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Restriction Fragment Length Polymorphism Analysis of Isolates of Infectious Bursal Disease Viruses from Southern Region of India

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ABSTRACT

The Restriction Fragment Length Polymorphism (RFLP) is used for the differentiation of classical virulent (cv), virulent (v) and very virulent (vv) strains of Infectious Bursal Disease Virus (IBDV) isolates collected from recent outbreaks in southern region of India. In the present study, five different isolates (BGE15, EDE14, RPM14, MDI14 and THI14) of IBDV strains were subjected for genotyping along with vaccine (Georgia, intermediate strain) by performing RT-PCR for amplification of a 743 bp from the hypervariable region of VP2 gene followed by restriction enzyme digestion with seven different enzymes (HhaI, SacI, SspI, StyI, BspMI, StuI and TaqI) for the differentiation of classical, virulent and very virulent strains of IBDV. The RT-PCR product obtained from all the five isolates were not cleaved by SspI and SacI enzyme and thus revealed the absence of restriction enzyme (RE) site for SspI and SacI enzyme, respectively. The HhaI enzyme cleaved vaccine and field isolates with similar restriction profiling pattern. The StuI enzyme did not digest vaccine strain whereas field isolates were cleaved by this enzyme. The BspM1 was not able to differentiate the field isolates from vaccine strain. TaqI enzyme cleaved both vaccine and field isolates of IBDV with different profile pattern. The StyI enzyme showed single RE site on vaccine strain and other field isolates with similar RE pattern. Thus, from the present study, it may be concluded that all the isolates belonged to vvIBDV and they do not have site for SspI marker.

Keywords: Infectious Bursal Disease virus; Reverse-transcription polymerase chain reaction; Restriction fragment length polymorphism; VP2 gene; Genotyping

Infectious Bursal Disease (IBD) is caused by an acute, highly contagious nature of birna virus that results in mortality and immunosuppression of young chickens (Dobos, 1979). Infectious bursal disease virus (IBDV) is a single-shelled, non-enveloped virus that contains a bi-segmented, double-stranded RNA genome (Macdonald, 1980; Muller and Nitschke, 1987). IBDV affects 3–6 weeks-old young chicks, has predilection for bursa of Fabricius and causes prolonged immunosuppression leading to concurrent viral and bacterial infections along with vaccination failures (Saif, 1991). Serotype I strains are classified as classic, variant or very virulent IBDV strains and differ in their virulence, antigenic, and pathogenic properties (Rosenberger *et al.,* 1985; Jackwood and Saif, 1987; Lin *et al*., 1994).The IBDV genome is divided into segments A and B. The segment A is about 3.4 kb and B is about 2.8 kb. The large segment A encodes 4 viral proteins, the two capsid proteins VP2 (48 kDa) and VP3 (32–35 kDa), the viral protease VP4 (24 kDa), and a nonstructural protein VP5 (17–21 kDa), while the smaller segment B encodes VP1 (90 kDa), an RNA-dependent RNA polymerase. Among five proteins of IBDV, the VP2

is the major host-protective antigen that induces serotype-neutralizing antibodies (Fahey *et al.,* 1989), the middle third of which contains a highly variable region (HVR) that ranges from amino acid (aa) position 206 to 350 (Bayliss *et al*., 1990). Mutations within these hydrophilic coding regions are thought to be responsible for the evolution of antigenically variant and virulent serotype 1 strains (Heine *et al.,* 1991; Schnitzier *et al.,* 1993).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is a molecular tool frequently applied in IBDV diagnosis. Most of RT-PCR protocols are based on VP2 nucleotide sequences. VP2 is the major host protective immunogen of IBDV and contains the determinants responsible for causing antigenic variation. RT-PCR followed by digestion with multiple restriction enzymes (RE) or restriction fragment length polymorphism (RFLP) and nucleotide sequencing of VP2 gene have been used for differentiation of IBDV strains (Lin *et al.,*1993; Dybing and Jackwood, 1996; Jackwood and Nielsen, 1997; Ture *et al.,* 1998).

