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The genome segment B encoding the RNA-dependent RNA polymerase protein VP1 of very virulent infectious bursal disease virus (IBDV) is phylogenetically distinct from that of all other IBDV strains

Brief Report

M. R. Islam*, **K. Zierenberg,** and **H. Müller**

Institute of Virology, Faculty of Veterinary Medicine, University of Leipzig, Leipzig, Germany

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Summary. A full-length cDNA clone of the segment B of the very virulent infectious bursal disease virus (IBDV) strain BD 3/99 was constructed and the full-length nucleotide sequence was established. The nucleotide sequence encoding VP1, an RNA-dependent RNA polymerase, of BD 3/99 was aligned with that of 17 other IBDV strains including six very virulent, three classical virulent, five classical attenuated, one antigenic variant and two serotype 2 strains. The VP1 genes of all very virulent strains were 97.5% to 99.8% identical. With the exception of an atypical Australian strain, 002-73, all of the classical virulent or attenuated and antigenic variant strains were also 97.5% to 100% identical. Serotype 2 strains showed only 4–6% divergence from serotype 1 classical virulent or attenuated strains; in contrast, however, the very virulent strains were 10.5% to 12.5% divergent from the classical virulent or attenuated strains as well as serotype 2 strains. Analysis of the deduced amino acid sequence of VP1 revealed 17 common, including 8 unique amino acid substitutions in the very virulent strains. In the phylogenetic tree the very virulent strains formed a distinct cluster and all other strains including classical virulent, attenuated and antigenic variant strains and even serotype 2 strains were grouped together. It is suggested that the VP1 of very virulent IBDV is phylogenetically distinct

*Permanent address: Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh.

from that of all other IBDV strains and probably originated from a hitherto unidentified source.

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Infectious bursal disease virus (IBDV) is a dsRNA virus belonging to the genus *Avibirnavirus* within the family *Birnaviridae* [4, 11]. There are two distinct serotypes of IBDV [22]. The Serotype 1 strains cause immunosuppression as well as an acute fatal disease of young chickens while Serotype 2 viruses are avirulent for chickens. Serotype 1 viruses are further categorized as classical virulent, antigenic variant and very virulent strains depending on their pathogenicity and/or antigenicity. Many attenuated vaccine strains have been derived from the classical virulent strains.

Non-enveloped icosahedral IBDV particles contain the bi-segmented dsRNA genome. The major open reading frame (ORF) in the larger genome segment A encodes a polyprotein which is co-translationally and autocatalytically cleaved into the two structural viral proteins VP2 and VP3, and a viral protease VP4 [1, 16, 24]. A second ORF in the segment A encodes a nonstructural protein, VP5 [24], the exact function of which is still unknown. The smaller segment B encodes the VP1, which is an RNA-dependent RNA polymerase (RdRp) [33].

RdRp is an essential protein for the replication of RNA viruses. RdRp genes of different viruses have been analysed with regards to their phylogenetic and taxonomic relationship [20, 27, 35]. The active domain of RdRp maintains a high degree of homology among the RNA viruses belonging to different species, genera, and even families. Sequence analysis of the RdRp gene could provide some insight into the phylogenetic relationship of different viruses.

The sequences of segment A, particularly the variable domain of the VP2 gene, of different IBDV strains have been aligned and several amino acid residues unique to very virulent IBDV have been identified [5, 13, 17, 34, 37, 39]. However, only a couple of reports on the alignment of segment B sequences are available [6, 37, 38]. In these studies a small number of sequences were compared. The present communication reports on the establishment of full-length sequence of segment B of an IBDV strain (BD 3/99) antigenically and genetically related to very virulent IBDV and its alignment with that of 17 other IBDV strains. The results revealed that the VP1 of very virulent IBDV is genetically distinct from that of other IBDV strains.

