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Original Article

Molecular characterization of two Bangladeshi infectious bursal disease virus isolates using the hypervariable sequence of VP2 as a genetic marker

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Two Bangladeshi infectious bursal disease virus (IBDV) isolates collected in 2007, termed GB1 and GB3, were subjected to comparative sequencing and phylogenetic analyses. Sequence analysis of a 474-bp hypervariable region in the VP2 gene revealed that among four major amino acid substitutions observed in the strains, two were unique to GB1 and GB3 (Ser217Leu and Ala270Thr) while one substitution was only found in GB1 (Asn299Ser). Among IBDVs from Bangladesh including GB1 and GB3, the rate of identity and homology was around 97～99%. The amino acid sequences of GB1 and GB3 differ from those of previous Bangladeshi IBDV isolates and contain amino acid substitutions Pro222Ala and Asn299Ser (in GB3 only). Phylogenetic analysis revealed that GB1 and GB3 are grouped with other very virulent IBDVs of European and American origin in contrast to two previously isolated Bangladeshi IBDV strains (GenBank accession Nos. AF362776 and AF260317), which belong to the Asian group. It was concluded that GB1 and GB3 belong to a very virulent group of IBDVs. However, amino acid sequences of GB1 and GB3 differ from those of the other Bangladeshi IBDVs by one or two amino acids encoded in the hypervariable region of the VP2 gene.

Keywords: chickens, infectious bursal disease virus, molecular characterization

Introduction

Infectious bursal disease virus (IBDV), also known as Gumboro virus [4], has become an economically important pathogen spread throughout the world. The resulting disease can take several forms and has generally been observed as subclinical, classic virulent, or very virulent cases [33]. Among the two distinct serotypes of IBDV [16], serotype 1 strains are pathogenic and cause lytic infection of immature B lymphocytes [23,24]. Gumboro disease caused by serotype 1 viruses usually occurs in 3- to 6-week-old chickens with acute signs of clinical disease [15]. Susceptible chickens younger than 3 weeks old do not exhibit clinical signs but suffer from subclinical infections. This disease is economically important because it results in severe immunosuppression that develops in young chickens after infection [15]. Currently, there is a co-existence of epidemiologically mixed Gumboro viruses with different levels of pathogenicityin every country of the world [6]. The viruses might spread from different parts of world through trade and other means.

The IBDV is a non-enveloped, icosahedral double-stranded RNA (dsRNA) virus that belongs to the genus Avibirnavirus in the family Birnaviridae [13]. The dsRNA genome consists of two segments, namely A and B [19]. The smaller B segment (approximately 2.9 kb) encodes a single 91-kDa protein, VP1, with RNA-dependent RNA polymerase [29] and capping enzyme [28] activities. The larger A segment (approximately 3.3 kb) contains two overlapping open reading frames (ORFs). The first ORF encodes the nonstructural protein VP5 (17 kDa). This protein is nonessential for viral replication and infection [17]. The

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second ORF encodes a 110-kDa polyprotein, which is autocatalytically cleaved [12,26] to produce viral proteins VP2 (40 kDa), VP4 (28 kDa), and VP3 (32 kDa). Using sequence analysis and reverse genetics, VP2 from segment A [34,36], VP1 from segment B [14], or both [2,3] have been found to contribute to virulence.

The VP2 gene has a region of about 500 bp encoding approximately 150 amino acids including a major conformational neutralizing antigenic domain situated between amino acids 206 and 350 with two minor hydrophilic peaks [27,32]. This domain is known as a hypervariable region. Due to its characteristic antigenic structure, this region has been most often used for the molecular characterization of IBDV [35]. It has been confirmed that the residues 253 and 284 in VP2 contribute to the cell tropism and virulence of the virus [22].

