Sequence analysis of VP2 hypervariable region of the field isolates of infectious bursal disease viruses from southern region of India

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Summary. – Infectious bursal disease virus isolates obtained from southern parts of India were subjected to comparative sequencing and phylogenetic analysis of 743bp hypervariable region of VP2. The sequence analysis showed that among eight isolates, only HY12 showed the characteristic conserved amino acid residues at 256I, 294I, and 299S of vvIBDV. Six isolates BGE14, PY12, NKL14, VCN14, RPM14 and EDE14 had conserved amino acid residues at 256I and 299S, whereas at residue 294, isoleucine was substituted by valine. The remaining isolate MB11 had leucine at residue 294 and asparagine at residue 299 similar to classical strain 52/70. The serine-rich heptapeptide sequence SWSASGS adjacent to the second hydrophilic region was conserved in all seven Indian IBDV isolates except isolate MB11. Conservation of this sequence was earlier reported to be an indication of a virus isolate being pathogenic in nature. The reported heptapeptide sequence of the classical strain is 'SWSARGS'. In the present study, 'SWSARGS' heptapeptide sequence was observed in MB11 isolate. The pathogenicity trials conducted with these isolates further confirmed the genome analysis in classification. This study further reveals that the circulating IBDV strains in India could be diverse in nature.

Keywords: infectious bursal disease virus; Indian isolates; reverse-transcription polymerase chain reaction; VP2 gene; sequence analysis; amino acid substitutions

Introduction

Infectious bursal disease (IBD) is caused by an acute, highly contagious nature of birnavirus 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-week-old chicks, has predilection for bursa of Fabricius and causes prolonged immunosuppression, leading to concurrent viral and bacterial infections along with vaccination failures (Saif, 1991). So far two serotypes of IBDV have been identified; however, only serotype I viruses are naturally pathogenic to chickens (Jackwood *et al.,* 1985). 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). The IBDV genome is divided into segments A and B, 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),

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Abbreviations: IBD = infectious bursal disease; IBDV = infectious bursal disease virus; cvIBDV = classical virulent IBDV; vvIBDV = very virulent IBDV

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). It includes two hydrophilic regions referred to as VP2 major hydrophilic peak A (aa 212–224) and peak B (aa 314–324) (van den Berg *et al.,* 1991; Schnitzier *et al.,* 1993; Delmas *et al.,* 2005). Mutations within these hydrophilic coding regions are thought to be responsible for the evolution of antigenic variants and virulent serotype 1 strains (Heine *et al.,* 1991; Schnitzier *et al.,* 1993). Three additional minor hydrophilic peaks at aa positions 248–252, 279–290, and 299–305 are considered to influence IBDV antigenicity (van den Berg *et al.,* 1991). At aa position 326–332, there is a serine-rich heptapeptide (SWSASGS), which might have correlation with the virulence of IBDV strains (Heine *et al.,* 1991); this might be one of the virulence markers of IBDV strains. The aa residues at positions 253, 279, and 284 of VP2 are also involved in the virulence (Brandt *et al.,* 2001). VP1 is considered as another important virulence marker of IBDV (Liu and Vakharia, 2004). Most exchanges of amino acid residues in VP2 occur in the four hydrophilic loops of the viral capsid (Coulibaly *et al.,* 2005). These exchanges indicate that the selective pressure for evolution of IBDV is directly focused on the capsid regions that are immediately exposed to the immune system (Durairaj *et al.,* 2011). Amino acid changes that occur within the variable region of VP2 can lead to variations in antibody recognition, antigenicity, immunogenicity, virulence, and tissue tropism of IBDV strains (Fahey *et al.,* 1989; Heine *et al.,* 1991).

Materials and Methods

Ethics statement. This experiment was approved by the Institutional Animal Ethical Committee of Tamil Nadu Veterinary and Animal Sciences University (Approval Lr. No. 3028/DFBS/B/2014 dt, 09.10.2014). All procedures related to the chickens and their care conformed to the internationally accepted principles in the Committee for the Purpose of Controlled Supervision on Experiments on Animals (CPCSEA) Guidelines for Laboratory Animal Facility.

Processing of tissue samples. The bursal tissue samples were collected from various suspected outbreaks during 2011 to 2014 in poultry flocks located in Southern states of India from birds with symptoms of IBDV. The respective observed morbidity and mortality rates ranged from 24–41% and 3–16%, were confirmed by random samples, which were screened for the presence of IBDV by agar gel immunodiffusion test and reverse transcriptase PCR (RT-PCR) for VP2 gene. Part of the bursal samples were stored in 10% formalin for histopathological examination and the remaining tissues were minced into fine pieces and suspended in 1 ml of PBS (pH 7.4). The suspensions were centrifuged at 2,000 x *g* for 30 min and the supernatants were collected, dispensed into aliquots and stored in -70ºC until further processing.

In vitro propagation of IBDV. The positive samples collected from eight different outbreaks of IBD 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 monolayers 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.

In vivo propagation of IBDV. The bursa-derived IBDV isolates (MB11, HY12, PY12, BGE14, VCN14, NKL14, RPM14 and EDE14) were inoculated in chickens for assessing the virulence of the IBDV strains as described by Rodriguez-Chavez *et al.* (2002). Eight groups of four-weeks-old native chickens (5 chickens per group) were subjected to the mortality study with these isolates and one group was kept as control. The chickens were inoculated with the virus isolates at a titer of 1 x 10^6 calculated in terms of $TCID_{50,}$ both by intraocular and intranasal routes. The control group was inoculated with PBS alone. Chickens died up to five days post-inoculation (p.i.) were necropsied and the samples (bursa and spleen) were collected. The average bursal/bodyweight (B/B) and spleen/bodyweight (S/B) ratios were calculated by the following formula (Tanimura *et al.,* 1995):

Bursa weight or spleen weight in grams / Body weight of individual bird in grams x 1000.

