NUCLEOTIDE SEQUENCE ANALYSIS OF VARIABLE REGION OF VP2 GENE OF TWO INFECTIOUS BURSAL DISEASE VIRUS ISOLATES FROM COMMERCIAL POULTRY FARMS

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Summary. – Two infectious bursal disease virus (IBDV) isolates were obtained from commercial poultry farms with a history of severe outbreaks. A 474-bp product encompassing hypervariable region of IBDV VP2 gene was amplified by reverse transcription–polymerase chain reaction (RT-PCR). The nucleotide sequences of two isolates, VMB1 and VMB2, were determined and compared with those of twenty IBDV strains, including seven very virulent, four classical virulent, four classical attenuated, three antigenic variants and two avirulent serotype 2 strains. The two isolates showed a similarity of 96.5–98.4% with very virulent strains, 84.6–94.6% with classical virulent strains, 90.0–91.4% with classical attenuated strains, 83.0–91.9% with antigenic variants and 65.8–68.7% with avirulent strains. The deduced amino acid sequences of the two isolates showed amino acid substitutions of V256I, N279D, L294I and N299S, specific for very virulent strains. Phylogenetic analysis showed that the two isolates, along with a reported very virulent Indian strain, were closely related to European, Japanese and Chinese very virulent strains indicating their evolutionary origin.

Key words: infectious bursal disease virus; virus isolates; RT-PCR; nucleotide sequence; amino acid sequence; phylogenetic analysis

Introduction

Infectious bursal disease (IBD), also known as the Gumboro disease, is a fatal, immunosuppressive disease of young chickens, characterized by severe lesions in bursa of Fabricius, followed by death, causing heavy economic losses to the poultry industry (Hirai *et al.*, 1974; Saif, 1991; Lin *et al.*, 1993). IBD virus (IBDV) (species *Infectious bursal disease virus*, genus *Avibirnavirus*, family *Birnaviridae*) is the causative agent of the disease. The virus genome consists of two segments, A and B, of double stranded RNA (Dobos *et al.*, 1979). Segment A (3.2 kbp) codes for two structural proteins, VP2 and VP3, and for two non-structural proteins, VP4 and VP5. Segment B codes for a non-structural protein VP1, the viral transcriptase. Two serotypes, 1 and 2, of IBDV have been reported, of which those belonging to serotype 1 are pathogenic to chickens, while those of serotype 2 are non-pathogenic (Jackwood *et al.*, 1982).

VP2 is considered the major host-protective virus antigen containing at least three neutralizing epitopes and the sites that determine the virus virulence (Eterradossi *et al.*, 1998; Pitcovski *et al.*, 1998; Chai *et al.*, 2001). The variable region of VP2 gene comprises a tight cluster of amino acid changes, which may be responsible for generation of antigenic variants among the strains (Bayliss *et al.*, 1990). This region, referred to as hypervariable one (HVR), is therefore an ideal site for

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Abbreviations: cDNA = complementary DNA; DNTPs = deoxyribose nucleotide triphosphates; DTT = dithiothreitol; HVR = hypervariable region; IBD = infectious bursal disease; IBDV = IBD virus; IPNV = infectious pancreatic necrosis virus; MMLV = Moloney murine leukemia virus; PCR = polymerase chain reaction; RNase = ribonuclease; RT = reverse transcription

determining the antigenic variation by sequence analysis of different IBDV strains (Proffitt *et al.*, 1999). Molecular characterization of isolates and identification and characterization of differences among the isolates from different geographical regions, may help in developing a correct and effective vaccine and in understanding the evolution of viruses (Pitcovski *et al.*, 1998).

This study was aimed at molecular characterization of IBDV isolates obtained from outbreaks in commercial farms with a history of proper vaccination measures in Karnataka State of India.

Materials and Methods

IBDV isolates. Intact bursae were collected from the affected birds in commercial poultry farms, which had severe outbreaks of IBD. IBDV isolates obtained from these bursal specimens, designated as VMB1 and VMB2, were used for genomic characterization.

Total RNA was extracted from bursal specimens using Trizol LS reagent (Life Technologies, USA), according to manufacturer's protocol. Briefly, 500 µl of Trizol reagent was added to approximately 100 mg of a bursal specimen and homogenized. The homogenate (500 µl) was mixed with 500 µl of Trizol reagent and centrifuged at 5,000 x g for 3 mins. The supernatant was collected and mixed with 200 µl of chloroform. The aqueous phase was separated by centrifugation at 12,000 x g for 15 mins at 4°C. RNA in the aqueous phase was precipitated with 500 µl of isopropanol and collected by centrifugation at 12,000 x g for 10 mins. After washing with 70% ethanol by centrifugation, the pellet was dissolved in RNase-free water and stored at -70°C until used.

