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Genetic analysis of VP1 gene reveals involvement of vvIBDVs in disease outbreaks in vaccinated flocks of Kerala

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Abstract
Recurrent outbreaks of Infectious bursal disease (IBD), become a major threat to poultry industry, worldwide. The disease mainly affects the pre developing B lymphocytes of bursa of Fabricius, causing severe immunosuppression in young chickens. Many outbreaks of IBD were observed in Kerala. The current study was carried out with the aim to analyse the involvement of genetic nature of infectious bursal disease virus which caused outbreak in vaccinated flocks of Kerala. Total 14 bursal samples were collected from flocks suspecting for IBD which were vaccinated against IBD. The amino acid analysis of VP1 gene of these isolates reveals that out of 14 isolates, 11 isolates showed TDN amino acid triplet in their sequences which is characteristic of vvIBDV. Three isolates showed NEG amino acid triplet, which is characteristic of non vIBDV. This is the first genetic analysis of VP1 gene revealing the involvement of vvIBDVs from the vaccinated flocks of Kerala.

Keywords: vvIBDV, VP1 gene, RT-PCR, ORF

1. Introduction
Infectious bursal disease (IBD) was first identified in Gumboro, southern Delaware, USA and hence named as 'Gumboro disease' (Cosgrove, 1962) [3]. The disease is caused by Infectious bursal disease virus (IBDV). This double stranded RNA genome is classified under family *Birnaviridae* of genus *Avibirnavirus* (Tiwari *et al*., 2003) [5]. IBDV particles possessed a non-enveloped icosahedral capsid but unlike other dsRNA virus single shelled with a diameter of about 60 nm (Martinez- Torrecuadrada *et al.*, 2002; Müller *et al*., 2003) [12, 13]. The IBD is a highly contagious disease and consists of two serotype. Serotype 1 is pathogenic which causes immunosuppression and sometimes acute fatal disease in chickens, whereas Serotype 2 is non-pathogenic (Liu and Vakharia, 2004) [11]. IBD virus mainly affects pre developing B lymphocytes present in bursa of Fabricius of 3-6 weeks old aged chickens (Gao *et al*. 2007) [16]. The bisegmented genome consist of two segments, segment A and B (Einem *et al.*, 2004). The larger open reading frame (ORF) of Segment A encodes two structural protein VP2 (outer capsid) and VP3 (inner capsid). It also encodes VP4 protease (Alfonso- Morales *et al*., 2015) [1]. The smaller ORF encodes the non-structural protein, VP5 (Alkie and Rautenschlein, 2016) [2]. Segment B encodes RNA dependent RNA polymerase VP1 gene (Jackwood, 2011) [8]. It exsists as both genome linked protein as well as a free polypeptide (Dey *et al.*, 2019) [4]. The gene plays an important role in viral replication, encapsidation of viral particles, and synthesis of m-RNA (Liu and Vakharia, 2004; Dey *et al*., 2019; Fan, *et al*., 2019) [11, 4, 5]. For the detection of IBDV, VP2 gene amplification is a specific target (Singh *et al*., 2014) [14]. Nouën *et al*. (2012) [10] suggested that according to the experimental and epidemiological evidence, for expression of pathogenic and vvIBDV phenotypes, both genome segments might be required.
The current study was carried out with the objective to analyse the genotype of IBDV based on VP1 gene of the isolates which caused field outbreaks of IBD in vaccinated flocks of Kerala.

2. Materials and Method
2.1 Sample collection and processing
Total 14 bursal samples were collected from vaccinated flocks suspecting for IBD, after post mortem examination of birds. The suspected flocks of birds belongs to various districts of Kerala. The samples were collected from 3-6 weeks old age chickens. The collected samples were from 11 layers and 3 broiler flocks.

The samples were stored in – 80 °C upto further processing. RNA was isolated from the suspected bursal tissue using TRIzol reagent, as per the manufacturer's protocol.