In Bangladesh, chickens are produced for national consumption and export. The poultry industry is negatively affected by the damage caused by IBDV [1,8]. After initial recognition of IBDV in the country in 1992, studies on characteristics of this immunosuppressive virus and protection of chickens from its damage by vaccination have been conducted [7,8]. In the last two decades, antigenically variant strains have been detected in a number of geographically distinct countries [6]. A molecular and antigenic study of numerous previously isolated Bangladeshi IBDV strains have demonstrated their close similarities with very virulent IBDV (vvIBDV) recognized earlier in Europe, Asia, and Africa [8]. Here, we present updates for the molecular characterization of two recent IBDV isolates from Bangladesh.

Materials and Methods

Field viruses

Two IBDV isolates, BML-2/07 designated as GB1 (originating from swollen bursa from a 20-day-old layer chicken in Mymensingh, Bangladesh) and BTB-4/07 designated as GB3 (obtained from severe hemorrhagic bursa from a 17-day-old broiler chicken in Tangail, Bangladesh), were subjected tomolecular characterization in this study.

Genomic RNA extraction

Total genomic RNA and viral RNA was directly isolated from 20% bursal tissue homogenates using a QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The eluted viral RNA was stored at −80°C until being used for reverse transcription - polymerase chain reaction (RT-PCR).

RT-PCR

A One Step RT-PCR kit (Qiagen, Germany) was used to amplify the VP2 variable region located in the central area of the VP2 coding region using the primers GF (forward: 5′ GCCCAGAGTCTACACCATA 3′) and GR (reverse: 5′ GATCCTGTTGCCACTCTTTC 3′). The 50-μL reaction mixture contained 10 μL 5× Qiagen One Step RT-PCR reaction mixture, 2 μL of a 10-mM dNTP mix, 2 μL of each primer (10 pmol/μL), 2 μL enzyme mix, 2 μL template RNA, 2.5 μL DMSO, and 27.5 μL nuclease-free water. Reverse transcription was performed on a thermal cycler (Bio-Rad, USA) at 55°C for 30 min followed by one cycle at 95°C for 15 sec. This was followed by 35 cycles of denaturation at 94°C for 20 sec, annealing at 55°C for 20 sec, and elongation at 72°C for 1 min. A final extension step was performed at 72°C for 10 min. The RT-PCR products were then kept at 4°C until purification.

Amplicon purification, cloning, and DNA sequencing

Purification of the amplicons was carried out using a QIAquick Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. A TA cloning kit (Invitrogen, USA) was then used to clone the VP2 fragment into a pCR2.1-TOPO vector (Invitrogen, USA). Screening for positive clones was conducted as previously described by Sambrook and Russell [25]. Recombinant plasmid DNA harboring the RT-PCR product was sequenced with an automated 377 ABI DNA Sequencer (Applied Biosystems, USA) using a Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA).

Data analysis and phylogenetic construction

For each IBDV strain, identical sequencingdata from two positive clones containing the expected VP2 DNA insert were recorded and analyzed. The sequences were edited with SeqEd v1.03 (Applied Biosystems, USA), aligned using AssemblyLIGN v1.9c (Accelrys, USA), and analyzed using the MacVector 8.2 package (Accelrys, USA) with a Macintosh computer system (Apple, USA). Specific identification of sequences was confirmed by comparison with known VP2 sequences in GenBank (National Center for Biotechnology Information, USA). Amino acid sequences were deduced by multiple alignments using GeneDoc (ver. 2.7) [21]. Phylogenetic trees were constructed using the MEGA 4.1 package [30]. Distance matrices were constructed using the Kimura's two-parameter model and trees were constructed using the neighbor-joining (NJ) algorithm. The dataset was resampled 1,000 times using the bootstrap method. Sequences of 15 strains obtained from GenBank were used for comparison (Table 1) and 75 strains were selected for phylogenetic tree construction.