The aim of this study was to investigate the genetic variation among IBDV isolates from commercially reared chickens in southern parts of India with the use of restriction enzyme in RT-PCR-RFLP in order to establish a genetic relationship for these viruses and also to assess their strain type as virulent, very virulent or classical strains.

MATERIALS AND METHODS

Collection of samples

Bursal tissue samples were collected from suspected outbreaks of IBD in various poultry farms in Southern states of India during 2014- 2015 was used. In the laboratory, the bursa was cut into fine pieces and suspended in 1 ml of phosphate buffered saline (PBS, pH 7.4). The suspension was then centrifuged at 10000 rpm for 5 min for initial clarification. The supernatant was removed, transferred to new 1.5 ml eppendorf tubes and stored in -70ºC until further use.

In vitro propagation of IBDV

The positive samples collected from different outbreaks of IBD (BGE15, EDE14, RPM14, MDI14 and THI14) in chickens of 3–5 weeks of age were processed for isolation in cell culture. The samples were inoculated in nine to 10 days old chicken embryos and passaged in primary chick embryo fibroblast (CEF) cultures with antibiotics (10,000 µg/l streptomycin sulphate and 10,000 IU/l penicillin G) and antimycotics (Fungizone containing 250 µg/ml Amphotericin B and 205 µg/ml sodium desoxycholate) (Rodriguez-Chavez *et al.,* 2002). Briefly, CEF mono layers were inoculated with each IBDV isolate directly using the growth medium. The IBDV-infected CEF cells were incubated for 3 to 4 days and the development of cytopathic effect (CPE) was assessed daily. The infected cells were frozen when approximately 50% CPE was observed. They were subjected to freeze-thawing at -70°C three times and then centrifuged at 2,000 x *g* for 10 min. The supernatants containing IBDV were aliquoted and frozen at -70°C until used.

RNA extraction

For RNA extraction, 250 µl of the bursal supernatant samples of the five IBDV isolates (BGE15, EDE14, RPM14, MDI14 and THI14) and vaccine strain (Georgia intermediate) as control were taken in separate eppendrof tubes. To the samples, 750 µl of Trizol reagent (RNA isoplus, Takara) was added and pipetting was done for about 10-15 times (in order to mix the contents evenly). After adding 200 µl of chloroform, the aqueous phase was separated by centrifugation at 12000 rpm for 15 min at 4ºC. The supernatant was precipitated by adding 0.5 ml of isopropanol at -20ºC for about half an hour. The RNA was pelleted at 12000 rpm for 20 min, washed with 70% ethanol, dried and dissolved in 10µl of nuclease free water and stored in -70ºC until use.

Reverse transcription

A commercial cDNA synthesis kit (RevertAid First strand H- cDNA synthesis, Thermoscientific, USA) was used to prepare the cDNA. The procedure was done as instructed by manufacturer. Briefly, 8 µl of RNA extract (50 ng), 1 µl of random hexamer primer, and nuclease free water was added upto 10 µl and incubated at 65ºC for 5 min and snap chilled. Further 4 µl of Reaction buffer, 2 µl of 10 mM dNTP, 2 µl of DMSO, 1 µl of Ribolock RNase Inhibitor and 1 µl of Revert Aid H Minus M-MuLV reverse Transcriptase enzyme were added to the mixture

and incubated for 5 min at 25ºC, followed by 60 min at 42ºC. The reaction was terminated by heating at 70ºC for 5 min, cooled on ice and stored at-20ºC.