2.2 RT-PCR

The isolated RNA was treated with DMSO followed by c-DNA synthesis. The c-DNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instruction. Reverse transcriptase polymerase chain reaction (RT-PCR) was used for quick detection of Infectious bursal disease virus targeting VP1 gene yielding amplicons of size ~1051 base pairs, using primers - Univ-F (5'-AATGAGGAGTATGAGACCGA-3') and Univ- R (5'-CCTTCTCTAGGTCAATTGAGTACC-3').
The gel was visualized to identify the size of the product in a gel documentation system under UV illumination.

2.3 Sequence analysis

Purification and extraction of gel was carried out by PCR gel extraction Kit (GeneJET Gel Extraction Kit, Thermo Scientific, USA) as per the manufacture's protocol and sent to AgriGenom Lab Private Limited, Cochin, India for sequencing. Amino acid analysis of obtained sequences was carried out using MEGAX software.

3. Result and Discussions

Total 14 bursal tissue were collected from vaccinated flocks in which birds showed post mortem lesion such as enlarged or in some cases hemorrhagic Bursa of Fabricius, hemorrhages on breast and thigh muscles, hemorrhages and erosions at the proventricular- gizzard junction. These samples were confirmed by RT-PCR targeting VP1 gene, yielding amplicons of size ~1051 base pairs (fig1). The obtained VP1 gene sequences of these isolates were analysed for amino acid using MEGAX software. The sequence of the present isolates were compared with Indian and foreign IBDV sequences. The amino acid (aa) sequence of VP1 protein were analysed for total 324 aa from 82 to 406 and compared to different IBDV strains such as very virulent (BD 3/99, UK661, IL3, IL4, PY12), classical attenuated (D78, VCN14/ ABT/MVC/India, Winterfield 2512,CEF49), and classical virulent (Cu. 1wt, Edgar). UK661 strain was considered as reference strain. Amino acid triplet at 145/146/147 and the amino acid of position 242 in VP1 have been identified as the virulent marker for vvIBDV (Kasanga *et al*. 2013; Gao *et al*. 2014) [9, 7].

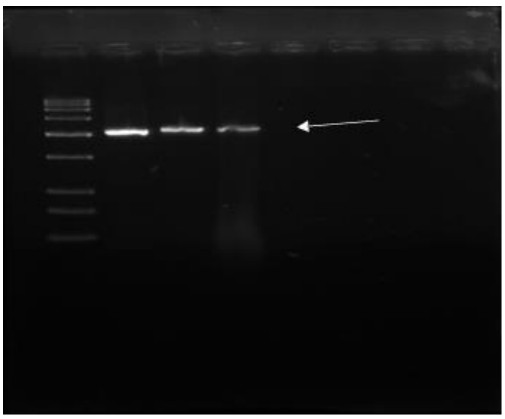

Fig 1: Confirmation of IBD virus by RT-PCR showing amplification of hypervariable region of VP2 gene yielding amplicons of size 604 bp amplicon.

Lane M - 100 bp DNA marker Lanes 1 - positive control
Lane 7 - Negative control (cDNA from healthy IBD free birds)
Lane 2 to 6 - samples

In the present study, the amino acid analysis of 14 isolates revealed that 11 isolates had TDN amino acid triplet while 3 isolates had NEG amino acid sequence in their sequence at 145/146/147 position. Eleven isolates showing TDN amino acid triplet had glutamic acid (E) at 242 position. The isolates having NEG amino acid triplet showed aspartic acid (D) at 242 position.
Among 14 isolates obtained from vaccinated flock, 11 isolates had TDN amino acid triplet while the 3 isolates had NEG amino acid triplet. This shows the occurrence of IBD with vvIBDV even after proper vaccination using classical attenuated and intermediate plus vaccine strains. Vaccine failure may occur as the IBDV have the intrinsic property develop rapidly, non-sufficient induction of protective immune response and/or reversion of the vaccine strains to virulence. Conventional live IBDV vaccines prepared from classical virulent strains shows low efficiency against vvIBDV (Alfonso-Morales *et al*., 2015) [1].

4. Conclusion

Thus, it is concluded from the present study that IBD outbreaks in Kerala showed the involvement of vvIBDV strains, even after vaccination. Therefore, new generation vaccine or vaccine strains having new genetic background must be used to prevent IBDV infection.

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