Results

Amino acid sequence analysis of the hypervariable VP2 region of GB1 and GB3 isolates

A 474-bp sequence encoding the hypervariable region of the VP2 protein (position: 606～1080 bp) from the GB1

Table 1. Key amino acids in the hypervariable region of the VP2 polypeptide of infectious bursal disease virus (IBDV) strains

No.	Stain	Country/territory of isolation	Accession No.*	Key amino acids								
				222	242	253	256	279	284	294	299	329
1	52-70	UK	D00869	P	I	Q	V	D	A	L	N	A
2	D78	USA	AF499929	P	I	H	V	D	T	L	N	A
3	CU1	Germany	D00867	P	I	H	V	D	T	L	N	A
4	GLS	USA	AY368653	T	I	Q	V	D	A	L	N	A
5	STC	USA	D00499	P	I	Q	V	D	A	L	N	A
6	GB3	Bangladesh	This study	P	I	Q	V	D	A	L	N	A
7	GSG4	Vietnam	AY841902	P	I	Q	V	D	A	L	N	A
8	GB1	Bangladesh	This study	P	I	Q	V	D	A	L	S	A
9	BLRI94/B551	Bangladesh	AF260317	A	I	Q	V	D	A	L	S	A
10	NP2K	Nepal	AY367560	A	I	Q	V	D	A	L	S	A
11	NP1SSH	Nepal	AY605264	A	I	Q	V	D	A	L	S	A
12	BD3-99	Bangladesh	AF362776	A	I	Q	V	D	A	L	S	A
13	VMB	India	EU788042	A	I	Q	V	D	A	L	S	A
14	GHUT1	Vietnam	AY841901	A	I	Q	V	D	A	L	S	A
15	HK46	Hong Kong	AF092943	A	I	Q	V	D	A	L	S	A
16	SH92	Korea	AF533670	A	I	Q	V	N	A	L	S	A
17	UAF06	Pakistan	EF529700	T	I	Q	V	D	A	L	S	A

*GenBank accession number. A: alanine, D: aspartic acid, H: histidine, I: isoleucine, L: leucine, N: asparagine, P: proline, Q: glutamine, S: serine, T: threonine , V: valine.

Table 2. Pairwise comparison (%) of nucleotide identity (upper diagonal) and amino acid homology (lower diagonal) for 17 representative IBDV strains

No.	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	52/70		99	99	97	98	98	98	98	96	96	95	96	96	96	96	97	97
2	D78	99		99	98	98	98	98	98	96	96	95	97	96	96	97	97	97
3	CU1	98	98		97	98	98	98	98	96	96	95	96	96	96	96	97	97
4	GLS	97	97	97		98	98	98	97	96	96	95	97	96	96	97	97	97
5	STC	97	97	98	97		99	99	98	97	96	96	97	97	97	97	97	97
6	GB3	97	97	98	97	98		100	99	97	96	97	95	97	97	96	97	97
7	GSG4	97	97	98	97	98	100		99	97	96	95	97	97	96	97	97	97
8	GB1	97	97	97	97	98	99	99		97	97	96	97	97	97	97	98	98
9	BLRI94/B551	96	96	96	96	97	98	98	98		98	97	98	98	98	98	99	98
10	NP2K	96	96	96	96	96	97	97	97	99		98	98	98	98	98	98	98
11	NP1SSH	93	94	93	94	94	95	95	95	96	97		97	97	97	97	97	97
12	BD3-99	96	96	96	96	97	98	98	98	99	99	97		99	98	98	99	98
13	VMB	96	96	96	96	97	98	98	98	99	98	96	99		98	98	99	98
14	GHUT1	96	96	96	96	97	97	97	98	99	98	96	99	99		98	98	98
15	HK46	96	97	96	97	97	98	98	98	99	99	96	99	99	99		99	98
16	SH92	97	97	97	97	97	98	98	98	99	98	96	99	99	99	99		98
17	UAF06	96	96	96	96	97	98	98	98	99	98	96	98	98	98	99	98	

and GB3 isolates was obtained. These sequences were used as entry data to search GenBank to confirm the VP2 sequence identity. Sequence from 15 strains in the GenBank database, including two classical isolates [52/70 (D00869), Cu-1 (D00867)] as well as very virulent strains from Europe, Asia, and North America were aligned