RT-PCR amplification of partial VP2 region

The VP2 hypervariable region of 743 bp was amplified using the primers 743-FP-(5'-GCCCAGAGTCTACACCAT-3') and 743-RP-(5'-CCCGGATTATGTCTTTGA-3') (Jackwood and Sommer-Wagner, 2005). The amplification was carried out in 25 µl reaction volume consisting of 12.5 µl of 2X Mastermix-Red Dye (Ampliqon), 1 µl of each primer (20 picomoles), 8.0 µl of nuclease free water and 2.5 µl cDNA of each IBDV isolate and vaccine as template. The reaction was carried out in a thermalcycler (Veriti, Applied Biosystems) as follows: initial denaturation of 94ºC for 2 min, followed by 30 cycles of 94ºC for 1.5 min, 53ºC for 1 min, 72ºC for 1 min and a final extension at 72ºC for 5 min. Negative control (addition of nuclease free water instead of cDNA) and positive control (vaccine, Georgia intermediate) were included in all the reactions. The amplification products were analysed in 1.2% agarose gel electrophoresis in Tris-Acetate-EDTA buffer (TAE buffer, pH 8.0). A 100 bp DNA ladder (GeNei, Bangalore) was used as molecular weight marker in each electrophoresis reaction.

RT-PCR-RFLP analysis

The PCR amplified product were purified using a PCR purification kit (Bio Basic Inc, Canada) and digested with the restriction enzymes (HhaI, SacI, SspI, StyI, BspMI, StuI and TaqI) according to the manufacturer's instructions (New England Biolabs, UK). For the restriction enzymes, BspMI, StuI and SspI, 2 µl of restriction enzyme buffer (for 20 µl reaction), 1µl of restriction enzyme (10U), 10 µl of the PCR amplicon of the six isolates and Vaccine strain were added and incubated at 37ºC for 2 hours. For the restriction enzymes SacI, HhaI,StyI and TaqI 2 µl of restriction enzyme buffer (for 20 µl reaction), 1 µl of restriction enzyme (20U), 10 µl of the PCR amplified product from the isolates and Vaccine strain, and 2 µl of Bovine Serum Albumin (10X) were added, incubated at 37ºC for 2 hours. The reactions were inactivated at 65ºC for 10 min and electrophoresis was carried out in 1.5% agarose gel for about 30 min at 100V.

RESULTS AND DISCUSSION

The re-emergence of the IBDV in the form of antigenic variants and very virulent strains has been the cause of significant loses to the poultry industry. The high mutation rate of the RNA polymerase of RNA viruses, due to lack of proof reading generates a genetic diversity that could lead to emergence of viruses with new properties, allowing them to persist in immune population (Lukert and Saif, 2003).

Differentiation between classical virulent (cv) IBDV and very virulent (vv) IBDV is very important to poultry industry with regard to choosing the appropriate vaccination programmes. The application of RT-PCR combined with restriction enzymes has been documented to rapidly differentiate between classical and vv IBDV standard strains (Zierenberg *et al*., 2001). Most of the methods for IBDV strain differentiation focus on the VP2 capsid protein of IBDV, as this protein contain most of the variation observed between IBDV strains (Bayliss *et al.,* 1990). In particular, a hyper variable region (HVR) between nucleotides 762 to 1151 has been identified in the VP2 gene that enables IBDV strains to be differentiated into genetic groups that correspond to classical, variant and very virulent IBDV phenotypes (Heine *et al.,* 1991; Brown *et al.,* 1994).

In the present study, the VP2 hypervariable region of 743 bp was amplified using the specific primers for five different isolates (BGE15, EDE14, RPM14, MDI14 and THI14) (Fig.1). The RFLP analysis was carried out to track the evolutionary changes at molecular level. The results showed that all the field isolates are very virulent strains of IBDV.

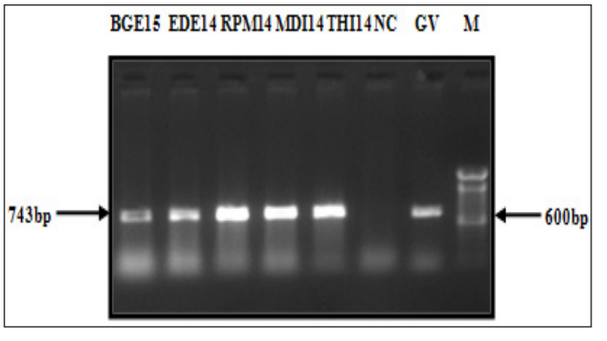

Fig. 1: RT-PCR amplification of partial VP2 gene of the IBDV isolates

The HhaI enzyme digestion of PCR amplified product of VP2 gene showed the presence of RE sites with similar restriction profile for vaccine strain as well as field isolates of IBDV (Fig. 2).