Statistical analysis. The average B/B and S/B indices between different groups were analyzed statistically by one-way analysis of variance using SPSS version 17.0 software.

Histopathology. Bursa obtained from the inoculated and control groups of chickens were fixed in 10% buffered formalin solution for at least 48 h and processed for histopathological examination according to the method described by Tanimura *et al.* (1995). Briefly, tissues were trimmed to a thickness of 0.5 cm and the blocks were subsequently dehydrated in series of alcohol, cleared with xylene and embedded in paraffin wax using an automatic tissue processor. The tissues were sectioned at about 5 mm using a microtome and mounted on glass slides, dewaxed and stained with Hematoxylin and Eosin (H & E) (Lillie, 1965). A modified lesion scoring method previously established by Muskett *et al.* (1979) was used to grade the tissues for histopathological changes.

RNA extraction and reverse transcription-PCR. A 20% (w/v) bursal homogenate was prepared in PBS from both uninfected and infected birds. The supernatants containing bursa-derived IBDV

strains were aliquoted and stored at -70°C until used. For RNA extraction from the tissue samples, 250 µl of the lysate collected from bursal tissues positive for IBDV (MB11, HY12, PY12, BGE14, VCN14, NKL14, RPM14 and EDE14) were added with 750 µl of Trizol reagent (RNA isoplus, Takara) and mixed by pipetting for 10–15 times. Chloroform, 200 µl was added, mixed and the aqueous phase was separated by centrifugation at 15,294 x *g* for 15 min at 4°C. The supernatant was separated and added with 500 µl of ice chilled isopropanol, kept for overnight incubation at -70°C. The RNA was pelleted by centrifuging at 15,294 x *g* for 20 min, the pellet was washed once with 70% ethanol and air dried. The final pellet was dissolved in 10 µl of nuclease free water and stored in -70°C until used.

One-step cDNA synthesis was carried out using cDNA synthesis kit (Thermoscientific, USA) with minor modifications. Briefly, nine µl of total RNA extract from each tissue sample was added with 1 µl of random hexamer primer, incubated at 65°C for 5 min and immediately chilled on ice. Four µl of reaction buffer, 2 µl of 10 mmol dNTP mix, 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 five min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for five min, cooled on ice and stored at -70°C until used.

To amplify a 743 bp region of VP2 hypervariable region, the primers 743-FP-(5'-GCCCAGAGTCTACACCAT-3') and 743-RP-(5'-CCCGGATTATGTCTTTGA-3') (Jackwood and Sommer-Wagner, 2005) were used. The amplification was carried out in 50 µl reaction volume consisting of 25 µl of 2x Mastermix-Red Dye (Ampliqon), two µl of each primer (20 picomoles), 2 µl of DMSO, 14 µl of nuclease free water and five µl of cDNA from each tissue sample. The amplification was carried out in a thermalcycler (Veriti, Applied Biosystems) with initial denaturation of 94°C for 2 min, followed by 30 cycles of 94°C for 1.5 min, 53°C for 1 min, 72°C for 1 min and a final extension at 72°C for 7 min. Negative control was included in all the PCR reactions. Vaccine strain (Georgia, intermediate strain) was used as positive control (Fig. 1).

Sequence analysis. The PCR amplified products were purified using PCR gel purification kit (Bio Basic Inc, Canada) and sequencing was performed at M/s. Shrimpex Biotech, Chennai-600019 (India). The nucleotide Sequence data was subjected to BLAST analysis (www.ncbi.nlm.nih.gov), assembled and analyzed using Seqman and MegAlign programs of Lasergene package (version 7.1.0) (DNA Star Inc. Madison, WI). Nucleotide sequence alignment was performed by ClustalW method with MegAlign™ program (DNA Star Inc), and the predicted amino acid sequence was analyzed by Protean™ program of Lasergene (DNA Star Inc). Phylogenetic analysis of 743 bp sequence containing the hypervariable region of VP2 gene, from nucleotide 881 bp to 1322 bp (numbering system according to Bayliss *et al.*, 1990) was performed using Maximum likelihood method with 1000 bootstrap replication in the MEGA software version 5.

Antigenic index. On the basis of formulations described by Jameson and Wolf (1988), potential antigenic sites of the deduced amino acid sequences were analyzed considering surface probability, regional backbone flexibility, and probable secondary structures of the predicted amino acid sequence using the Protean™ program (DNA Star Inc. Madison, WI).

Results

Clinical signs and gross pathological studies on native chickens

The chickens inoculated with isolate MB11 showed mild clinical signs and the mortality was 0% after 5 days post inoculation, whereas other isolates exhibited typical acute illness of IBDV and mortality from 20 to 40% after 3 to 4 days post inoculation. In control birds, no abnormalities were recorded throughout the trials. Severe bursal hemorrhages, congestion, paint brush hemorrhages in the muscle of the breast, thigh and legs were observed in chicks inoculated with isolates HY12, PY12, BGE14, VCN14, NKL14, RPM14 and EDE14 except MB11 (Fig. 2).