The IBDV strain BD 3/99 was isolated in 1999 from a chicken in Bangladesh. Nucleotide sequencing of the variable domain of the VP2 gene and antigenic characterization with a panel of monoclonal antibodies confirmed that BD 3/99 antigenetically and genetically related to very virulent IBDV isolated recently from Europe, Asia and Africa [17]. In the present study a full-length cDNA clone of the segment B of BD 3/99 was constructed. Briefly, cDNA corresponding to the whole genome segment was synthesized and amplified in two overlapping fragments by reverse transcription-polymerase chain reaction (RT-PCR). To this end, RNA was isolated from the infected bursal homogenate essentially as described [17]. RT-PCR reaction was performed using the Titan™ One Tube RT-PCR System

Table 1. Primers used for synthesis and amplification of cDNA corresponding to the segment B of BD 3/99

Primer	Primer sequence[a]	Primer characteristics
B1-sense	5′-cta g*tc tag a***ta ata cga ctc act ata** GGA TAC GAT GGG TCT GAC-3′	*Xba*I + T7 promoter + IBDV nt 1–18
B1-antisense	5′-GAT CCC GAG ATC TTT GCT GTA T-3′	IBDV nt 1860–1839
B2-sense	5′-AGA CAG CGA GGA GTT CAA ATC AAT TGA GGA-3′	IBDV nt 1647–1677
B2-antisense	5′-acc *gct cga gtc tag acc cGG G*GG CCC CCG CAG GCG AAG-3′	*Xho*I + *Xba*I + *Sma*I + IBDV nt 2827–2808

[a]IBDV-specific sequence is given in upper case, added restriction sites are in italics and the T7 promoter sequence is in bold face lower case

(Boehringer Mannheim, Germany) according to the manufacturer's instruction. Two pairs of primers (Table 1) based on the segment B sequence of IBDV strain Cu-1 were used in the RT-PCR. The B1-sense primer had an *Xba*I restriction site and a T7 promoter sequence tagged to its 5′-end, while *Sma*I, *Xba*I and *Xho*I restriction sites were tagged to the 5′-end of the B2-antisense primer. The overlapping region in the two cDNA fragments (B1 and B2) contained a unique *Bgl*II restriction site. The B1 fragment was first cloned in the pQE 60 vector (Qiagen, Germany) using *Xba*I and *Bgl*II restriction sites and then the B2 fragment was cloned in the same vector using *Bgl*II and *Xho*I restriction sites; thus a full-length clone of BD 3/99 segment B cDNA (pQE-BD3/B) was constructed. The cloned cDNA insert has a *Sma*I restriction site at the 3′-end and a T7 promoter at the 5′-end, to be used for linearising the plasmid and in vitro transcription of RNA, respectively. The insert is also flanked by two *Xba*I restriction sites, which can be used for its subsequent transfer to another vector, if required.

For sequencing, the cloned full-length cDNA was cut into six fragments such as *Xba*I-*Pst*I (321 bp), *Pst*I-*Kpn*I (482 bp), *Kpn*I-*Stu*I (521 bp), *Stu*I-*Kpn*I (465 bp), *Kpn*I-*Xma*III (514 bp) and XmaIII-XbaI (554 bp) fragments. Each of these fragments was subcloned in the pBluescript II Sk + vector (Stratagene, Germany). The subcloned cDNA fragments were sequenced from the both ends with M13 forward and reverse primers using Dye Terminator Cycle Sequencing Kit on an automated ABI 377 DNA Sequencer (Applied Biosystems Inc., USA). The sequence data were compiled using the computer programme Edit-Seq (DNASTAR Inc., USA). Subclones obtained from two independently constructed full-length clones were sequenced. In case of any sequence discrepancy between the duplicate clones, the respective region was amplified by RT-PCR from the original RNA sample using appropriate primers (data not shown) and then sequenced directly. The deduced amino acid sequence of the VP1 of BD 3/99 is presented in Fig. 1. The VP1 sequence of BD 3/99 was compared with that of other IBDV strains by the Clustal V multiple alignment method using the