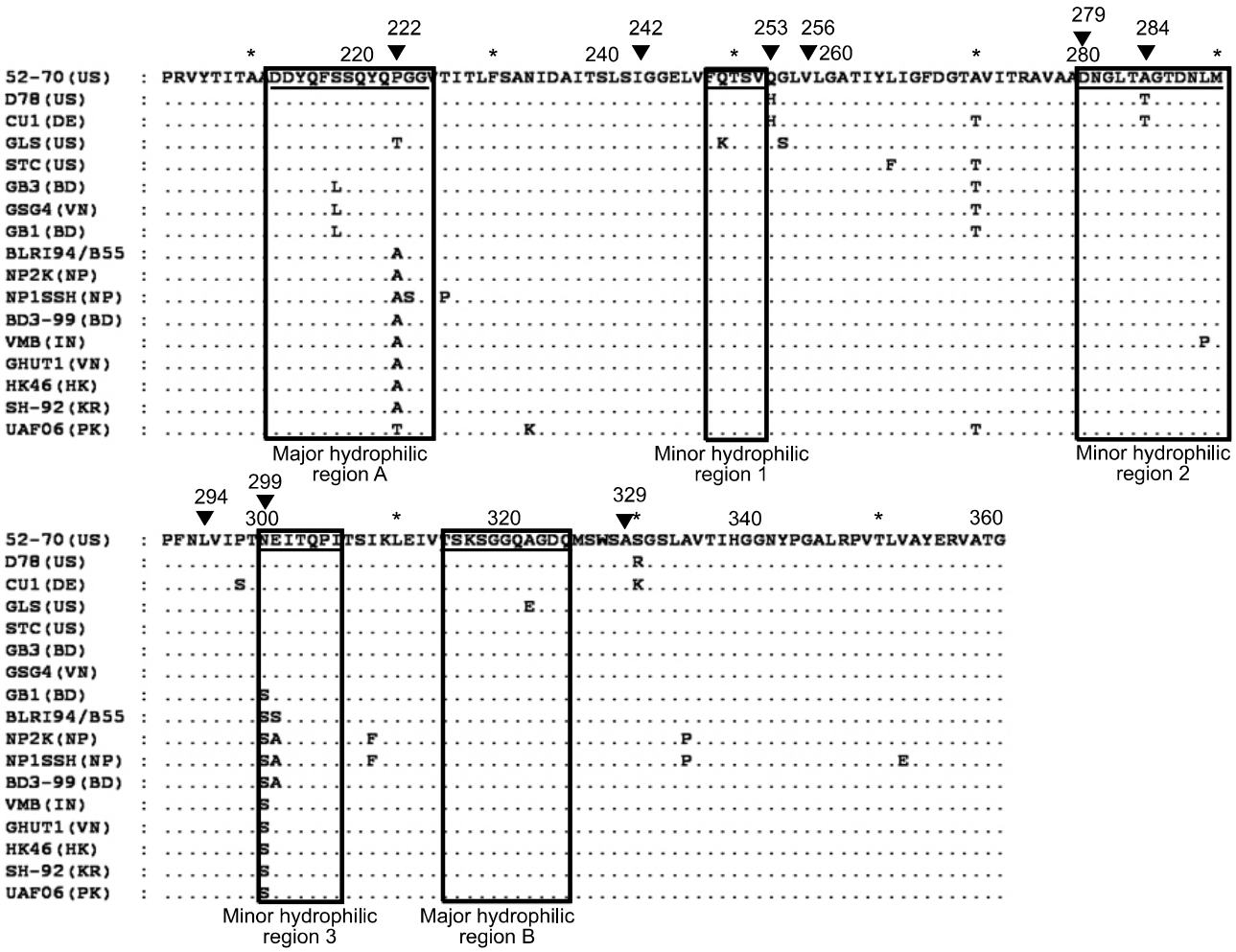

Fig. 1. Alignment and comparison of predicted amino acid sequences for the hypervariable region of the VP2 gene of 17 IBDV isolates. Amino acid sequence of the isolate 52-70 (US) is shown on top. Identities of the subsequently aligned sequences are indicated by dots and differences are represented by single letters. Key amino acids examined in this study are indicated by numbers and arrows. The amino acid sequence (underlined in the top sequence) containing the neutralizing epitopes including major (A and B) and minor (1, 2, and 3) hydrophilic regions in the 17 isolates (boxed regions) is shown. US: USA, DE: Germany, BD: Bangladesh, VN: Vietnam, NP: Nepal, IN: India, HK: Hong Kong, KR: Korea, PK: Pakistan.

(Table 2). Among the strains selected for comparative alignment, the sequences of VP2 genesfrom IBDVs of Bangladesh, Pakistan, and India were preferably considered in order to deduce the genetic relationship between viruses isolated in neighboring countries. Alignment of the predicted amino acid sequences encoded by nucleotides 606～1080 covering the VP2 hypervariable region is presented in Fig. 1.

The amino acid sequence of 52/70 (D00869) was used as a reference for comparison. A change in only one amino acid (serine at 299) was found in GB1 when comparing the same position for the 52/70 classical strain (Table 1 and Fig. 1). Amino acid substitutions in GB1 were similar to those found in the Asian IBDV strains. Among four major substitutions observed when comparing the strains (Fig. 1), two were unique to GB1 and GB3 (Ser217Leu and Ala270Thr). The Nepali isolates (NP2K and NP1SSH) had an alanine substitution at position 222, which has been identified as a marker of virulence. This was not observed in the isolates examined in the present study or other mostly classical isolates. This led to a speculation that the IBDV population in the South Asian geographic region is diverse and might influence vaccination programs in this region.

Nucleotide sequence identity and amino acid sequence homology

Table 2 shows results of the pairwise comparison conducted to evaluate nucleotide identity and amino acid homology between the 17 strains. Variation rate among the 17 strains was between 1～5% for the 474-bp nucleotide sequence and 1～7% for the corresponding 158 amino