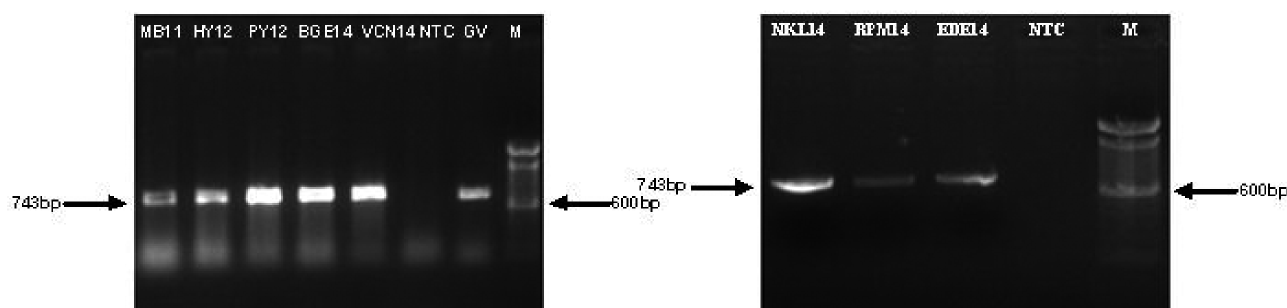

Fig. 1

Amplification of hypervariable region of VP2 gene from the suspected tissues

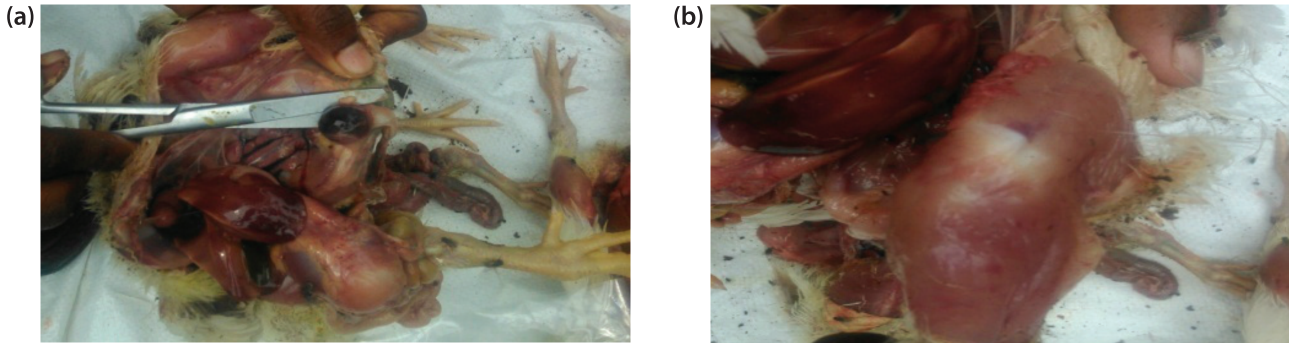

Fig. 2

Gross pathological changes of IBDV-infected birds
(**a**) Severe bursal hemorrhages; (**b**) Severe paint brush hemorrhages in the thigh muscle.

Histopathology

The average bursa: body weight ratios were markedly reduced in birds inoculated with MB11, HY12, PY12, VCN14, BGE14, and NKL14 compared with uninoculated control group as shown in Table 1. There were significant differences in the groups infected with isolates when compared to control group ($P < 0.05$). There were significant differences in the groups inoculated with PY12 and RPM14 when compared to the groups inoculated with isolate EDE14 ($P < 0.05$). The groups inoculated with BGE14, VCN14, HY12, NKL14 did not differ significantly from each other. In spleen index, there were significant differences in the groups infected with isolates HY12, EDE14, RPM14 when compared to control group. The histological evaluation of the bursa showed marked bursal lymphoid necrosis and depletion in the infected groups, and significant differences were found in bursal lesion scores when compared with the uninoculated control groups. No observable significant lesions and bursal scoring lesions were present in the control birds throughout the trial. The isolates HY12, PY12, BGE14, VCN14, NKL14, RPM14 and EDE14 at day 5 post inoculation showed severe lymphoid necrosis and depletion both in the medulla and cortex of the follicles in the bursal tissues. Mild hemorrhage was also present in some follicles and within the interstitial connective tissue (Fig. 3).

Nucleotide and amino acid sequence analysis of IBDV isolates

The region analyzed included 145 amino acid residues from 252 to 397 of the segment A polyprotein. When compared with European strain 52/70, the following amino acid changes were observed within the hypervariable region of the VP2. Glutamine was substituted by histidine at residue 253 in the classical MB11 isolate. The amino acid substitution of isoleucine for valine was observed in the isolates BGE14, HY12, PY12, NKL14, VCN14, RPM14 and EDE14, whereas MB11 isolate did not have this change at the position 256. Alanine was substituted by threonine at the amino

Table 1. Grading, Bursa:Body weight, Spleen:Body weight ratios and mortality following inoculation of 1 x 10^6 $TCID_{50}$ infectious bursal disease virus at 5th day of post inoculation (mean ± S.D.)

