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Research article

Partial molecular characterization of infectious bursal disease virus detected in Africa: potential evidence for virus recombination and genome segments A and B reassortment in nature

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Abstract

Genome reassortment and recombination mutations have been speculated to occur in infectious bursal disease virus (IBDV) and contribute to the emergence of new strains. However, evidence was lacking until recently when natural reassortant viruses were detected in China and Zambia. In this study, genome reassortment in African very virulent IBDVs was investigated by partial genomic sequencing, comparison of deduced amino acids and phylogenetic analyses. The findings revealed that the VP2 hypervariable region (VP2-HVR) and part of VP1 corresponding to 332 amino acids of the N-terminus were the best representative of the entire genome segments A and B of IBDV, respectively. The part of VP1, which spans the one-third of segment B to the 5'-end revealed 5 amino acid positions (61, 145, 147, 219 and 242) to be critical for the evolution of genome segment B. Taken together, these findings indicate that partial molecular characterization of segments A and B could be used for examination of genome reassortment and recombination mutations in IBDV.

Keywords: IBDV; Genome reassortment; recombination mutation; Africa

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Introduction

Infectious Bursal Disease (IBD) virus (IBDV), is a bi-segmented RNA virus, which belongs to the genus *Avibirnavirus* of the family *Birnaviridae*. In chickens, two serotypes of IBDV, 1 and 2 can be found. The serotypes 1 IBDVs, classified into very virulent (VV), classical virulent, mild and intermediate strains, and antigenic variants (Wu et al., 2007), are known to be the causative agents of IBD, while serotype 2 IBDVs are non-pathogenic (Ismail et al., 1988).

The IBDV genome segment A contains partially overlapping open reading frames (ORFs), ORF1 and ORF2. The small ORF1 encodes a non-structural protein VP5, whereas the large ORF2 encodes a precursor polyprotein, which is cleaved by autoproteolysis to produce VP2, VP4 and VP3 (Lejal et al., 2000). VP2 and VP3 are the major structural proteins of the virion, and VP4 is the viral protease (Da Costa et al., 2002). Genome segment B, on the other hand, contains one ORF encodes VP1, which is the RNA-dependent RNA polymerase (RdRp) responsible for viral genome replication and RNA synthesis (Shwed et al., 2002).

Many RNA viruses, including IBDV, have a high capacity to exchange genetic material(s) to one another,

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acquire genes from their hosts, and create and spread beneficial mutations efficiently (Worobey and Holmes, 1999). Genome reassortment has been demonstrated for many RNA viruses such as Rift valley fever virus (Bowen et al., 2001), Influenza virus (Walenstein et al., 2005), and Cucumber mosaic virus (Roossinck, 2002), as well as plant viruses such as Tobravirus (Robinson et al., 1987), Cucumovirus (White et al., 1995) and Rice grass stunt virus (Miranda et al., 2000). These studies indicated that reassortment contributes to the emergence and evolution of viruses with altered disease potential and host range.

Kasanga et al (2007) described mortality ranging from 10-62% in chickens naturally infected with VV-IBDVs. The past reports suggested that genomic recombination or reassortment might occur in IBDV (Brown and Skinner, 1996; Hon et al., 2006; Kong et al., 2004; Le Nouen et al., 2005; Le Nouen et al., 2006; Yamaguchi et al., 1997). In addition, previous findings demonstrated the existence of heterogeneous virus population in chickens in Tanzania and Zambia, ranging from classical attenuated, classical virulent and very virulent genotypes (Kasanga et al., 2007; Kasanga et al., 2008; Kasanga et al., 2013a) suggesting a high risk of occurrence of IBDV genome reassortment under the natural field condition. However, direct evidence was lacking, until recently (Kasanga et al., 2013b; Wei et al., 2006; Lu et al., 2015; Vera et al., 2015) when reassortant strains in Zambia, China and Argentina were described.

The aim of this study was to examine the molecular characteristics of IBDV field strains recovered from different areas of Africa. The author has investigated the potential recombination mutation(s) and genome reassortment of 10 IBDV field strains from Zambia and Tanzania. The partial nucleotide and deduced amino acid sequences of genome segments A and B of the field viruses were compared with other serotype 1 and 2 strains to identify their genetic relationship with other IBDVs. Furthermore, by constructing phylogenetic trees based on the nucleotide and deduced amino acid sequences, the relationship with other strains in terms of genetic evolution and origin was determined.

Materials and Methods

IBDV strains

Ten African-origin virus strains, namely KMRG-48, KMRG-79, KARS-53, KDSM-02, KMRG-00, KMZA-28, KZC-107, KZC-103, KZC-110 and KZC-104, primarily isolated in chickens from confirmed IBD outbreaks in Tanzania and Zambia were used. The description of these strains is shown in Table 1. The first strand complementary DNA (cDNA) was synthesized using Rever Tra Ace reverse transcriptase (RT) (Toyobo, Osaka, Japan) and random primer $Pd(N)_6$ (Toyobo). The cDNAs from each virus were used as templates for PCR. The other serotype 1 and 2 viruses were used for comparison purpose during the sequence and phylogenetic analysis of nucleotide and deduced amino acids of the African strains.