RT-PCR. The forward primer P_{23}(5′-CCCAGAGTCTACAC CATA-3′) and the reverse primer RP_{53} (5′-TCCTGTTGCCA CTCTTTC-3′), described by Lin *et al.* (1993), were used for the amplification of 474 bp amplicon corresponding to the nucleotide positions 738 to 1211 of the IBDV VP2 gene according to the numbering system of Bayliss *et al.* (1990). For the RT reaction the extracted total RNA (5 µl corresponding to approximately 5 µg of RNA) was mixed with 20 pmoles each of the forward and reverse primers, heated at 85°C for 5 mins and cooled on ice. The RNA was then reverse transcribed to cDNA by adding 4 µl of the 5X first strand buffer, 1 µl of 0.1 mol/l dithiothreitol (DTT), 1 µl of 10 mmol/l dNTPs, 2 µl of RNasin and 200 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies, USA) and 4 µl of distilled water. The RT reaction was carried out at 42°C for 90 mins, at 95°C for 5 mins and stopped at 4°C.

For amplification of HVR by PCR 5 µl of cDNA was incubated in total volume of 50 µl containing 5 µl of the 10x PCR buffer, 1.5 µl of 50 mmol/l $MgCl_2$, 1 µl each of the forward and reverse primers (20 pmoles each), 1 µl of 10 mmol/l dNTPs, 1 µl (3 U) of Taq DNA polymerase, and 34.5 µl of distilled water. The reaction conditions consisted of denaturation at 94°C for 30 secs, annealing at 56°C for 30 secs, and extension at 72°C for 1 min. These steps were repeated in 35 cycles. The PCR products were subjected to electrophoresis on 1.5% agarose gels and staining with ethidium bromide.

Nucleotide sequencing and analysis. The amplified products were purified from low melting point agarose gel and sequenced on an ABI prism 377 automated DNA sequencer at the Indian Institute of Science, Bangalore, India. The nucleotide sequences and deduced amino acid sequences were analyzed by the Editseq Program (DNAstar, Inc). The sequence comparison was carried

Table 1. Characteristics of IBDV strains used for sequence analysis

Strain	Serotype	Pathotype	Geographical region	Reference	Acc. No.
VMB1	1	Very virulent	India	This paper	AJ416444
VMB2	1	Very virulent	India	This paper	AJ416445
KT1/98	1	Very virulent	India	Kataria *et al.* (2001)	AJ249521
UK661	1	Very virulent	UK	Brown *et al.* (1994)	X92760
OKYM	1	Very virulent	Japan	Yamaguchi *et al.* (1996)	D49706
KK1	1	Very virulent	Korea	Kwon *et al.* (2000)	AF165150
BD3/99	1	Very virulent	Bangladesh	Islam *et al.* (2001)	AF362776
D6948	1	Very virulent	The Netherlands	Boot *et al.* (2000)	AF240686
HK46	1	Very virulent	China	Cao *et al.* (1998)	AF051838
002-73	1	Classical virulent	Australia	Hudson *et al.* (1986)	M64738
52/70	1	Classical virulent	UK	Bayliss *et al.* (1990)	D00869
Cu-1wt	1	Classical virulent	Germany	Unpublished	AF362747
STC	1	Classical virulent	USA	Kibenge *et al.* (1990)	D00499
Cu-1	1	Classical attenuated	Germany	Bayliss *et al.* (1990)	D00867
P2	1	Classical attenuated	Germany	Mundt and Muller (1995)	X84034
CEF94	1	Classical attenuated	The Netherlands	Boot *et al.* (1999)	AF194428
PBG-98	1	Classical attenuated	UK	Bayliss *et al.* (1990)	D00868
GLS	1	Antigenic variant	USA	Vakharia *et al* (1994)	M97346
K310	1	Antigenic variant	Korea	Kwon *et al.* (2000)	AF165149
Variant-E	1	Antigenic variant	USA	Heine *et al.* (1991)	D10065
23/82	2	Avirulent	UK	Bernstein (1994)	AF362773
OH	2	Avirulent	USA	Jackwood *et al.* (1982)	M66722