Virus isolates	Grading	Spleen index	Bursal index	Mortality
BGE14	Severe	0.455 ± 0.05^{ab}	0.472 ± 0.07^{abc}	40 (2/5)
NKL14	Severe	0.573 ± 0.07^{abc}	0.483 ± 0.12^{abc}	20 (1/5)
VCN14	Severe	0.526 ± 0.20^{abc}	0.482 ± 0.14^{abc}	20 (1/5)
EDE14	Severe	0.397 ± 0.33^{ab}	0.564 ± 0.06^{bc}	20 (1/5)
RPM14	Severe	0.455 ± 0.29^{ab}	0.365 ± 0.08^{a}	20 (1/5)
MB11	Mild to moderate	0.518 ± 0.13^{abc}	0.421 ± 0.03^{ab}	No mortality
HY12	Severe	0.350 ± 0.09^{a}	0.482 ± 0.12^{abc}	20 (1/5)
PY12	Severe	0.537 ± 0.13^{abc}	0.333 ± 0.07^{a}	No mortality
Control	Normal	0.749 ± 0.05^{c}	0.854 ± 0.10^{d}	No mortality

Results are expressed as mean ±S.D. Means in the same column with same superscript did not differ significantly ($P < 0.05$).

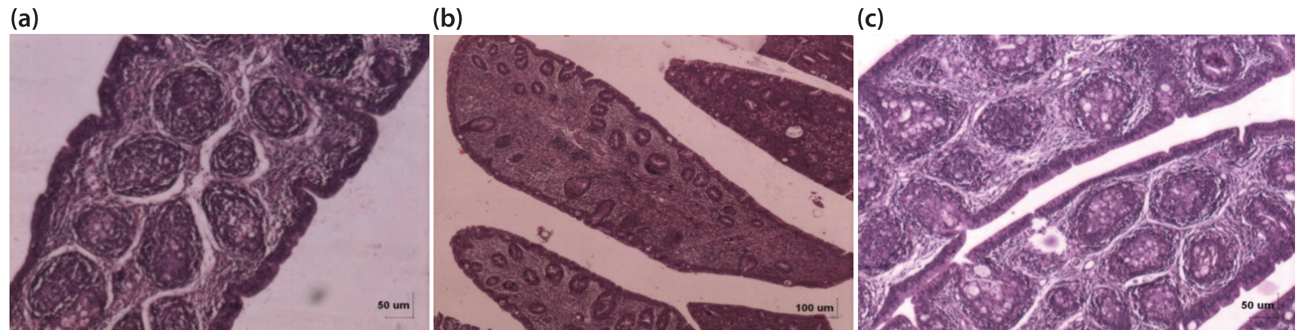

Fig. 3

Bursal tissue section stained with H & E

(a) BGE14 isolate caused severe lymphoid depletion in bursa; **(b)** NKL14 isolate produced follicular atrophy and interstitial cell fibrosis of bursa; **(c)** Normal lymphoid follicles from uninfected bird.

acid position of 270 in the MB11 isolate, and isoleucine was substituted by threonine at residue 272 in the HY12 isolate. Aspartic acid was substituted by asparagine at residue 279 in MB11 and in HY12 isolates of IBDV. Alanine was substituted by threonine at residue 284 in MB11 isolate. Leucine was substituted by isoleucine at residue 294 in PY12 isolate, other very virulent isolates have the substitution of valine and the classical isolate MB11 did not have this substitution. Asparagine was substituted by serine in all very virulent isolates and was not observed in the classical isolate of MB11 at residue 299. Glutamic acid was substituted by alanine in all very virulent isolates except very virulent isolate HY12 and classical isolate MB11 at residue 300. The serine-rich heptapeptide sequence SWSASGS adjacent to the second hydrophilic region was conserved among all the Indian IBDV isolates except MB11.

The identity matrix of nucleotides and amino acids of the isolate MB11 (classical strain) and other very virulent isolates of this study were compared to highly similar isolates 52/70 and UK661, respectively, from GenBank. Classical strain (MB11) had highest nucleotide similarity of 96.7% with both 52/70 and STC and had highest amino acid 96.3% with STC and 96% with 52/70. The pairwise sequence analysis of different very virulent isolates of IBDV shows highest nucleotide and amino acid similarity for BGE14 - 96.1%, EDE14 - 95.5%, HY12 - 98%, NKL14 - 95.5%, PY12 - 96.9%, RPM14 - 96.9% and VCN14 - 96.8% and BGE14 - 98.6%, EDE14 - 98.5%, HY12 - 98.8%, NKL14 - 95.1%, PY12 – 98.3%, RPM14 - 98.6% and VCN14 – 98.6%, respectively, in comparison with UK661. Nucleotide and amino acid sequence identity and divergence are shown in table 2 and 3, respectively.

Phylogenetic analysis

Based on the nucleotide sequence analysis, all isolates except MB11 were grouped in the same cluster along with already reported very virulent European and Japanese isolates, indicating their close evolutionary relationship. Among the classical strains, European strain 52/70 was closer to Indian field isolates than any other classical, variant or attenuated strain as reported by earlier works (Brown *et al.*, 1994; Yamaguchi *et al.*, 1997; Kataria *et al.,* 2001). Phylogenetic analysis revealed 5 distinct branches in the phylogenetic tree with 1000 bootstrap replication. Isolates RPM14, EDE14, VCN14, PY12, BGE14 and NKL14 formed a separate clad close to very virulent strains of other Indian isolates. Whereas isolate HY12 formed a separate clad, found close to other very virulent isolates such as OKYM, TN1/93, JK1/97, Is-KS, UK661 and HR1/96. The MB11 isolate was grouped with the classic strains such as STC, 52-70, Cu-1, PBG and variant strain GLS (Fig. 4).