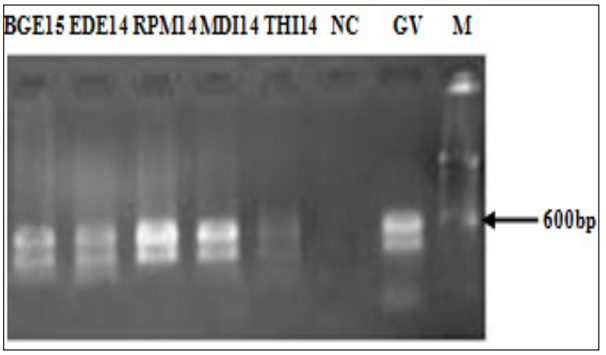

Fig. 2: HhaI digestion of RT-PCR amplified product of VP2 gene (The restriction profiles of all the isolates were similar to that of vaccine strain (GV))

The results of the present study are in agreement with Priyadharsini *et al.* (2016) who also reported that vvIBDV also had single RE site. However, contrary results have been reported by Kataria *et al.* (1999) who observed that HhaI analysis of IBDV had single site for vaccine strain and absence of site in the field isolates in India. Similarly, Shoshtari *et al*. (2004) also reported that field classical and vaccine strains had sites for HhaI enzyme and no sites were reported for field strains of very virulent IBDV. In the present study, presence of HhaI site in the genome of vvIBDV is reported with similar restriction profile among Indian isolates of vvIBDV and hence HhaI enzyme cannot be used as an appropriate enzyme to differentiate the cvIBDV from vvIBDV.

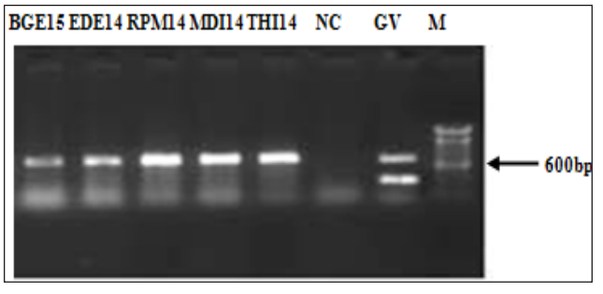

Fig. 3: SacI digestion of RT-PCR amplified product of VP2 gene (The restriction profiles of all the isolates differ from vaccine strain (GV). None of the isolates were digested by SacI enzyme)

The SacI digestion showed the presence of single RE site on vaccine strain. In contrast, the other field isolates were not digested by SacI enzyme and revealed the absence of RE site for SacI enzyme (Fig. 3). This indicated that these isolates are neither similar to vaccine strain (intermediate) nor classical strains of IBDV. Several other researchers have reported the ability of SacI to differentiate between classical IBDV from vvIBDV. The results of the present study are in agreement with Sellers *et al.* (1999), De Paula *et al.* (2003) and Shoshtari *et al.* (2004) who also opined that SacI site is present only in cvIBDV.

The SspI site was absent in both vaccine strain and other field isolates (Fig. 4). Our result is in contrary with Shoshtari *et al.* (2004), De Paula *et al.* (2004) and Juneja *et al.* (2008) who have reported that vvIBDV had single site for SspI enzyme and sites are present only in field isolates and not in vaccine strain. In addition, Roussen *et al.* (2012) reported that all the vvIBDV strains did not have this SspI site and some non-vvIBDV strains also had this site (Eterradossi *et al.,* 1999: Lim *et al.,* 1999; Sapats and Ianjatonic, 2002). However, the results of the present study are in agreement with Priyadharsini *et al.* (2016) who reported the absent of SspI site from vvIBDV from India. This study indicating that vvIBDV strains from current outbreaks did not possess site for SspI enzyme.