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        234                                                                                       322
VMB1    IDAITSLSIGGELVF-QTSVQGLILGATIYLIGFDGTAVITRAVAADNGLTAGTDNLMPFNIVIPTSEITQPITSIKLEIVTSKSGGQAG
VMB2    ...............-..........................................................................
KT1/98  ...............-..........................................................................
UK661   ...............-..........................................................................
OKYM    ...............-..........................................................................
KK1     ...............-.................L.......................V................................
BD3/99  ...............-...................................................A......................
D6948   ...............-..........................................................................
HK46    ...............-..........................................................................
002-73  .....N..V......-.......V.N.....V.....T.T......G..............L..........V.................
52/70   ...............-.......V.....................................L....N.......................
Cu-1wt  ...............-.......V.....................................L....N.......................
STC     ........V......-.......V......F......T.......................L....N............V..........
Cu1     ........V......-....H..V..............T........N....T.........L..S.N.......................
P2      ........V......-R...H.IV.......................N....T.........L....N.......................
CEF-94  ........V......-....H..V.......................N....T.....L...L....N.......................
PBG-98  ........V......-R...H..V..............T........NT...T.........L....N.......................
GLS     ........V......-K...HS.V.............S.........N....T.........L....N.....................E.
K310    T..T....V...P..-.....S.V.........S..ST.T......N.........PI...L.F..N.................T.....
Var-E   ........V......-K....S.V......................N......I.......L....N..................D....
23/82   ...L..F.V......S.VTI.SIEVDV..HF......D.AVK...T.F...T..N..V...L.V..N........M...V..Y.I..T..
OH      ...L....V.....IS.VTIHSIEVDV...F......E.TVK...T.F...T..N..V...LGG...........M...V..Y.R..T..
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        323                               356
VMB1    DQMSWSASGSLAVTIHGGNYPGALRPVTLVAYER
VMB2    ..................................
KT1/98  ..................................
UK661   ..................................
OKYM    ..................................
KK1     ..................................
BD3/99  ..................................
D6948   ..................................
HK46    ..................................
002-73  .....L...N........................
52/70   ..................................
Cu-1wt  ..................................
STC     ..................................
Cu1     .......K..........................
P2      .......R..........................
CEF-94  .......R..........................
PBG-98  ...L...R..........................
GLS     ..................................
K310    ..........P.A.....................
Var-E   E.................................
23/82   .PI..TV..T....V...................
OH      .PI..TV..T....V...................
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Fig. 1

Deduced amino acid sequences of VP2 at positions 234–356 of the IBDV isolates VMB1 and VMB2 and of some other IBDV strains

A dot indicates identity with the isolate VMB1 at the given position. The hydrophilic region B is underlined and the heptapeptide sequence is boxed.

out by the Clustal method and phylogenetic analysis was performed by the neighbor-joining method with the Megalign TM Software Package (DNAstar, Inc.). The phylogenetic tree was outrooted to an outgroup sequence of segment A of infectious pancreatic necrosis virus (IPNV). The Genbank accession numbers of the IBDV strains used for comparisons are given in Table 1. The nucleotide sequences of the isolates VMB1 and VMB2 were submitted to the EMBL nucleotide sequence database and were assigned Acc. Nos AJ416444 and AJ416445, respectively.

Results

HVRs of VP2 genes of IBDV isolates VMB1 and VMB2 were analyzed and compared with those of 20 different IBDV strains (Table 2). VMB1 and VMB2 showed a 98.1% similarity due to seven identical nucleotide substitutions T848C, C905T, T908C, C920T, C989T, C1010T, and G1163A. Isolates VMB1 and VMB2 showed a similarity of 96.5–98.4% with very virulent strains (KT1/98, UK661, OKYM, KK1, BD3/99, D6948, and HK46), 84.6–94.6% with classical virulent strains (002-73, 52-70, Cu-1wt, and STC), 90.0–91.4% with classical attenuated strains (Cu1, P2, CEF, and PBG-98), 83.0–91.9% with antigenic variants (GLS, K310 and Variant-E), and 65.8–68.7% with avirulent strains (23/82 and OH).