Antigenic index

Potential antigenic sites within the predicted 145 amino acid hypervariable region were compared by calculating an 'antigenic index' that reflects the influence of several different parameters such as hydrophilicity, surface probability, backbone flexibility and secondary structure prediction (Jameson and Wolf, 1988). Significant differences in the 'antigenic index' profile between classical, virulent and very virulent IBDVs have been previously determined in the hypervariable region. In our studies, classical strain 52/70 showed missing antigenic peak number 1, 8 and 9 when compared with MB11 isolate. Comparison of very virulent Indian isolates with UK661 revealed that except HY12, the other very virulent isolates showed missing antigenic site at number 1. The isolates NKL14, UK661 and EDE14 showed missing antigenic peak identified at number 7 and 8 and HY12 isolate showed missing antigenic peak at number 8 when compared with BGE14

Table 2. Nucleotide variations

Strain	Nucleic acid position																								
	882	**888**	**895**	**897**	**903**	**906**	**918**	**933**	**936**	**937**	**939**	**944**	**951**	**960**	**964**	**975**	**979**	**987**	**996**	**1009**	**1020**	**1023**	**1025**	**1028**	**1032**
52/70	C	A	G	A	C	C	T	G	T	G	G	T	A	C	G	G	G	C	T	C	A	C	A	A	A
BGE14/ABT	T	.	A	C	T	T	C	A	.	.	A	.	.	T	.	A	.	T	C	G	.	.	G	C	T
HY12/ABT	.	.	A	.	.	T	.	.	C	.	.	C	.	.	A	A	.	T	.	A	.	.	G	.	.
MB11/ABT	.	C	.	.	.	.	C	.	A	A	.	.	G	.	A	.	A	.	.	.	.	A	.	.	.
NKL14/ABT	T	.	A	C	T	T	C	A	.	.	A	.	.	T	.	A	.	T	C	G	.	.	G	C	T
PY12/ABT	T	.	A	C	T	T	C	A	.	.	A	.	.	.	.	A	.	T	C	G	.	.	G	C	.
RPM14/ABT	T	.	A	C	T	T	.	.	.	.	A	.	.	.	.	A	.	T	C	G	G	.	G	C	.
VCN14/ABT	T	.	A	C	T	T	C	A	.	.	A	.	.	.	.	A	.	T	C	G	G	.	G	C	.
EDE14/ABT	T	.	A	C	T	T	C	A	.	.	A	.	.	.	.	A	.	T	C	G	G	.	G	C	.
002-73	.	.	.	G	.	.	G	.	C	A	.	C	.	.	.	.	.	.	C	.	.	.	G	.	.
GLS	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	.	.	.
OKYM	.	.	A	.	T	T	.	.	.	.	.	.	.	.	.	A	.	T	.	A	.	.	G	.	.
PBG	.	C	.	.	.	.	C	.	A	A	.	.	G	.	A	.	A	.	.	.	.	A	.	.	.
STC	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
UK661	.	.	A	.	T	T	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	G	.	.

Strain	Nucleic acid position																
	1047	**1065**	**1092**	**1113**	**1119**	**1125**	**1130**	**1140**	**1143**	**1178**	**1179**	**1212**	**1215**	**1227**	**1251**	**1263**	**1281**
52/70	A	A	A	G	T	C	C	C	T	C	A	C	C	T	T	A	T
BGE14/ABT	.	.	G	A	.	.	.	A	C	.	.	T	.	C	.	G	C
HY12/ABT	.	.	G	A	.	.	.	.	C	.	.	T	.	C	C	.	C
MB11/ABT	.	.	.	.	A	T	.	.	.	T	G	.	T	.	C	.	.
NKL14/ABT	.	.	G	A	.	.	G	A	C	.	.	T	.	C	.	G	C
PY12/ABT	.	.	G	A	.	.	.	A	C	.	.	T	.	C	.	G	C
RPM14/ABT	.	.	T	A	.	.	.	.	C	.	.	T	.	C	.	G	C
VCN14/ABT	.	.	T	A	.	.	.	A	C	.	.	T	.	C	.	G	C
EDE14/ABT	G	T	G	A	.	.	.	A	C	.	.	T	.	C	.	G	C
002-73	.	.	T	.	.	.	.	T	.	.	.	T	T	.	C	G	C
GLS	.	.	.	.	.	.	.	T	.	.	.	T	.	.	C	.	.
OKYM	.	.	G	A	.	.	.	.	C	.	.	T	.	C	C	.	C
PBG	.	.	.	.	A	.	.	.	.	.	G	.	.	.	C	.	.
STC	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.
UK661	.	.	G	A	.	.	.	.	C	.	.	T	.	C	C	.	C

Table 3. Amino acid variations

Strain	Amino acid position											
	253	**256**	**270**	**272**	**279**	**284**	**294**	**299**	**300**	**330**	**334**	**350**
52/70	Q	V	A	I	D	A	L	N	E	S	A	T
BGE14/ABT	.	I	.	.	.	.	V	S	A	.	.	.
HY12/ABT	.	I	.	T	N	.	I	S	.	.	.	.
MB11/ABT	H	.	T	.	N	T	.	.	.	R	.	M
NKL14/ABT	.	I	.	.	.	.	V	S	A	.	G	.
PY12/ABT	.	I	.	.	.	.	V	S	A	.	.	.
RPM14/ABT	.	I	.	.	.	.	V	S	A	.	.	.
VCN14/ABT	.	I	.	.	.	.	V	S	A	.	.	.
EDE14/ABT	.	I	.	.	.	.	V	.	.	.	.	.
002-73	.	.	T	T	G	.	.	S	.	.	.	.
GLS	H	.	.	.	N	T	.	.	.	.	.	.
OKYM	.	I	.	.	.	.	I	S	.	.	.	.
PBG-98	H	.	T	.	N	T	.	.	.	R	.	.
STC	.	.	T	.	.	.	.	.	.	.	.	.
UK661	.	I	.	.	.	.	I	S	.	.	.	.