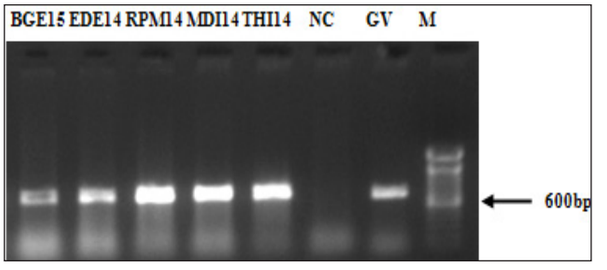

Fig. 4: SspI digestion of RT-PCR amplified product of VP2 gene (The SspI restriction site was not observed in any of the isolates as well as vaccine strain (GV))

The profile pattern with the enzyme BspMI did not show any differences between the vaccine strain and other field isolates in the agarose gel electrophoresis (Fig.5). However, Razmyar and Peighambari, (2007) reported the BspMI digested only very virulent strains and can be used to compare serotype I (classical and vvIBDV) and serotype 2, in which the BspMI cleaved the vvIBDV. To confirm this, the VP2 hypervariable region of vvIBDV

isolates and vaccine strain (GV) was sequenced and the BspM1 site was found at the position of 701 of 5' end in the vvIBDV isolates and not for vaccine strain (Data not shown). Upon digestion with BspM1 it produces two fragments in the size of 701 and 42 bp respectively which could not able to differentiate the field isolates from vaccine strain in agarose gel electrophoresis. The BspMI cleavage site found in vvIBDV strains was correlated with the aminoacid position 222 (proline to alanine) in the major hydrophilic peak A of the VP2 hypervariable region. This amino acid exchange was conserved in all the vvIBDVs (Brown *et al.,* 1994 and Kataria *et al.,* 2001). However, the present study revealed that BspMI enzyme site was not able to differentiate the field isolates from vaccine strain.

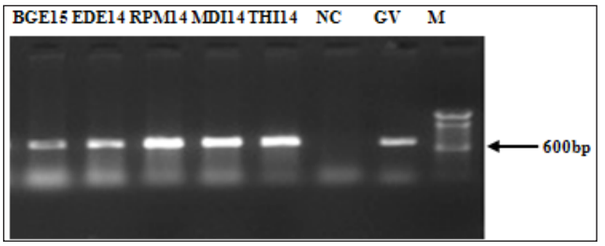

Fig. 5: BspMI digestion of RT-PCR amplified product of VP2 gene (The BspM1 was not able to differentiate the field isolates from vaccine strain (GV)

The StuI enzyme did not digest vaccine strain whereas the rest of the field isolates were cleaved by this enzyme indicated that these isolates belong to vvIBDV (Fig.6). Our results are also in correspondence with Shoshtari *et al.* (2004), Juneja *et al.* (2008) and Priyadharsini *et al*. (2016), who reported the presence of single RE site for StuI in vvIBDV and not in vaccine strains.

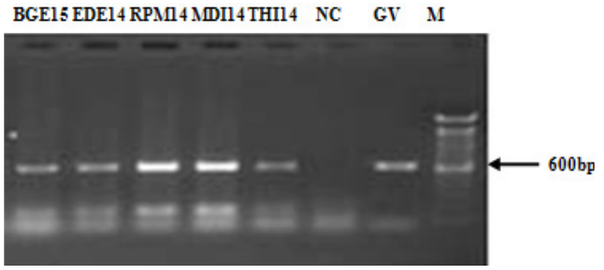

Fig. 6: StuI digestion of RT-PCR amplified product of VP2 gene (The restriction profile of vaccine strain (GV) reveals the absence of restriction site for StuI, whereas all other IBDV isolates had a single restriction site)

The StyI enzyme showed single RE site on vaccine strain and other field isolates with similar RE pattern. (Fig.7). Our results are in agreement Kataria *et al.* (1999), who reported the digestion with StyI had single site in all the five field isolates and they found vaccine strain also had a single site for StyI but at different position. Hoque *et al.* (2001) also reported that StyI had single site in all the field isolates except one field isolate which did not have site for StyI enzyme.