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MSDVFNSPQA RSKISAAFGI KPTAGQDVEE LLIPKVWVPP EDPLASPSRL  50
AKFLRENGYK ILQPRSLPEN EEYETDQILP DLAWMRQIEG AVLKPTLSLP 100
IGDQEYFPKY YPTHRPSKEK PNAYPPDIAL LKQMIYLFLQ VPEATDNLKD 150
                                                 **
EVTLLTQNIR DKVYGSGTYM GQATRLVAMK EVATGRNPNK DPLKLGYTFE 200
SIAQLLDITL PVGPPGEDDK PWVPLTRVPS RMLVLTGDVD GEFEVEDYLP 250
                                             *
KINLKSSSGL PYVGRTKGET IGEMIAISNQ FLRELSALLK QGAGTKGSNK 300
KKLLSMLSDY WYLSCGLLFP KAERYDKSTW LTKTRNIWSA PSPTHLMISM 350
ITWPVMSNSP NNVLNIEGCP SLYKFNPFRG GLNRIVEWIM APDEPKALVY 400
                                          *   *
ADNIYIVHSN TWYSIDLEKG EANCTRQHMQ AAMYYILTRG WSDNGDPMFN 450
QTWATFAMNI APALVVDSSC LIMNLQIKTY GQGSGNAATF INNHLLSTLV 500
LDQWNLMKQP SPDSEEFKSI EDKLGINFKI ERSIDDIRGK LRQLVPLAQP 550
GYLSGGVEPE QPSPTVELDL LGWSATYSKD LGIYVPVLDK ERLFCSAAYP 600
            *
KGVENKSLKS KVGIEQAYKV VRYEALRLVG GWNYPLLNKA CKNNASAARR 650
HLEAKGFPLD EFLAEWSELS EFGEAFEGFN IKLTVTPESL AELNRPVPPK 700
                                       *        *
PPNVNRPVNT GGLKAVSNAL KTGRYRNEAG LSGLVLLATA RSRLQDAVKA 750
KAEAEKLHKS KPDDPDADWF ERSETLSDLL EKADIASKVA HSALVETSDA 800
LEAVQSTSVY TPKYPEVKNP QTASNPVVGL HLPAKRATGV QAALLGAGTS 850
RPMGMEAPTR SKNAVKMAKR RQRQKESRQ.                      880
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Fig. 1. Deduced amino acid sequence of the VP1 of BD 3/99. Putative RdRp motifs are boxed with catalytically essential residues given in bold face. Amino acid residues unique for very virulent IBDV are indicated with asterisks

computer programme MegAlign (DNASTAR Inc., USA). In this method the sequences are grouped into clusters on the basis of the distance between each pairs. The clusters are first aligned as pairs and then collectively. After the alignment, the programme constructs a phylogenetic tree employing the neighbor-joining method. The VP1 of BD 3/99 and 17 other IBDV strains were aligned. These included three classical virulent, five classical attenuated, one antigenic variant, six very virulent and two serotype 2 strains (Table 2). The VP1 sequence of an aquabirnavirus, infectious pancreatic necrosis virus (IPNV) Jasper strain (GeneBank accession no. M58756) [12], was used as an outgroup in the construction of the phylogenetic tree.

The length of VP1 is variable (Table 2). The number of amino acids in VP1 is 881 in CEF 94, 879 in all very virulent strains and in Variant E, 878 in 002-73, Cu-1wt, Cu-1, Cu-1M, W2512, P2 and 23/82, and 876 in OH. For F 52/70, only a partial sequence was available. The discrepancy in length is due to three reasons: (i) the position of the stop codon is variable in different strains due to mutations of one or two nucleotides at the 3′-termimus of the VP1 gene; (ii) strains 002-73 and OH have scattered deletions of three nucleotides which also resulted in frameshift in the amino acid sequence between position 100 and 135; (iii) strain OH has additional deletions of two codons causing disappearance of two amino acids at positions 650 and 789 (numbering is according to Cu-1wt strain).

Table 2. Characteristics of IBDV strains used in multiple alignment analysis of segment B sequence

Strain	Serotype	Pathotype	No. of a.a.[a] in the VP1	Geographic origin [Reference]	GeneBank accession No.
Cu-1wt	1	Classical virulent	878	Germany [26]	
F 52/70	1	Classical virulent	N.A.	U.K. [7]	D12610
002-73	1	Classical virulent	878	Australia [14]	M19336
W 2512	1	Classical attenuated	878	U.S.A. [2]	AF083092
Cu-1	1	Classical attenuated	878	Germany [26]	AF362748
Cu-1M	1	Classical attenuated	878	Germany [26, 10]	AF362772
P2	1	Classical attenuated	878	Germany [32]	X84035
CEF 94	1	Classical attenuated	881	The Netherlands [28]	AF194429
Variant E	1	Antigenic variant	879	U.S.A. [31]	AF133904
BD 3/99	1	Very virulent	879	Bangladesh [17]	AF362770
UK 661	1	Very virulent	879	U.K. [5]	X92761
OKYM	1	Very virulent	879	Japan [36]	D49707
HK 46	1	Very virulent	879	China [8]	AF092944
D 6948	1	Very virulent	879	The Netherlands [3]	AF240687
IL 3	1	Very virulent	879	Israel [38]	AF083093
IL 4	1	Very virulent	879	Israel [38]	AF083094
OH	2	Avirulent	876	U.S.A [18]	U20950
23/82	2	Avirulent	878	U.K. [9]	AF362774