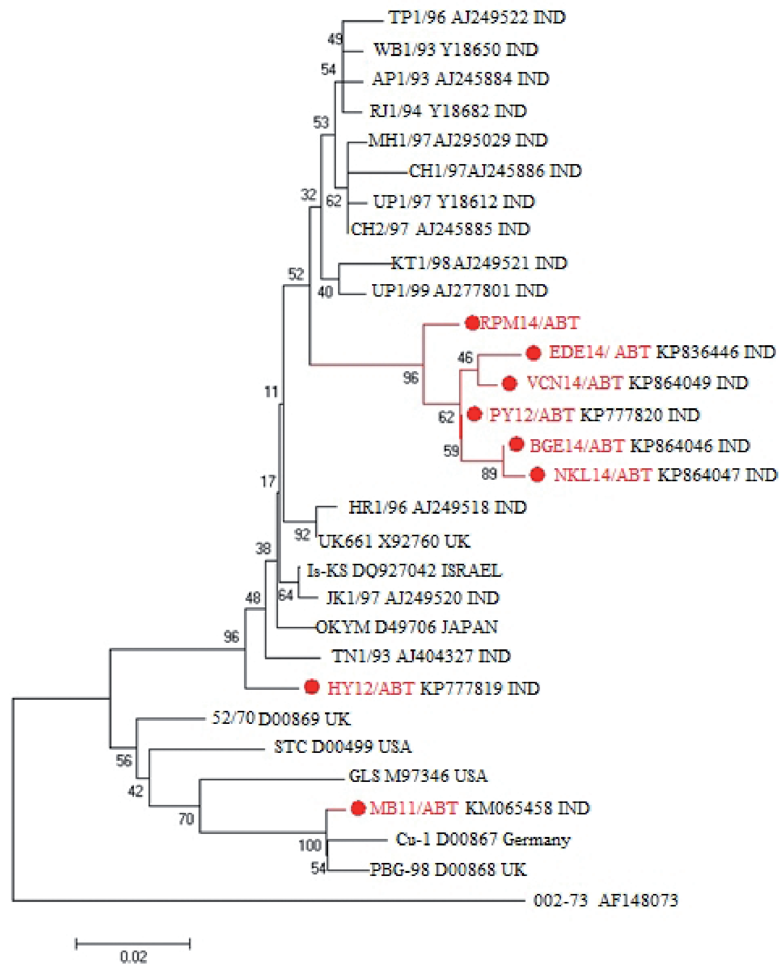

Fig. 4

Phylogenetic analysis based on partial segment A sequence of IBDV

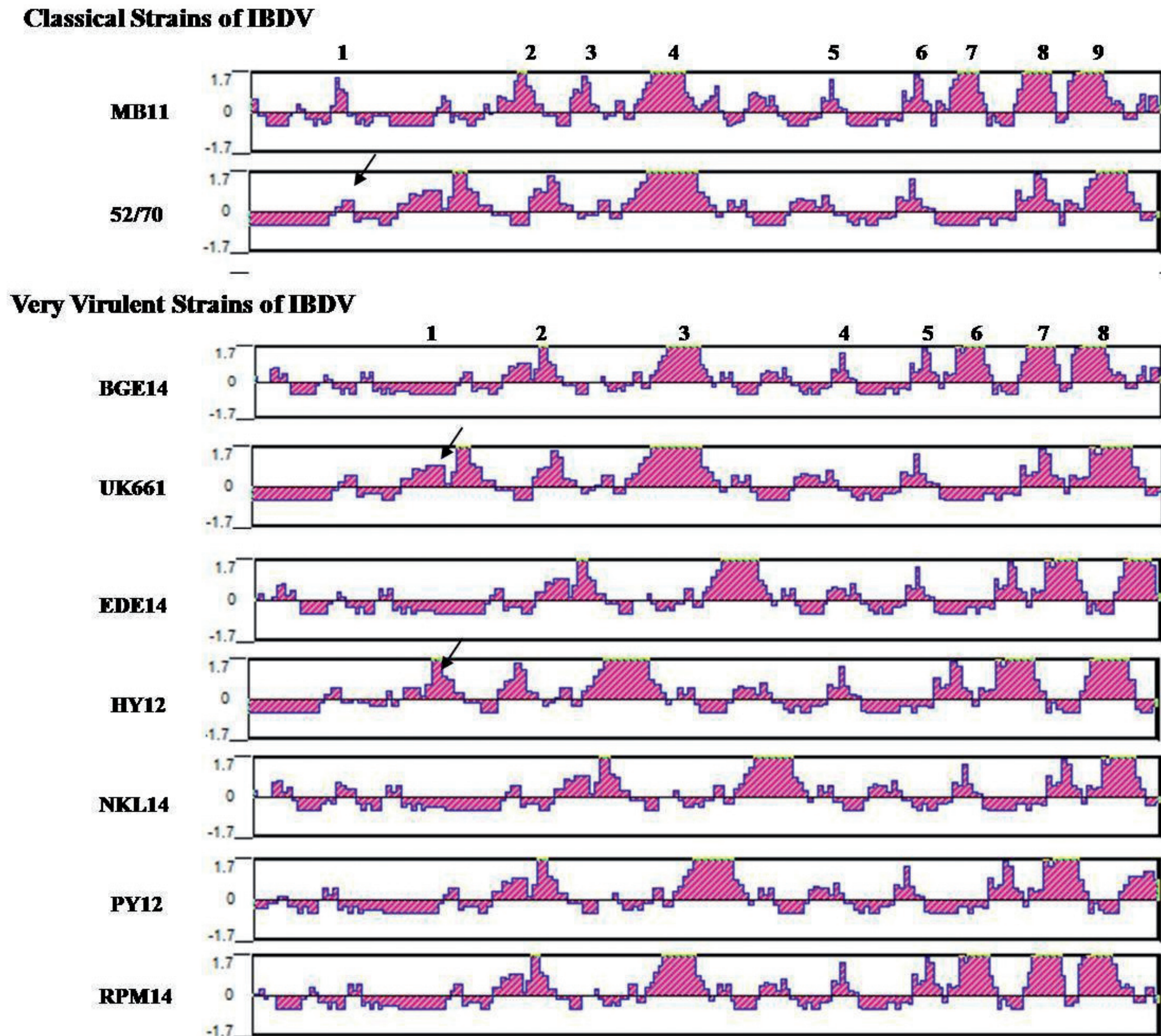

Fig. 5

Antigenic index analysis in VP2 gene of the IBDV isolates

Peaks identified as potential antigenic sites within the deducted amino acid sequence of the hypervariable region of VP2 gene from amino acid residues 252 to 397. Eight IBDV isolates are being compared with European classical virulent and UK 661 very virulent strains. The classical MB11 isolate has 9 major antigenic peaks. The classical 52/70 strains show missing antigenic peaks number1, 8 and 9 and there are changes in the topography of the major antigenic peak of MB11 isolate. The very virulent strain BGE14 shows 7 major antigenic peaks when compared to UK661 isolates. Except the very virulent UK661 and HY12 isolates, remaining vvIBDV isolates show the missing antigenic peak number 1 when compared to other very virulent isolates. The NKL14, UK661 and EDE14 show missing antigenic peak that was identified at number 7 and 8, HY12 isolate show missing antigenic peak at number 8.

isolate, which is having 7 major antigenic sites showed in Fig. 5.

Discussion

One of the major problems observed in the poultry farms of India are the frequent outbreaks of IBD in spite of the extensive use of available IBD vaccines. This failure in vaccination could be due to the genetic drift or mutations observed in VP2 (which is a major protective antigen of IBDV) that may result from immunological pressure or genetic reassortment between more than one strain (Hon *et al.,* 2006). In this study, bursal tissues collected from different states of southern India were subjected to RT-PCR using primers specific for variable region in the VP2 gene of IBDV, and isolations in CEF cells. The VP2 gene of IBDV was targeted to determine the prevalence and to track evolution-

ary changes at the molecular level by sequencing. Molecular evaluation of the hypervariable region of the chosen eight isolates were studied to compare their nucleotide and amino acid sequences with other Indian IBDV isolates and isolates from other parts of the world. The multiple alignment and phylogenetic analysis of the hypervariable domain of the VP2 region helped to group the IBDV isolates into different pathogenic subgroups.