The deduced amino acid sequences (corresponding to positions 234–356 according to the numbering system of Bayliss *et al.* (1990), of the isolates VMB1 and VMB2, seven very virulent, four classical virulent, four classical attenuated, three antigenic variants and two avirulent strains are shown in Fig. 1. Whereas the isolates VMB1 and VMB2 shared a 100% identity with each other and with very virulent strains KT1/98, UK661, OKYM, D6948 and HK46, they showed a divergence of 0.8% and 1.6% with the very virulent strains

Table 2. Nucleotide sequence similarities (upper triangle, %) and divergences (lower triangle, %) of variable regions of VP2 genes of two field IBDV isolates and other IBDV strains (for details see Table 1)

	VMB1	VMB2	KT1/98	UK661	OKYM	KK1	BD3/99	D6948	HK46	002-73	52/70	Cu-1wt	STC	Cu1	P2	CEF-94	PBG-98	GLS	K310	Var-E	23/82	OH
VMB1	*	98.1	98.1	97.6	97.0	96 5	96.8	97.6	97.3	84.9	94.3	93.5	92.2	90.6	91.4	91.4	90.3	91.4	83.0	91.6	66.8	68.7
VMB2	1.9	*	98.4	98.4	97.8	97.3	97.6	98.4	98.1	84.6	94.6	93.8	92.7	90.3	91.1	91.1	90.0	91.6	83.8	91.9	65.8	67.7
KT1/98	1.9	1.6	*	97.8	97.8	96.8	97.0	97.8	97.6	84.4	94.1	93 3	92.2	89.8	90.3	90.6	89.5	91.6	83.3	91.1	65.5	66.3
UK661	2.5	1.6	2.2	*	98.9	98.4	97.6	99.5	99 2	85.7	94.6	93.8	93.5	91.4	91.9	91.9	91.4	92.5	84.4	92.2	66.0	68.5
OKYM	3.1	2.2	2.2	1.1	*	98.4	97.6	99.5	99.2	85 2	94.1	93.3	93.0	90.8	91.1	91.4	90 8	91.9	83.8	91.6	65.8	67.4
KK1	3.6	2.8	3.3	1.6	1.6	*	97.0	98.9	98.7	83.8	93.5	92.7	92.5	90.3	90 8	91.1	90.3	91.4	83.6	91.1	65.8	67.7
BD3/99	3.0	2.2	2.8	2.2	2.2	2.8	*	98 1	97.8	83.8	93.5	92.7	92.5	90.3	91.1	91.1	90.3	91.6	83.6	90.8	65.5	67.7
D6948	2.5	1.6	2.2	0.5	0 5	1.1	1.6	*	99.7	85.2	94.6	93.8	93.5	91.4	91.9	91.9	91.4	92.5	84.4	92.2	66 0	68.2
HK46	2.8	1.9	2.5	0.8	0.8	1.4	1.9	0.3	*	84.9	94.3	93.5	93.3	91.1	91 6	91.6	91.1	92.7	84.1	91.9	65.5	67.7
002-73	15.4	15.7	16.1	14 6	15.4	16.8	16 8	15.4	15.7	*	88.1	87.6	87.1	86.3	86.0	86.5	86 0	86.5	84 9	87.3	64.7	68.7
52/70	5.7	5.4	6.0	5.4	6 0	6.6	6.6	5.4	5.7	12.6	*	99.2	97.0	94.3	94 9	94.9	94.1	95 7	87.3	96.0	68.2	69.3
Cu-1wt	6.5	6.2	6 8	6 2	6.8	7.5	7 4	6.2	6.5	13.2	0 8	*	96.2	93.5	94.1	94.1	93.3	94.9	86.5	94.9	67.7	69.3
STC	7.2	6.9	7.5	6.3	6.9	7.5	7.5	6 3	6.6	13.6	3.0	3.9	*	94.6	94.6	94 6	94 6	94.9	87.1	94.6	70.1	70.1
Cu1	8 7	9 0	9.7	8.4	9.0	9.7	9.6	8.4	8.7	14.5	5.6	6.5	5.4	*	98.4	98 4	98.4	94.6	86.5	94 1	69 3	70.4
P2	8.1	8.4	9.0	7.7	8.4	9.0	9.0	7.7	8.1	14.5	5.0	5.9	5.3	1.6	*	98.9	98.9	95.1	86 0	94.6	69.8	71.2
CEF-94	8 1	8.4	9.0	7.7	8.4	8.7	9.0	7.7	8.1	14.2	5 0	5.9	5.3	1.6	1 1	*	98 4	95.1	85 7	94.6	70.1	71.4
PBG-98	8.7	9.0	9.6	8.4	9.0	9.6	9.6	8.4	8.7	14 5	5 6	6.5	5.3	1.6	1.1	1.6	*	94 6	86.0	94.1	68 7	70.1
GLS	8.4	8.1	8.1	7.5	8.1	8.7	8.7	7.5	7.1	14.2	4.2	5.0	5.1	5.3	4.7	4.7	5.3	*	89.5	96.2	67.4	67.7
K310	16.9	15.8	16.5	15.8	16 5	16.5	17.2	15 8	16.2	16.4	13 0	13.9	13.3	13.9	14.6	14.6	14.6	11 3	*	88.7	62.8	64.2
Var-E	8.1	7.8	8 7	7.8	8.4	9.1	9.0	7.8	8 1	13.6	3.9	4.7	5 4	6.2	5.6	5.6	6.2	3.9	11.9	*	67.9	67.9
23/82	35.8	36 8	37.3	36.3	36.3	36.8	37.2	36.3	36 8	37.6	34.0	34 0	31.8	33.1	31.8	32.2	33.1	34.7	39.8	34.4	*	88.5
OH	34.1	35.0	36.4	33.6	35 0	34.5	34.9	34 1	34.5	33.5	32.7	32 3	31.4	31.5	30.2	30.6	31 5	33 5	37.8	33.5	10.1	*