[a] *a.a.* Amino acids
N.A. Complete VP1 sequence is not available

Sequence distances data (Table 3) derived from the multiple alignment of the VP1 encoding nucleotide sequences revealed that all the very virulent strains are 97.5% to 99.8% identical. Similarly, with the exception of strain 002-73, all the classical virulent and attenuated strains are 98.2% to 100% identical. A very virulent strain differed from a classical virulent or attenuated strain within a range of 10.9% to 12.5%. The early Australian strain 002-74 was almost equally divergent (10.2% to 12.3%) from the other classical virulent or attenuated strains as well as very virulent strains. The Variant E strain was more close to the classical virulent or attenuated strains (97.5% to 97.9% identity). The VP1 of two serotype 2 strains, OH and 23/82, is 92.7% to 96.1% similar to the classical virulent or attenuated strains but only 87.5% to 89.9% similar to the very virulent strains.

The alignment of the deduced amino acid sequences is summarized in Table 4. The VP1 sequence of the classical virulent strain Cu-1wt was used as the reference. Scattered mutations unique to a particular strain were not included in the table. Eighteen positions were identified where 5 to 12 strains had an amino acid residue different from that of Cu-1wt. Among the 3 classical virulent strains, 002-73 appeared to be highly divergent having as many as 11 amino acid substitutions as compared to Cu-1wt. Only a partial sequence was available for F 52/70. In general, the VP1 sequence of the attenuated strains and the antigenic variant strain were similar to that of Cu-1wt. Only at position 13, all of the attenuated

Table 3. Distances between the VP1 gene nucleotide sequences of very virulent (BD 3/99, UK 661, OKYM, HK 46, D 6948, IL 3, IL 4), classical virulent (002–73, F 52/70, Cu-1 wt), classical attenuated (Cu-1, Cu-1M, W 2512, P 2, CEF 94), US variant (Variant E), and serotype 2 (OH, 23/82) strains