The serine-rich heptapeptide sequence SWSASGS adjacent to the second hydrophilic region was conserved among all the Indian IBDV isolates except MB11. Conservation of this sequence was earlier reported to be an indication of a virus isolate being pathogenic in nature (Vakharia *et al.,* 1994). Lim *et al.* (1999) identified the minimal changes (279D-N and 284A-T) responsible for tissue culture adaptation and concomitant attenuation of vvIBDV by *in vitro* mutagenesis without need to change the 'SWSASGS' heptapeptide. In the present study, the isolates HY12 and MB11 showed mutational changes at 279D-N and isolate MB11 had changes also at 284A-T. It was found that the isolates BGE14, HY12, PY12, NKL14, VCN14, RPM14 and EDE14 are not of vaccinal or attenuated origin due to the absence of 253 Histidine and 284 threonine mutations that are typically found in attenuated vaccine strains (Jackwood *et al.,* 2008). Yuwen *et al.* (2008) reported that the serine rich heptapeptide SWSASGS was the conserved marker amino acids of vvIBDVs, which was different for classical, attenuated, vaccine and variant strains as 222T (P or Q), 256V, 294L and 299N. The heptapeptide of the attenuated and vaccine strain is SWSARGS (Eterradossi *et al.* 1999). In the present study, 'SWSARGS' heptapeptide sequence was observed only in the isolate MB11.

All the isolates had glycine at residue 254 as this was the characteristic of standard IBDV strains. Leucine was substituted by isoleucine at residue 294 in PY12 isolate. But other Indian very virulent isolates and isolates from other parts of the world are having valine residue in place of leucine. Substitution of isoleucine at residue 294 had not been reported earlier from India in any pathotypes of IBDV viruses. Similar amino acid change has also been reported by Banda *et al.* (2001) in isolate 9109 from United States and it was concluded that the clinical significance of this substitution at residue 294 in the protein sequence of the 9109 isolate was not clear. The poor performance and recurrent respiratory problems of broiler flock were correlated with the presence of IBDV 9109 isolate in the flock, most likely as the consequences of immunosuppression. However, the birds did not exhibit clinical signs or mortality rates characteristic of vvIBDV strains. The preliminary clinical studies in SPF birds and commercial broilers with 9109 isolate produced a subclinical form of IBDV with bursal atrophy.

