ISOLATES OF INFECTIOUS BURSAL DISEASE VIRUS FROM INDIA ARE OF VERY VIRULENT PHENOTYPE

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Summary. – Four Indian field isolates, a classical virulent and an attenuated vaccine strains of Infectious bursal disease virus (IBDV) have been characterized by sequence analysis of part of the VP1 gene (from nucleotide 1538–1979) comprising one of viral RNA dependent RNA polymerase motifs. Sequence alignment of these viruses with reported viruses of other countries revealed Indian IBDV field isolates to be 100% similar to very virulent Japanese (OKYM), European (UK661) and Bangladesh (BD3/99) IBD viruses at amino acid level, whereas they had 0.2–0.9% divergence at nucleotide level. Out of the total 24 nucleotide changes found in the Indian field isolates, as well as reported very virulent viruses, only one resulted in amino acid change S-P at 562 position. The Indian field isolates displayed nucleotide divergence of 10.6–11.6% and amino acid divergence of 2.8–3.5% from the classical virulent and attenuated vaccine strains. The RNA dependent RNA polymerase motif from amino acid 528–541, present in the sequence analyzed, was conserved among all the viruses, irrespective of pathotype and serotype. In the phylogenetic tree, based on nucleotide sequence, Indian field viruses were grouped with reported very virulent viruses in one lineage whereas, classical virulent, attenuated vaccine and serotype 2 strains formed part of the second lineage. But in the phylogenetic tree based on amino acid sequence alignment, the serotype 2 strain OH grouped with Indian field isolates and reported very virulent viruses in one lineage and classical virulent and attenuated vaccine strains formed the second lineage.

Key words: Infectious bursal disease virus; VP1 gene; nucleotide sequence

Introduction

Infectious bursal disease virus (IBDV) is responsible for a highly contagious, immuno-suppressive disease in young chickens, destroying dividing B-lymphocytes in the bursa of Fabricius (Allan *et al*., 1972; Hirai *et al*., 1974). Out of the two serotypes of the virus reported, only serotype 1 viruses cause clinical disease in chickens whereas, serotypes 2 viruses are apathogenic in nature (Jackwood *et al*., 1985). With in serotype 1 viruses, various antigenic/ pathogenic subtypes like classical virulent, attenuated, antigenic variants and very virulent viruses have also been reported (Jackwood and Saif, 1987; Nunoya *et al*., 1992).

IBDV, classified under genus *Avibirnavirus* in the family *Birnaviridae*, is non-enveloped, icosahedron having a double stranded, bi-segmented RNA genome (Murphy *et al*., 1995). Segment A (3.3 kb) encodes a 110-K polyprotein (NH2-VP2-VP4-VP3-COOH), which is auto cleaved into VP2, VP4 (NS) and VP3 proteins (Hudson *et al*., 1986). Another non-structural protein, VP5 is encoded by an overlapping ORF present at the 5' end of the segment A (Mundt *et al*., 1995). Whereas, segment B (2.8 kb), which has single ORF, encodes for 90 K VP1 protein, which is the putative, virion associated RNA dependent RNA polymerase (RdRp) (Nagy

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Abbreviations: cDNA = complementary DNA; CEF = chicken embryo fibroblasts; IBD = infectious bursal disease; IBDV = IBD virus; ORF = open reading frame; PCR = polymerase chain reaction; RdRp = RNA-dependent RNA polymerase; RT = reverse transcription; MMLV = Moloney murine leukemia virus

et al., 1987; Spies *et al*., 1987). Various other functions of VP1 protein include transcriptase and methyl and guanyl transferase activities (Spies *et al*., 1987; Spies and Muller, 1990).