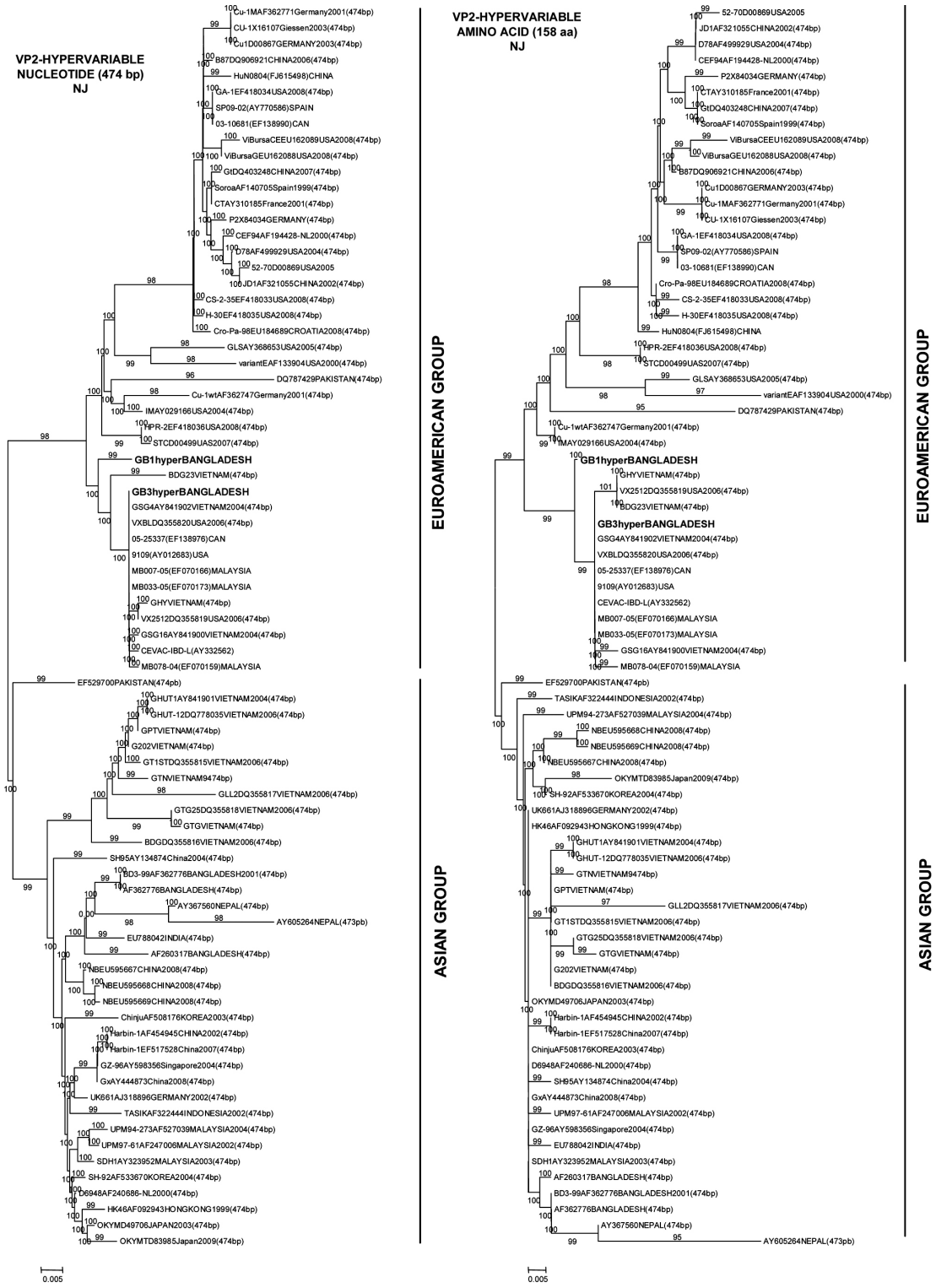

Fig. 2. Phylogenetic tree constructed with MEGA 4.1 illustrating the relationships among Bangladeshi IBDV isolates (GB1 and GB3) and 75 other strains of different geographical origins. Distance matrices were constructed using the Kimura's two-parameter model and trees were constructed using the neighbor-joining (NJ) algorithm based on the pairwise nucleotide differences in the VP2 variable region. Units at the bottom of the tree indicate the number of substitution events. The length of each pair of branches represents the distance between sequence pairs. The dataset was resampled 1,000 times using the bootstrap method. Accession numbers of the sequences and countries of origin are marked at end of strains when known.

acids. The GB1 and GB3 Bangladeshi isolates differed less than 3% from other strains in terms of nucleotide sequence identity and amino acid homology. Interestingly, the GB3 isolate had a 100% identity with the nucleotide and amino acid sequences of the GSG4 isolate from Vietnam (AY841902). Among the IBDVs from Bangladesh, the rates of identity and homology were 97～99% compared to each other and to previously isolated strains (Table 2).

Assessment of the key amino acids of the Bangladeshi isolates

Table 1 shows key amino acids responsible for virulence for each strain at nine positions within the hypervariable region of the VP2 polypeptide. A very important amino acid, glutamine, responsible for virulence of IBDV was found at position 253. It is known that isolates with a glutamine at position 253 have increased pathogenicity while those with a histidine at position 253 are much less pathogenic. As shown in Table 1, the CU1 and D78 isolates had a histidine at this site while the remaining strains including GB1 and GB3 had glutamine at this position. However, unlike the previously obtained Bangladeshi IBDV isolates, both GB1 and GB3had a proline at position 222 while GB3 had asparagine at position 299.

