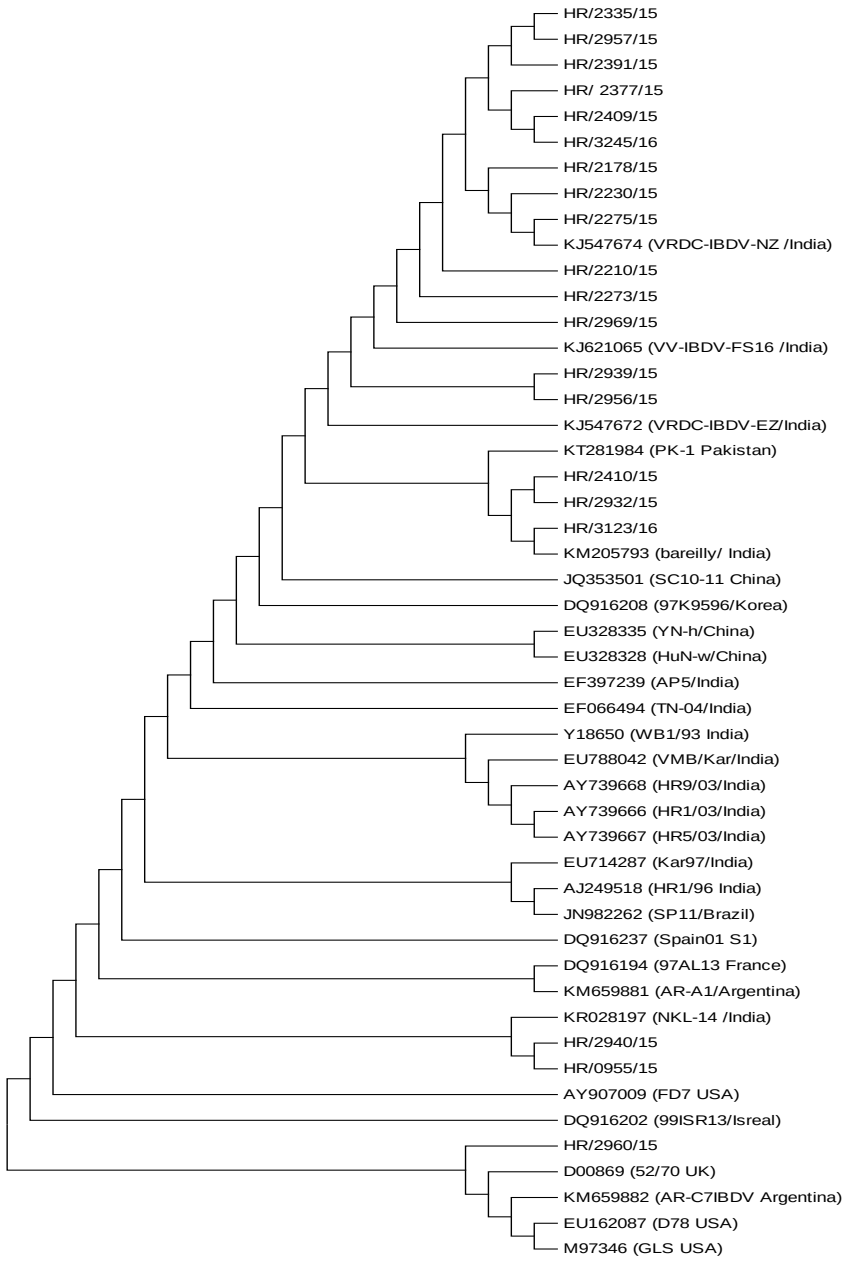

Fig 1: Phylogenetic analysis based on partial amino acid sequences of VP2 gene of infectious bursal disease virus (The evolutionary tree was using Neighbor-Jonining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to inter the phylogenetic tree in MEGA 6.0. The sequences with GenBank accession numbers are already published sequences, whereas the sequences of the present study do not have the accession number.

Remaining two field strains showing amino acid sequence (272T (Therionine), 279N (Asparagine), 284A, 294I, 299S and 330S which are characteristic to intermediate plus variant strains of IBD viruses. The serine rich heptapeptide sequence (326SWSASGS332) was observed in all 20 IBD strains. These studies were also reported by Morla *et al.*, 2016 [17]. Substitutions of glutamic acid (E) to alanine (A) at amino acid position 300 in a field strain HR/2960/15, threionine (T) to lysine (K) at position 359 in a field strain HR/3123/16, isoleucine (I) to valine (V) at position 384 in field strains HR/0955/15, HR/2960/15 and HR/2940/15 were observed in this study.

Table 1: Amino acids at different positions in VP2 gene of infectious bursal disease virus

Genotype	222	242	249	253	254	256	272	279	284	294	299	330
Very virulent (vv)	A	I	Q	Q	G	I	I	D	A	V/I	S	S
Intermediate plus variant (IPV)	A	I	Q	Q	G	I	T	N	A	I	S	S

Table 2: Amino acid changes observed in infectious bursal disease virus strains of the present study.