Many workers have exploited sequence variations in the VP2 gene of IBDV isolates for their molecular characterization (Brown *et al*., 1994; Cao *et al*., 1998; Kataria *et al*., 2001; Viswas *et al*., 2002). As the origin of very virulent IBDV strains is still not known, sequence characterization of other functionally important viral genes, including VP1 may help in evolutionary characterization of these newly emerged strains. We report here, the characterization of Indian IBDV field isolates, classical and vaccine strains by sequence analysis of part of VP1 gene encoding one of the functionally important RdRp motifs.

Materials and Methods

Viruses and cell culture. Four field isolates, collected as infected bursal tissues from different geographic locations of India, a vaccine strain (Intermediate plus, IM+) and a cell culture adapted Indian classical strain (Poona) were used in the study. Details of these and other viruses used for sequence comparison are given in Table 1. The vaccine and cell culture adapted Indian classical strains, were propagated in chicken embryo fibroblasts (CEF), grown and maintained in medium M-199 supplemented with 10% new born calf serum.

RNA extraction and RT-PCR. Total RNA from infected CEF cells or bursal tissue homogenates was extracted using TRIZOL reagent (Life Technologies Inc., USA) as per the manufacturer's protocol. The VP1 gene sequence from all the viruses were amplified by RT-PCR, as described earlier (Indervesh *et al*., 2002)

Sequence analysis. Both the strands of the PCR products were sequenced after cloning them in pGEMT vector (Promega, USA) by T7 sequencing kit (Pharmacia-Amersham, USA) following the manufacturer's protocol using $\alpha[^{35}S]$dATP. After electrophoresis in 6% polyacrylamide gel, all the sequences were read manually and analyzed using 'Lasergene' software (DNASTAR, Inc. USA). EMBL accession numbers of these sequences and reported sequences used for comparison are given in Table 1. Sequences were aligned by Clustal method and analyzed phylogenetically using the 'MegAlign' programme of the same software. In this programme, sequences are clustered on the basis of distances between pairs, using K-tuple value 1, Gap penalty 3, Window 5 and Diagonal saved 5, where K-tuple value is expressed as the unit's length in nucleotides, window specifies the range of programme searches for all possible or alternate K-tuple values. After aligning clusters pair wise, all the sequences are aligned in the groups.

Results and Discussion

For molecular characterization and phylogenetic analysis, complete genome segments, as well as parts of VP1 and VP2 gene sequences of IBDV have been exploited by many workers (Brown and Skinner, 1996; Yamaguchi *et al*., 1997; Kataria *et al*., 2001; Viswas *et al*., 2002; Bais *et al*., 2003). In this study, RT-PCR amplified 442 bp VP1 gene sequence (position 1538 to 1979) of four Indian IBDV field isolates, a vaccine and a classical strain were analyzed. This region was selected for molecular characterization and phylogenetic analysis, as variation in this region has been demonstrated earlier by restriction enzyme analysis of RT-PCR amplified products (Tiwari *et al*., 2003) and also it contains one of the RdRp motifs. Sequence characterization of other important regions of the viral genome may throw some light on the evolution of newly emerged strains, which is still not clear.

The sequence analysis revealed that nucleotide changes, in any of the viruses as compared to consensus sequence, varied from 1 (very virulent virus) to 44 (IM+ vaccine strain) out of which 6 nucleotide changes 1449A-G, 1559T-A, 1582C-T, 1663C-T, 1710T-C and 1920T-C resulted in change in amino acids 508K-R, 511S-R, 519S-L, 546P-L, 562S-P and 632W-R, respectively. Similar to reported very virulent strains, Indian field isolates had 24 nucleotide changes as compared to consensus, resulting in only one amino acid change S-P at position 562.