Phylogenetic associations

For the phylogenetic analysis, sequences of the VP2 hypervariable region from 75 isolates obtained from GenBank along with those of the GB1 and GB3 Bangladeshi isolates were included in the study. The sequences were compared using GeneDoc (ver. 2.7) and MEGA 4.1 with 1,000 bootstrap resamplings and the NJ method. Phylogenetic trees were created based on data from nucleotide sequences along with the amino acid sequences (Fig. 2). Two major clusters formed in all constructed trees; these clusters were considered to represent Euro-American and Asian groups. GB1 and GB3 Bangladeshi IBDVs were grouped with 40 other strains of European and American origin. This is in contrast to the previously isolated Bangladeshi IBDV strains marked BD3-99 (GenBank accession Nos. AF362776 and AF260317) that wereplaced into the Asian group (Fig. 2). Among the Euro-American viruses in the first group, there were a number of IBDV strains that had been isolated in Asia. Likewise, there were several viruses isolated in Europe and other parts of the world that were in the Asian group of isolates.

Discussion

Residues present in the VP2 region at positions 222, 242, 253, 256, 279, 284, 294, 299, and 329 are key amino acids believed to be responsible for viral antigenicity and pathogenicity [5,10,11,20,38]. Any amino acid changes at these positions may result in variability of pathogenic potential and antigenicity. Recently, special attention was given to amino acids at position 253 wherehistidine or glutamine is found [10]. Because of the intensive vaccination programs performed in the field with live attenuated viruses, there is a possibility that the viruses used mutate and subsequently change their pathogenic potential [10].

Molecular evaluation of the hypervariable region of two isolates was conducted to compare their nucleotide and amino acid sequences with those of other strains fromneighboring countries and other parts of the world. A comparative alignment and phylogenetic analysis of the hypervariable domain of the VP2 region helped to group the IBDV isolates into different pathogenic subgroups and assess geographical subtypes. Results obtained from molecular analysis of the recent IBDV isolates demonstrated that these strains have similarities with previously characterized Bangladeshi isolates in terms of molecular features and virulence level. Amino acids found at positions 253 and 284 [10,11,37] indicated that these isolates have a very virulent pathotype, similar to IBDVs previously isolated in 1999 [8].

A glutamine residue at position 253 and alanine at position 284 were found in the two isolates examined in the present study as well as the previously isolated Bangladeshi IBDV and a number of recently isolated strains of European and Asian origin. These two residues are responsible for pathogenicity andare unique to highly virulent IBDVs. Our findings demonstrated that GB1 and GB3 are of a virulent nature [10]. However, the GB1 and GB3 viruses differed from the previous Bangladeshi IBDV isolates by having a proline residue at position 222 instead of alanine. One of these viruses, GB3, had an aspargine at position 299 while the other isolates contained a serine. This may be indicative of genetic variation between the recent two isolates and the previously isolated Bangladeshi IBDVs although all of these viruses fall into same virulence group.

The origin of vvIBDVs and how these viruses spread throughout various countries including Bangladesh are not clear [31,32]. Nowadays, strains of viruses with every virulence level and different pathogenicities exist, and live attenuated vaccines have been introduced in many countries as a result [9,18]. Over the last 20 years, the IBDV has changed and new pathogenic variants have evolved so that the virus exists as low, mild, and vvIBDVs in the field. Results of our phylogenetic analysis based on the VP2 hypervariable sequences demonstrated the contrasting relationships between the previously and recently isolated Bangladeshi IBDVs. Sequence analysis of the hypervariable region of VP2 gene, typically used for phylogenesis, is crucial but the full length of the protein-coding sequence of the A segment might reveal more of the fine differences between strains. Comparative

alignment and phylogenetic analysis of the full genome or at least the entire VP2 sequence would provide better molecular and functional comparisons.

Based on our findings, we concluded that that the two recent IBDV isolates evaluated in the present study belong to a very virulent group similar to the previously isolated Bangladeshi IBDVs. However, amino acid sequences of the hypervariable VP2 region of the two recent isolates differed from those of the other IBVDs by one or two key amino acids.

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