Amino acids position	Amino acids in reference strain	Amino acids in present study
242	Isoleucine (I)	Isoleucine (I)
249	Glutamine (Q)	Glutamine (Q)

253	Glutamine (Q)	Glutamine (Q)
254	Glycine (G)	Isoleucine (I)
256	Isoleucine (I)	Isoleucine (I)
272	Isoleucine (I)	Isoleucine (I), Threionine (T)
279	Aspartic acid (D)	Aspartic acid (D), Asparagine (N)
284	Alanine (A)	Alanine (A)
294	Isoleucine (I)	Isoleucine (I)
299	Serine (S)	Serine (S)
300	Glutamic acid (E)	Alanine (A)
330	Serine (S)	Serine (S)
359	Threionine (T)	Lysine (K)
384	Isoleucine (I)	Valine (V)

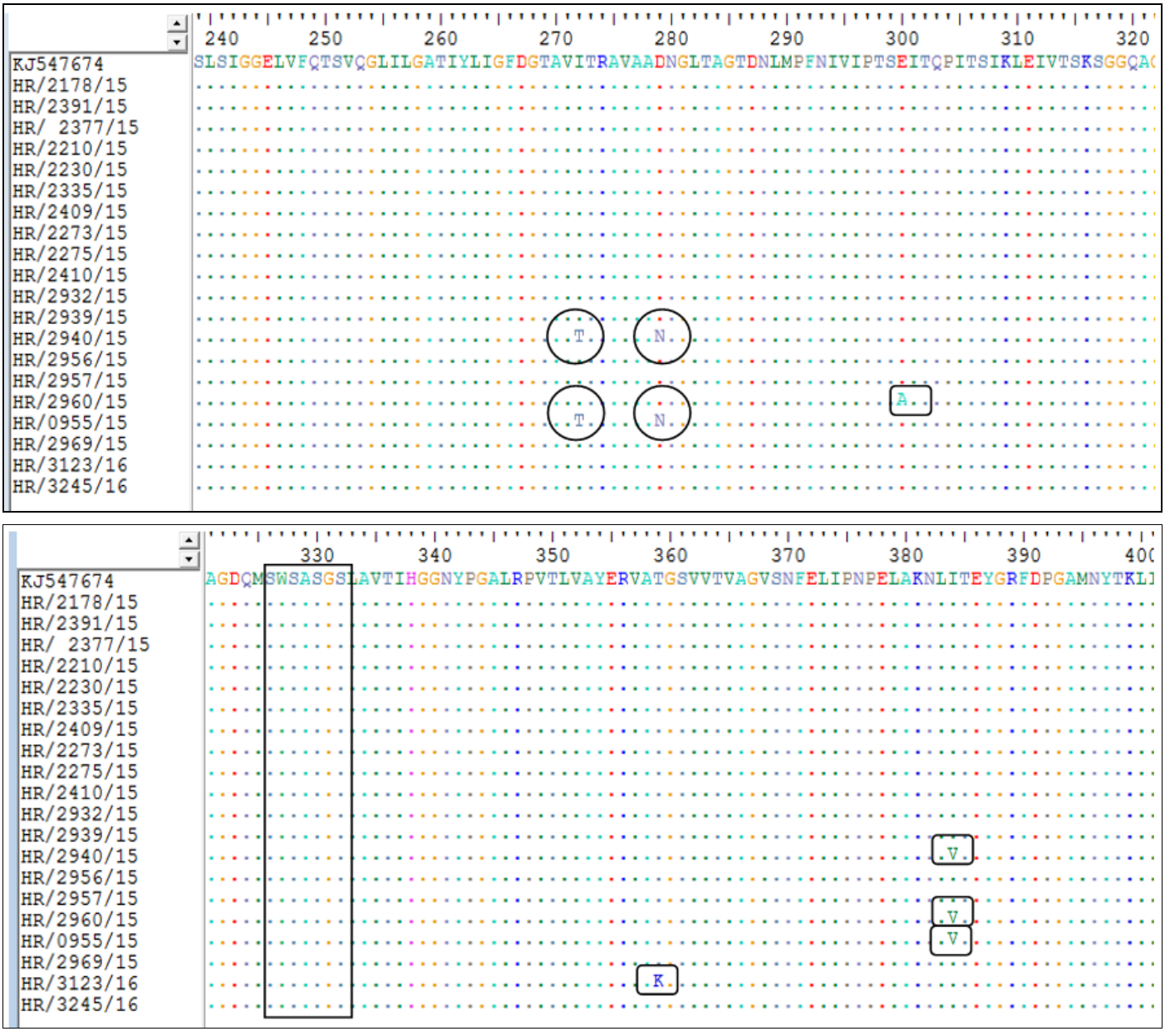

Fig 2: Diagram showing amino acid sequences of the VP2 gene (239-400 amino acids position of KJ547674) of infectious bursal disease virus (the sequences without accession numbers are partial sequences of VP2 gene of this study and the accession number of reference strain is of already published sequence of VP2 gene of IBD virus).

In the present study all the field strains had 96.7-99.9% similarity within themselves at nucleotide level. However, homology of field strains with the already published Indian strains was 92.3-97.4%. Mittal (2004) [13] reported that all four field strains had 99.3-99.8% similarity within themselves at the nucleotide level, however, their similarity with other reported vvIBDVs was between 97.1-98.3%. Mor *et al.* (2013) [16] reported that all 10 field strains had 92.1-100% similarity within themselves and 92.1-99.8% similarity with the Indian strains used for comparison. Owolodun *et al.* (2015) [21] reported that Nigerian field strains showed 99.6-100% similarity with each other at nucleotide level. Similar findings have been reported by various workers (Kasanga *et al.*, 2013; Liu *et al.*, 2013; Amin and Jackwood, 2014;

Mohamed *et al*., 2014; Li *et al*., 2015) [9, 12, 1, 14, 11].

4. Conclusion

Sequencing of hypervariable region of VP2 gene suggested the circulation of very virulent strains of IBDVs in commercial broiler chickens in Haryana state. Continuous surveillance in all poultry regions of the state is essential to understand better the epidemiology of IBDVs. Strict biosecurity measures are required so that the impact of immunosuppression due to IBD vaccine and concurrent infections can be minimized.

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