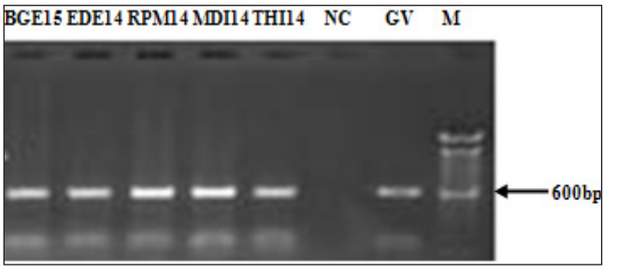

Fig. 7: StyI digestion of RT-PCR amplified product of VP2 gene (The StyI enzyme showed single RE site on vaccine strain and other field isolates)

The TaqI enzyme showed single RE site with vaccine and all other field isolates have two RE site with similar profile pattern. The profile pattern clearly indicated difference between vaccine strains and field isolates (Fig. 8). However, earlier Kataria *et al.* (1999), who reported the digestion with TaqI yielded two fragments with the same pattern for the field strains, but no site was noticed in vaccine strains.

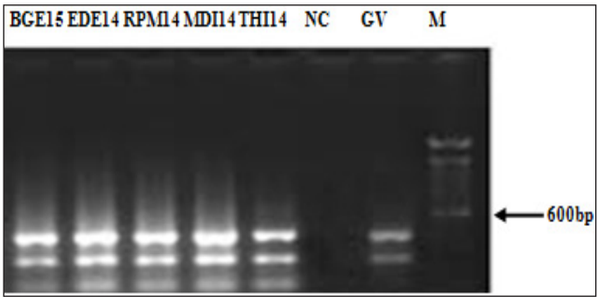

Fig. 8: TaqI digestion of RT-PCR amplified product of VP2 gene (The TaqI enzyme showed single RE site with vaccine and all other field isolates have two RE site with similar profile pattern)

The profile patterns produced by all the restriction enzymes in different isolates are summarized in Table 1.

Table 1: Differentiation of IBDV field isolates by RT-PCR – RFLP of partial VP2 region

ISOLATES	RESTRICTION ENZYME							Classification of isolates
	HhaI	SacI	StuI	SspI	BspMI	StyI	TaqI	
Vaccine	+	+	-	-	-	+	+	Intermediate
BGE15	+	-	+	-	+*	+	++	Very virulent
EDE14	+	-	+	-	+*	+	++	Very virulent
MDI14	+	-	+	-	+*	+	++	Very virulent
RPM 14	+	-	+	-	+*	+	++	Very virulent
THI 14	+	-	+	-	+*	+	++	Very virulent

Symbol '+' indicates presence of RE site and '–' symbol indicates absence of RE site; * symbol indicates based on the sequencing of VP2 hypervariable region, the BspM1 site was found only on the vvIBDV and not on the vaccine strain.

CONCLUSION

This study indicates that vvIBDV strains from southern parts of India could be more diverse in nature. All the five isolates was not cleaved by SspI and SacI enzyme and the HhaI and StyI enzymes cleave the field isolates and vaccine strain in same profile pattern. The TaqI enzyme cleave the field isolates and vaccine strain in different profile pattern. As earlier reported by several researchers the StuI cleave all the five isolates not on vaccine strain. It indicates this isolates belongs to vvIBDV. Sametimes the BspM1 was not able to differentiate the vvIBDV filed isolates from vaccine strain in agarose gel electrophoresis. In this study, all the five isolates did not possess site for SspI enzyme site which indicates the IBDV-VP2 gene is continuously evolving in nature which is linked with genetic variation of IBDV. Though, RT-PCR combined with RFLP is genotypic method of characterization, further confirmation to differentiate the vvIBDV from cvIBDV and vaccine strain has to be carried out either by sequencing of VP2 hypervariable region or pathogenicity studies.

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