Percent identity

	BD 3/99	UK 661	OKYM	HK 46	D 6948	IL 3	IL 4	002-73	F 52/70	Cu-1wt	Cu-1	Cu-1M	W 2512	P 2	CEF 94	Var. E	OH	23/82
BD 3/99		97.9	97.5	97.9	98.6	98.5	98.4	88.1	89.9	88.9	89.0	89.2	89.1	89.1	89.2	88.8	88.1	89.8
UK 661	2.1		97.9	98.2	99.2	99.0	98.8	87.9	89.0	88.4	88.5	88.7	88.6	88.6	88.7	88.3	87.8	89.6
OKYM	2.5	2.1		98.1	98.6	98.5	98.3	87.9	88.6	88.1	88.2	88.2	88.1	88.1	88.2	87.9	87.5	89.2
HK 46	2.0	1.8	2.0		98.9	98.9	98.6	87.9	89.3	88.3	88.4	88.5	88.5	88.5	88.5	88.3	87.7	89.4
D 6948	1.4	0.8	1.4	1.1		99.6	99.4	88.3	89.5	88.7	88.8	89.0	88.9	88.9	89.0	88.6	88.1	89.9
IL 3	1.5	1.0	1.5	1.2	0.4		99.8	88.3	89.6	88.8	88.8	89.0	89.0	88.9	89.0	88.7	87.9	89.9
IL 4	1.6	1.2	1.7	1.4	0.6	0.2		88.3	89.5	88.5	88.6	88.8	88.7	88.7	88.8	88.5	88.1	89.8
002-73	12.1	12.2	12.2	12.3	11.8	11.7	11.8		90.3	88.9	89.3	89.2	89.3	89.1	89.1	88.6	90.3	90.0
F 52/70	10.5	11.6	11.8	11.2	10.9	10.9	11.0	10.2		98.3	98.3	98.4	98.3	98.2	98.2	97.5	94.1	96.1
Cu-1wt	11.6	12.2	12.5	12.2	11.8	11.8	12.0	11.2	1.7		99.1	98.9	98.9	98.8	98.8	97.5	93.4	95.4
Cu-1	11.5	12.1	12.4	12.2	11.7	11.7	11.9	10.9	1.7	0.9		99.8	99.8	99.7	99.7	97.7	93.4	95.5
Cu-1M	11.3	12.0	12.4	12.0	11.6	11.6	11.7	10.9	1.7	1.0	0.2		100.	99.9	99.8	97.9	93.4	95.5
W 2512	11.3	12.0	12.4	12.1	11.6	11.6	11.8	10.8	1.7	1.0	0.2	0.0		99.8	99.8	97.9	93.4	95.5
P 2	11.3	12.0	12.4	12.1	11.6	11.6	11.8	10.9	1.8	1.1	0.3	0.1	0.2		99.8	97.9	93.3	95.5
CEF 94	11.3	12.0	12.4	12.0	11.6	11.6	11.7	10.9	1.8	1.1	0.3	0.2	0.2	0.2		97.9	93.4	95.5
Var. E	11.6	12.3	12.7	12.3	11.9	11.8	12.0	11.6	2.5	2.5	2.3	2.1	2.2	2.2	2.1		92.7	94.8
OH	11.5	11.8	12.0	11.9	11.4	11.4	11.5	9.7	5.0	5.8	5.9	5.8	5.9	6.0	5.9	6.7		94.3
23/82	10.7	10.8	11.3	11.1	10.5	10.6	10.7	10.0	4.0	4.7	4.6	4.6	4.6	4.6	4.6	5.3	5.0	

Percent divergence

Table 4. Amino acid exchanges in the VP1 among classical virulent (Cu-1 wt, F 52/70, 002-73), classical attenuated (W 2512, Cu-1, Cu-1M, P2, CEF 94), US variant (Variant E), very virulent (BD 3/99, UK 661, OKYM, HK 46, D 6948, IL 3, IL 4) and serotype 2 (OH, 23/82) strains. Scattered mutations have been omitted

Strain	Amino acid exchanges in VP1 at position[a]																	
	4	12	13	61	145	146	147	242	287	390	393	508	511	546	562	646	687	695
Cu-1wt	I	R	K	V	N	E	G	D	T	L	E	R	R	L	S	G	S	K
F 52/70	–	–	–	–	–	–	–	–	–	·	·	·	·	P	·	·	·	·
002-73	V	T	·	I	T	·	S	·	A	·	·	K	N	P	·	S	R	·
W 2512	·	S	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Cu-1	·	T	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Cu-1M	·	S	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P2	·	S	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CEF 94	·	S	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Variant E	·	S	·	·	·	·	·	·	·	·	·	·	·	P	·	·	·	·
BD 3/99	V	S	·	I	T	D	N	E	A	M	D	K	S	P	P	S	P	R
UK 661	V	S	·	I	T	D	N	E	A	M	D	K	S	P	P	S	P	R
OKYM	V	Q	·	I	T	D	N	E	A	M	D	K	S	P	P	S	P	R
HK 46	V	S	·	I	T	D	N	E	A	M	D	K	S	P	P	S	P	R
D 6948	V	S	·	I	T	D	N	E	A	M	D	K	S	P	P	S	P	R
IL 3	V	S	·	I	T	D	N	E	A	M	D	K	S	P	P	S	P	R
IL 4	V	S	·	I	T	D	N	E	A	M	D	K	S	P	P	S	P	R
OH	V	T	·	·	·	·	·	·	·	·	·	·	·	P	·	S	·	·
23/82	V	T	·	·	·	·	·	·	·	·	·	·	·	P	·	·	·	·
						*	*	*		*	*				*		*	*