Table 1. Characteristics of the viruses used in this study

Virus strain	Strain type	Origin or source	Acc. No.
KT1/98	Field isolate	Karnataka, India	AJ295022
RJ1/94	Field isolate	Rajasthan, India	AJ295021
TN1/93	Field isolate	Tamil Nadu, India	AJ295023
UP1/93	Field isolate	Uttar Pradesh, India	AJ295027
IBDV (Poona)	Cell culture adapted classical strain	India	AJ295024
Intermediate plus (IM+)	Vaccine	Commercial vaccine used in India	AJ295025
BD 3/99	Very virulent	Bangladesh	AF362770
OKYM	Very virulent	Japan	D49707
UK661	Very virulent	UK	X92761
52/70	Classical virulent	UK	D12610
OH	Serotype 2	USA	U20950
QC-2	Classical virulent	Australia	U62661

Table 2. Nucleotide sequence similarities (%, upper triangle) and divergences (%, lower triangle) of an IBDV VP1 gene motif in different strains (for details see Table 1)

	KT1/98	RJ1/94	TN1/93	UP1/97	BD3/99	OKYM	UK661	POONA	IM+	52/70	QC2	OH
KT1/98	***	99.5	99.1	99.3	98.0	99.8	99.5	90.1	89.9	89.4	89.4	90.0
RJ1/94	0.5	***	99.1	99.3	98.0	99.8	99.5	90.1	89.9	89.4	89.4	90.0
TN1/93	0.9	0.9	***	99.3	98.0	99.3	99.1	90.3	90.1	89.6	89.2	89.6
UP1/97	0.7	0.7	0.7	***	98.2	99.5	99.3	89.9	89.6	89.2	89.2	89.8
BD3/99	1.8	1.8	1.8	1.6	***	98.2	98.0	90.1	89.9	89.4	89.4	90.0
OKYM	0.2	0.2	0.7	0.5	1.6	***	99.8	90.3	90.1	89.6	89.6	90.2
UK661	0.5	0.5	0.9	0.7	1.8	0.2	***	90.1	89.9	89.4	89.4	90.0
POONA	10.8	10.8	10.6	11.1	10.6	10.6	10.8	***	99.3	97.3	97.5	93.4
IM+	11.1	11.1	10.8	11.4	10.9	10.8	11.1	0.7	***	97.5	97.7	93.7
52/70	11.4	11.4	11.1	11.6	11.1	11.1	11.4	2.5	2.3	***	98.2	93.9
QC2	11.3	11.3	11.6	11.6	11.1	11.0	11.3	2.3	2.1	1.8	***	94.1
OH	9.8	9.8	10.3	10.1	10.1	9.5	9.8	5.7	5.5	5.2	5.0	***

The amino acid changes 508K and 511S were present in Indian field isolates as well as reported very virulent viruses UK661 and OKYM, but not in other serotype 1 viruses. However, presence of similar amino acid in serotype 2 strain OH indicates these changes may not be related to virus pathogenicity. The amino acid change 546P-L in Indian classical strain was similar to attenuated vaccine strain IM+. Earlier also, based on VP2 gene hypervariable region sequence, the same Indian classical strain (Poona) was found to be similar to attenuated vaccine strains (Kataria, 1999). This could be because this classical strain has been passaged in cell culture for more than 20 times. However, IM+ vaccine strain had a unique amino acid R at position 632.

All the four Indian field isolates showed 100% similarity with Japanese (OKYM) and European (UK661) very virulent viruses at amino acid level, whereas these viruses had divergence of 0.2 to 0.9% at nucleotide level (Table 2). Similar to reported very virulent viruses, Indian field isolates had 10.6 to 11.6% divergence at nucleotide and 2.8 to 3.5% at amino acid levels from the classical and attenuated vaccine strains. As compared to classical and attenuated vaccine strains having divergence of 5.0–5.7%, Indian field isolates had more divergence (9.8–10.3%) from serotype 2 strain OH, at nucleotide level. But at amino acid level, classical and attenuated strains were more diverged (2.8–3.5%) from serotype 2 strain as compared to reported very virulent and Indian field isolates having only 1.4% divergence. Earlier, similar findings have also been reported by other workers (Yehuda *et al*., 1999; Islam *et al*., 2001). Despite variations in different regions, the RdRp motif, present in the region studied, was found to be conserved, both at nucleotide (1608–1649) and amino acid (528–541) levels, irrespective of pathotypes or serotypes. Islam *et al*. (2001) also reported similar findings for one of the very virulent field isolates, BD3/99 from Bangladesh.