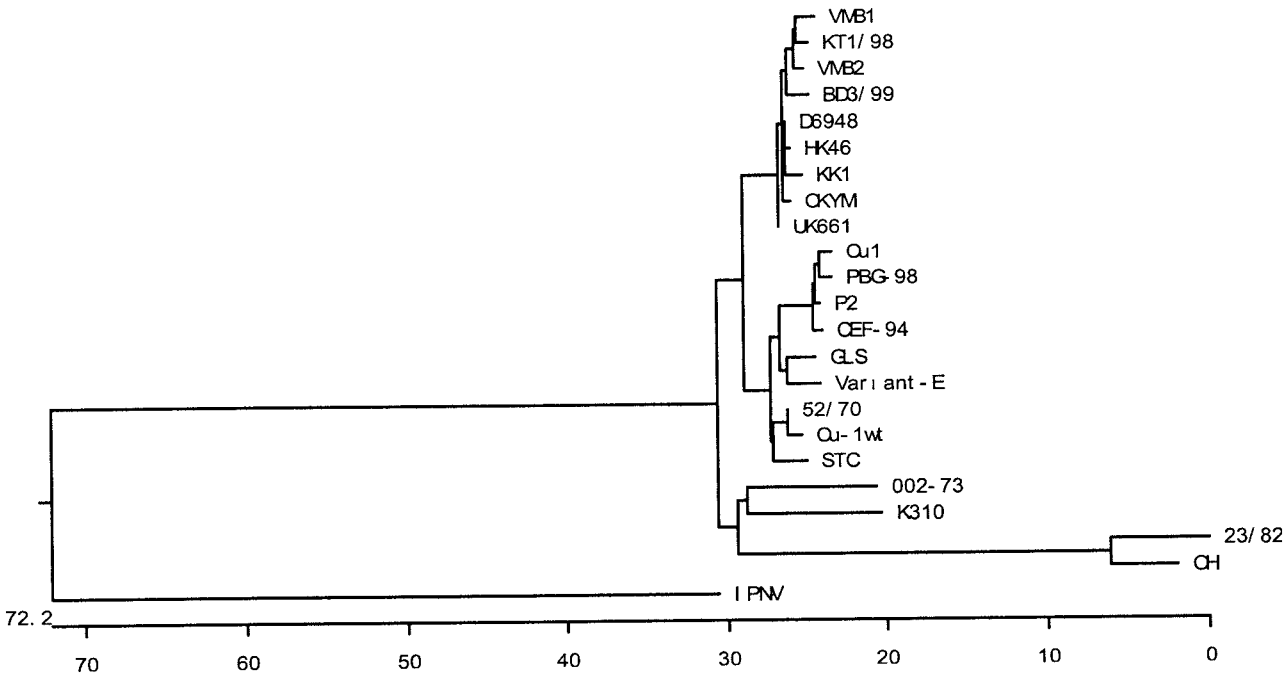

Fig. 2

Phylogenetic tree of HVR of VP2 gene of IBD viruses

The neighbor-joining tree was constructed from pairwise nucleotide differences in VP2 gene. Branched distances correspond to sequence divergence. Units at the bottom of the tree indicate the number of substitution events.

BD3/99 and KK1, respectively. The two isolates diverged by 2.5–10.5% from classical virulent strains, by 7.7–10.5% from classical attenuated strains, by 8.6–17.3% from the antigenic variants and by 38.4% from avirulent strains.