[a]Amino acid residues similar to that of Cu-1wt are indicated as dots (·), a dash (–) indicates that the sequence information is not available
*Mutations unique and common to all very virulent strains

strains have unique Threonine instead of Lysine present in all other strains. At position 546, the attenuated strains and Cu-1wt have Leucine but all other strains have Proline. The amino acid residue at position 12 is highly variable irrespective of pathotype or serotype. It is interesting to note that as compared to Cu-1wt all the very virulent strains have 17 common amino acid substitutions. Out of these, 8 substitutions (Glu146Asp, Gly147Asn, Asp242Glu, Leu390Met, Glu393Asp, Ser562Pro, Ser687Pro and Lys695Arg) are unique to the very virulent strains only. The remaining 9 substitutions are also shared by either a classical virulent strain 002-73 and/or serotype 2 strains.

In the phylogenetic tree (Fig. 2) derived from the multiple alignment analysis all the very virulent strains formed a cluster away from all other strains. All classical virulent and attenuated strains including Variant E and serotype 2 strains formed another cluster. Interestingly, however, the Australian strain 002-73 is more distantly related to other classical virulent strains as compared to the serotype 2 strains.

The findings of the present study, as described above, can be summarized as follows: (1) the VP1 sequence of BD 3/99 is very similar to that of other very virulent IBDV strains, (2) the VP1 sequences of very virulent IBDV strains are genetically distinct from that of classical virulent or attenuated strains, (3) the VP1 of the early Australian strain 002-73 is equally divergent from the very virulent strains and the classical virulent/attenuated strains.

The VP1 of IBDV is an RdRp [33]. It is a genome-linked protein [23] located inside the viral capsid and thus is not subjected to immune pressure. Therefore,

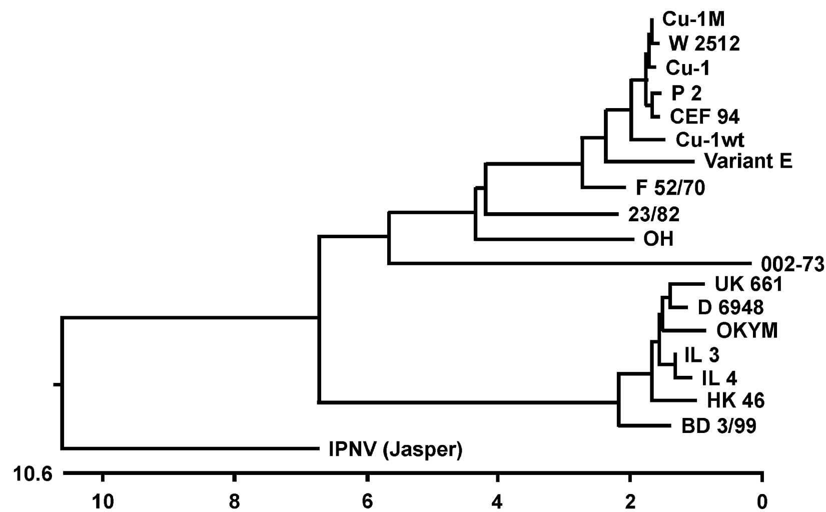

Fig. 2. Phylogenetic tree showing the relatedness among the VP1 gene of very virulent (BD 3/99, UK 661, OKYM, HK 46, D 6948, IL 3, IL 4); classical virulent (002-73, F 52/70, Cu-1 wt), classical attenuated (Cu-1, Cu-1M, W 2512, P 2, CEF 94), US variant (Variant E), and serotype 2 (OH, 23/82) strains. The neighbour-joining tree was constructed from the pair-wise nt differences in the VP1 gene. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events

one would expect that the VP1 gene should remain well conserved. In fact, this is the case when the VP1 gene of serotype 1 classical virulent and attenuated strains and serotype 2 strains are compared. They show only 4–6% divergence (Table 3). Surprisingly, the very virulent strains differ from the classical virulent and attenuated strains as well as serotype 2 strains at a level of 10.5% to 12.5% (Table 3). However, a multiple alignment of the segment A polyprotein gene of the very virulent strains and classical virulent/attenuated strains would show less than 5% variation, while serotype 1 and serotype 2 strains would have as much as 18–20% divergence (unpublished observation). The 002-73 sequence was not taken into consideration for the statements made above because of its unique divergence from all other IBDV sequences.