Based on nucleotide sequence alignment, all the IBD viruses were phylogenetically grouped in two major genetic lineage (Fig. 1). The first major lineage included four Indian

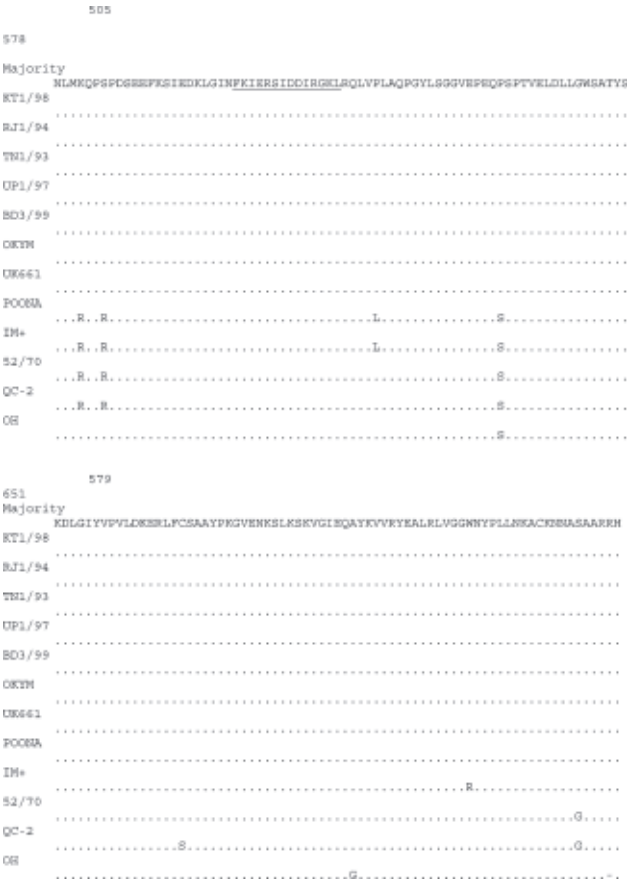

Fig. 1

Comparison of deduced amino acid sequences of VP1 protein from positions 505 to 651 of different IBDV strains

For the details of virus strains see Table 1. The amino acids identical to the majority are indicated by a dot. The reported conserved amino acids 528–541 are underlined.