When compared with the very virulent strain KK1, the amino acid substitutions included L266F and V290M, whereas, only one amino acid substitution (A300E) was observed when compared with the very virulent strain BD3/ 99. The amino acid substitutions N239S, V242I, V256I, N258G, V264I, T270A, T272I, G279D, L294I, V305I, L328S, and N332S were observed when the isolates were compared with the classical virulent Australian strain 002-73. When compared with the antigenic variant K310, the amino acid substitutions included T234I, T237I, V242I, P246L, S254G, V256I, S266F, S269T, T270A, T272I, N279D, P289L, I290M, L294I, F296I, N299S, T317S, P333L, and A335V.

Phylogenetic analysis of nucleotide sequences of the two isolates, seven very virulent, four classical virulent, four classical attenuated, three antigenic variants, and two avirulent strains is shown in Fig. 2. The isolates VMB1 and VMB2 were grouped with the very virulent strains. The antigenic variants, except for K310, branched with the classical attenuated strains. All the classical virulent strains, except for 002-73, branched with the antigenic variants and classical attenuated strains. Surprisingly, K310 and 002-73 branched separately with the avirulent strains 23/82 and OH. IPNV, which was used as an outgroup in this analysis, branched out separately as expected.

Discussion

The repeated occurrence of IBD in commercial farms in Karnataka State, India, is of major concern to the poultry breeders. In spite of regular vaccinations, the disease outbreaks have been reported in these farms. In order to reason out the incidence quickly and proper measures, genomic characterization of field isolates was essential. Since the nucleotide and amino acid sequence analyses of VP2 HVR can reveal the characteristics of the IBDV strains (Banda *et al.*, 2001), it was carried out for the isolates VMB1 and VMB2.

The nucleotide sequence analysis revealed a 98.1% similarity between the isolates VMB1 and VMB2. The two isolates showed a similarity of 96.1–98.4% with the very virulent strains. The specific nucleotide substitutions 833C, 857C, 897A, 905T in VMB2, 908T in VMB1, 1011A and

1094G, reported earlier (Kataria *et al.*, 2001) along with substitutions 830T, 866A and 1115A were conserved in the two isolates indicating their very virulent nature. All the classical virulent strains, except for the Australian strain 002-73, showed a 92.5–94.6% similarity with the very virulent strains. The Australian strain 002-73 diverged by 14.6–16.8% from the very virulent strains.

The deduced amino acid sequence analysis showed a 100% similarity of the two isolates with most of the very virulent strains compared in this study. The amino acid substitutions, typical for the very virulent strains V256I, L294I, N299S (Cao *et al.*, 1998; Eterradossi *et al.*, 1999; Kataria *et al.*, 2001) and N279D (Ikuta *et al.*, 2001) were maintained in the isolates VMB1 and VMB2. Our PCR product did not include the hydrophilic region A. The analysis of the hydrophilic region B, which spans from amino acid positions 314 to 324, important for the binding of neutralizing monoclonal antibodies (Eterradossi *et al.*, 1999; Kataria *et al.*, 2001), did not reveal any substitutions in the isolates VMB1 and VMB2. The isolates showed the presence of a serine-rich heptapeptide sequence 'SWSASGS' at the amino acid positions 326-332, a marker for pathogenic strains (Vakharia *et al.*, 1994; Kataria *et al.*, 2001) and alanine at position 284, another marker for wild type, potentially pathogenic strains (Jackwood *et al.*, 2001).

The phylogenetic analysis of HVR of VP2 gene suggests that very virulent IBDVs from Europe (UK661), Japan (OKYM), China (HK46), the Netherlands (D6948), Bangladesh (BD3/99) and Korea (KK1) are closely related to the isolates VMB1 and VMB2, indicating their evolutionary relationship. The Australian strain 002-73 is more closely related to the avirulent strains 23/80 and OH, and more distantly related to the very virulent strains than other classical virulent strains. This finding strengthens the similar observation made by Islam *et al.* (2001) on the basis of the VP1 sequence analysis. Kataria *et al.* (2001) analyzed the HVR of VP2 gene of Indian very virulent isolates including KT1/98 and concluded that very virulent IBDV strains prevalent in India might have entered India from northern and eastern neighbor parts spreading from China to Southeast Asia.

In the present study, the phylogenetic analysis revealed the closest relationship of the isolates VMB1 and VMB2 to the very virulent strain KT1/98, further strengthening the hypothesis proposed by Kataria *et al.* (2001).

In conclusion, the nucleotide and amino acid sequence analyses have showed that the isolates VMB1 and VMB2 are of very virulent nature and might have originated from Europe, Japan or China.

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