Nucleic acid polymerases show fundamental structural similarities reflected by distinct sequence motifs [19, 27]. Three or four most commonly found RdRp motifs are now considered to be involved in the polymerase activity in RNA viruses [15, 21, 29]. The first three well-conserved motifs are **D**XXXXD, S/T**G**XXXTXXXN, and G/S**D**D; the fourth motif is relatively less conserved but contains a single conserved residue **R/K** (essential residues are given in bold face, residues not in bold face are quite preserved but mutations are tolerated, X indicates any residue). These motifs are located in the central part of the protein. The first two motifs have been identified in birnaviruses, although the second D in the first motif is not conserved [12]. The GDD motif is fully conserved in the RdRp of most viruses, although this motif also appears as SDD in some viruses such as coronaviruses and orthomyxoviruses [27]. Among birnaviruses IBDV has IDD while IPNV has either LKN or LKD at the corresponding positions. It is not known if the IDD motif of IBDV is involved in the polymerase function. The fourth motif has not yet been reported for birnaviruses. However, the residue K at position 579 in the VP1 of IBDV could represent the essential residue of the fourth motif. IPNV has R at the corresponding position. A region immediately upstream to this residue is highly conserved in all IBDV and IPNV strains (data not shown). All four putative RdRp motifs, as shown in the BD 3/99 VP1 sequence (Fig. 1), are fully conserved in all IBDV strains. However, mutational experiments would be necessary to confirm the role of these putative motifs in polymerase activity.

The genetic basis of enhanced virulence of very virulent IBDV is not clear. Although several very virulent IBDV-specific mutations have been identified in the capsid proteins, none has so far been implicated in enhanced virulence. The role of VP1 in the virulence of IBDV also has not yet been established. It is likely that the efficiency of the viral polymerase would influence the replication rate and, thus, the pathogenic potential of a virus. In an earlier study 17 common amino acid substitutions, including 8 unique substitutions, were observed in two very virulent IBDV strains (OKYM and UK 661) [37]. Interestingly, these substitutions are also observed in all the very virulent IBDV strains sequenced so far (Table 4). The significance of these mutations is not known. None of these mutations is within any putative RdRp motif. However, the essential domain of the RdRp motif may extend beyond the consensus region and mutations in the vicinity

of the RdRp motifs can influence the polymerase activity [30]. Out of 8 amino acid substitutions unique to very virulent IBDV, 3 were in the central region of the VP1 sequence where putative RdRp motifs are located (Fig. 1, Table 4). These are Leu390Met, Glu393Asp and Ser562Pro. All three mutations predict a minor shift in the hydrophilicity of the protein (data not shown). Site-directed mutational experiments will be required to elucidate if these mutations play any role in possible enhancement of the polymerase activity and, therefore, virulence of the virus. It is interesting to note that at amino acid position 13 all classical attenuated strains have a unique mutation (Lys13Thr). Any possible role of this mutation in attenuation of these strains deserves further investigation.

The analysis of the sequence distances (Table 3) and pathotype-specific amino acid mutations (Table 4) suggest that the VP1 of very virulent IBDV constitutes a genetic lineage distinct from that of classical virulent or attenuated strains and serotype 2 strains as well. This is further obvious from the phylogenetic tree based on the nucleotide sequences of the VP1 gene (Fig. 2), where all the very virulent strains formed a distinct cluster and all other strains including classical virulent, attenuated and antigenic variant strains and even serotype 2 strains were grouped together. The origin of very virulent IBDV still remains obscure. It appears very unlikely that the very virulent IBDV acquired the VP1 from a classical virulent strain; rather they might have derived the VP1 from a hitherto unidentified source, possibly by segment reassortment, as already stated by Yamaguchi et al. [37]. This anthropological point of interest demands extensive search for IBDV or other birnaviruses in domestic and wild birds of diverse geographical origin and detailed analysis of their VP1 gene using modern bioinformatics tools.

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Author's address: Prof. Dr. H. Müller, Institute of Virology, Faculty of Veterinary Medicine, University of Leipzig, An den Tierkliniken 29, D-04103 Leipzig, Germany; e-mail: virology@vetmed.uni-leipzig.de

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