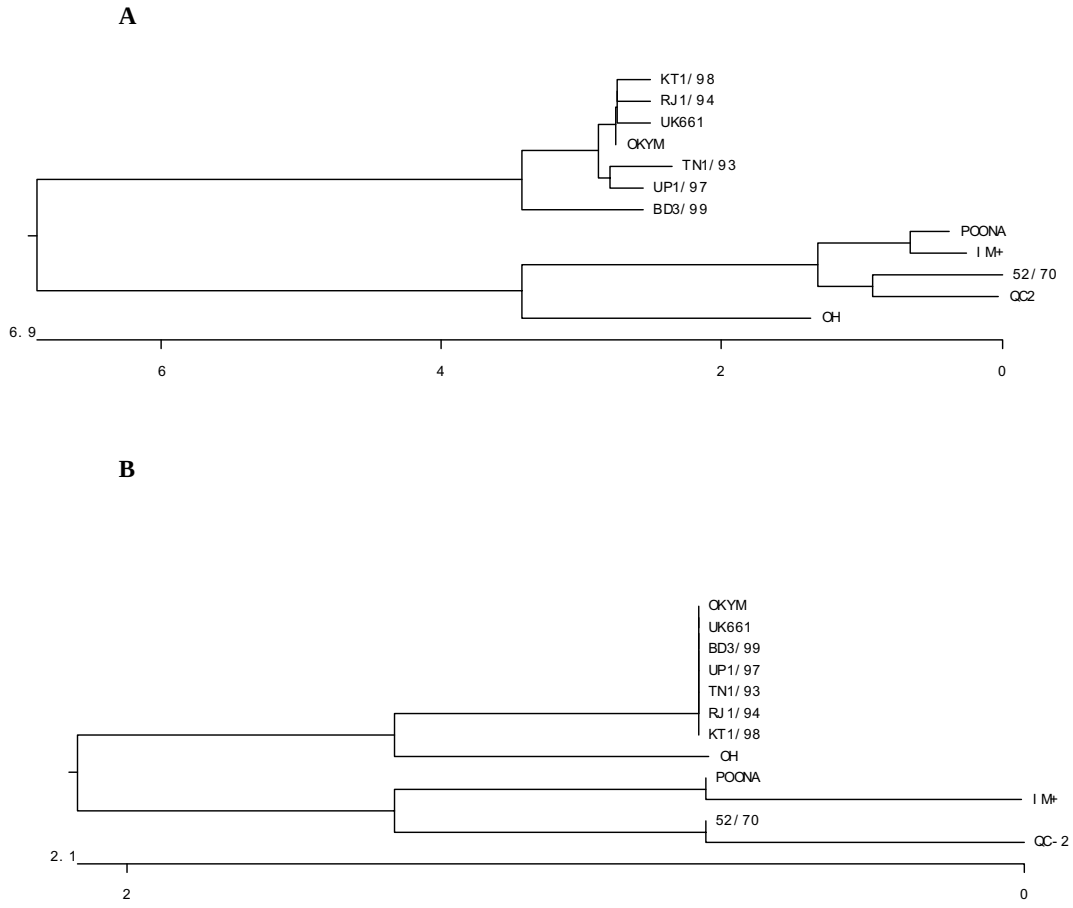

Fig. 2

Phylogenetic trees constructed on the basis of nucleotide (A) and deduced amino acid sequences (B) of an IBDV VP1 gene motif demonstrating the relationship of Indian to published IBDV strains

For the details of virus strains see Table 1.

field isolates (KT1/98, RJ1/94, TN1/93 and UP1/97) and reported very virulent viruses, whereas classical and attenuated viruses along with serotype 2 strain were placed in second lineage. On the basis of both partial and complete VP1 gene sequence, Yamaguchi *et al*. (1997) also reported that Japanese and European very virulent viruses forming separate lineage away from classical and serotype 2 viruses. Similar to this, based on VP2 gene sequence, earlier also, we have reported phylogenetic grouping of Indian field viruses with reported very virulent viruses (Kataria *et al*., 2001; Viswas *et al*., 2002).

Two major genetic lineages were also observed in the phylogenetic tree based on amino acid sequence alignment (Fig. 2). Contrary to nucleotide sequence, in the phylogenetic tree based on amino acid sequence, serotype 2 strain was placed along with all Indian field isolates. This could be because albeit classical viruses have high degree of nucleotide sequence divergence (5.0 to 5.7%) from serotype 2 strain, these changes being synonymous, did not result in amino acid change. Unlike phylogenetic grouping of Indian field isolates, based on VP1 gene sequence, the grouping based on both nucleotide and amino acid sequence of VP2 gene hypervariable region showed separate lineage for serotype 2 strain OH away from serotype 1 viruses (Kataria *et al*., 2001; Viswas *et al*., 2002).

Though, the inconsistent phylogenetic tree patterns, based on VP1 gene sequence with respect to serotype 1 and 2 viruses was evident in our study but nevertheless, other than VP2 gene sequence, based on VP1 gene sequence also it was possible to group serotype 1 IBD viruses as very virulent and other pathotypes, which is very important for epidemiology point of view.

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