The mutations in the very virulent isolates reported earlier by Cao *et al.* (1998) and Eterradossi *et al.* (1999) at 256V-I, 294L-I and 299N-S were conserved in all Indian isolates of vvIBDV including the isolates of present study, whereas MB11 isolate had valine at 256, like other classical strains of IBDV. Asparagine was substituted by serine in all very virulent isolates at residue 299 and the same was not observed in the classical isolate MB11.

All vvIBDV isolates have alanine residue at 300, except vvIBDV isolate HY12 and classical isolate MB11. The HY12 and MB11 isolates have glutamic acid at residue 300. Mohamed *et al.* (2014) also reported the presence of alanine residue at 300 in the vvIBDV isolates from Bangladesh, Nigeria, India and Nepal. Glutamic acid is characterized by negatively charged (acidic) R group, whereas, the R group in alanine is non polar (hydrophobic). It is probable that this change from negative charge to non-polar may modify the protein folding or the interaction with other molecules that may change the topography of the neutralizing epitopes that may lead to vaccination failure (Jackwood *et al.,* 2011).

De Paula *et al.* (2004) also reported the presence of serine at position 299 in BR-GO strain (Brazil). However, they didn't observe this change exclusively in all vvIBDV strains. On the other hand, Chong *et al.* (2001) stated that the asparagine to serine substitution at the amino acid position 299 and alanine to glutamic acid substitution at position 300 were not related to pathogenicity of the strains, although serine at position 299 was observed in many vvIBDV strains.

Raus *et al.* (2013) reported that among five amino acid residues, only A (222) was located at hydrophilic region of VP2 variable region. Other amino acids I (242), I (256), I (294) and S (299), though they were closely located, are not directly in hydrophilic regions and are believed to be still in the projection domains in VP2. Amino acid residues A (222), I (256) and I (294) were reported to be unique residues, which had been used as the indication of the very virulent subtypes. IBDV isolates, which carry at least these 3 amino acid residues, are categorized as very virulent subtypes. When compared with attenuated viruses, there were differences at the positions 253, 279, 284, 290 and 330 of very virulent viruses. Among these five, the amino acids at positions 253, 279 and 284 were confirmed to be important marker for the virulence, cell tropism and pathogenic phenotypes (Brandt *et al.,* 2001). The same finding was also reported by van Loon *et al.* (2002), and they observed the change of the amino acids at positions 253 and 284 in both tissue culture adaptation and virus attenuation in SPF chickens. According to Coulibaly *et al.* (2005), these three amino acids at positions 253, 279 and 284 were located in the most exposed loops of the projection domain that had direct interaction with immune system. However, Boot *et al.* (2000) found that the changes of VP2 in attenuated IBDV with very virulent pathotypes showed that no mortality and morbidity could be observed. Previous studies indicated that few of Indian IBDV isolates were similar to very virulent IBDV from European and other Asian countries and it was

postulated that vvIBDV strains would have entered India from northern and eastern parts, probably from China and South East Asia (Kataria *et al.,* 2001). Complete genome sequencing of these isolates and classical viruses would provide more information on virus characteristics.

Phylogenetic analysis revealed that most of the isolates are more closely related to the very virulent UK661 isolate than any other classical and vaccine strains. As reported earlier by Ramadass *et al.* (2003), the sequence analysis indicated that presence of mixed type of IBDV infection in southern states of India poses a problem in the institution of vaccination regimen in the control of IBD. Jenberie *et al.* (2013) reported that the Ethiopian vvIBDV clustered with African IBDV genetic lineage, independent of the Asian / European IBDV lineage. Nwagbo *et al.* (2016) studied the genetic diversity of IBDV in Nigeria by sequencing the VP2 and VP1 region and they reported a new lineage of Nigerian reassortant IBDV strains.

The potential antigenic sites were also reported by Lana *et al.* (1992), who found that the variant A virus was missing a peak in the antigenic index within the hypervariable domain (amino acids 284–288) that is present in all other classical viruses. Banda *et al.* (2001) reported that five isolates (619, 625, 849, 850 and 11153) exhibited identical antigenic profile indexes to that of variant E strain and another isolate (9109) exhibited a profile similar to that of the STC strain. Mohamed *et al.* (2014) reported the missing antigenic peaks number 4 and 2 in strains 3 and 4, respectively, as well as changes in the topography of major antigenic peaks in strains 4 and 7 when compared to UK661.

In conclusion, the results show that the eight isolates from different states of southern India share similar origins with vvIBDVs of Asia and Europe. The isolate MB11, which has the serine rich heptapeptide sequence 'SWSARGS', shares the origin with classical virulent IBDV as indicated by other determinants in the partial VP2 hypervariable region. Though the isolate HY12 carries the heptapeptide 'SWSASGS', it also exhibits variations in the VP2 determinant regions. The *in vivo* study also reveals the very virulent nature of all the isolates except MB11, which exhibits the classical virulent feature of IBDV. Furthermore, complete genome sequencing of these isolates and classical viruses would provide more information on virus characteristics and evolution.

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