Amplification and cloning of VP2-HVR and part of VP1 cDNAs

In order to investigate the existence of recombination mutation(s) and/or reassortment between genome segments A and B among IBDV field strains, part of segment A representing the VP2-HVR and part of segment B encoding the one-third of VP1 to the N-terminus were used. The VP2-HVRs were amplified by polymerase chain reaction (PCR) using V1 forward primer (5'-CCA GAG TCT ACA CCA TAA-3') and V2 reverse primer (5'-CCT GTT GCC ACT CTT TCG TA-3') (Yamaguchi et al., 1996). The parts of VP1 corresponding to 998 nucleotides (332 amino acids) of the N-terminus of segment B were amplified by PCR using BF1 forward primer (5'-CCT CTT CTT GAT GAT TCT ACC A-3') and BR1 reverse primer (5'-GAC CAT ATG TTA CGG GTC TT-3') designed in this study. The cDNA templates for VP2-HVR and part of VP1 were amplified using Thermus aquaticus Ex-Taq DNA polymerase (Takara, Shiga, Japan) in a TAKARA PCR Thermo Cycler GP (Takara, Shiga, Japan).. The procedure for cDNA synthesis, PCR, DNA purification and cloning were performed as previously described (Kasanga et al., 2013a).

Sequencing of cDNA clones

To verify PCR products, five independent clones for each fragment of VP2-HVR and part of VP1 were sequenced at Doragon Genomics center (TAKARA Bio, Mie, Japan) with a Templiphi DNA sequencing Template Amplification Kit, DYEnamic ET dye terminator kit, and MegaBACE 1000 (Amersham Biosciences Co., Piscataway NJ, USA). The sequencing was conducted according to Sanger et al., (1977).

Sequence assembly, genetic and phylogenetic analysis

The partial nucleotide and deduced amino acid sequences of KMRG-48, KMRG-79, KARS-53, KDSM-02, KMRG-00, KMZA-28, KZC-107, KZC-103, KZC-110 and KZC-104 strains were compared to the published cDNA sequences of IBDV. cDNA sequence data were analysed using GENETYX software Version 14.0.1 (Software Development Co., Tokyo, Japan).

Prior to the phylogenetic analysis, cDNA sequences were aligned using the CLUSTAL W program (Thompson et al., 1997). The trees for partial sequences were constructed with 30 strains, including the Tanzanian and Zambian serotype 1 viruses. The topological accuracy of each tree was estimated from

the bootstrap values using the Kimura two-parameter option with 1,000 replicates created by the neighbor-joining (NJ) method (Kimura, 1980). The tree constructed using full genome segments A and B of selected serotype 1 viruses was used for comparison (Kasanga et al., 2013b).

Alignment of deduced amino acids

The deduced amino acid sequences of 10 strains were aligned and compared to that of other serotype 1 and 2 IBDVs including that of classical strains, antigenic variants, classical attenuated, and VV-IBDVs. Amino acids alignment was created by the parallel editor option of Genetyx-mac software version 14.0.1 (Software Development Co., Tokyo, Japan).

Results

cDNA sequence analyses of VP2-HVR and part of VP1

The sequence analyses were performed to determine the correctness of nucleotide arrangement in segments A and B of the studied field IBDVs. The sequences were derived from five independent clones for each cDNA fragment. No differences were observed between the clones. The VP2-HVRs of all ten strains were 434 nucleotides-long (144 amino acids) and showed no deletions or insertions in any of the sequences. The nucleotide sequence similarity among ten sequences ranged between 96 – 99.3%. The parts of VP1 (corresponding to 332 amino acids of the N-terminus) were 998 nucleotides-long without deletion or insertions with similarities ranging between 95.5 – 99.7%. On BLAST search, none of the sequences showed 100% homology to any of the VP2-HVR nucleotide sequences. On the other hand, the BLAST search for nucleotide sequences corresponding to part of VP1 showed 100% nucleotide sequence homology between KZC-104 and D78. The GenBank accession numbers for VP2-HVR and part of VP1 are shown in Table 1. The sequence analyses indicated that all ten field viruses were genetically different with varying degree of divergences in VP2-HVR and part of VP1.

Analyses of amino acid sequences for VP2-HVR and part of VP1

The analyses of amino acid sequences were performed to examine the antigenic and evolutionary pathways of genome segments A and B in IBDV. The deduced amino acid sequences of the VP2-HVR and part of VP1 of ten sequenced African strains were aligned with other VP2-HVR and part of VP1

Table 1: Description of African IBDV strains used in this study

Strains	Geographic origins	Year isolated	References	GenBank accession numbers	
				VP2-HVR	Part of VP1
KMRG-48	Tanzania	2002	Kasanga et al., 2007 and this study	AB200983	AB368952
KMRG-79	Tanzania	2004	Kasanga et al., 2007 and this study	AB200986	AB368953
KARS-53	Tanzania	2004	Kasanga et al., 2007 and this study	AB200984	AB368954
KDSM-02	Tanzania	2004	Kasanga et al., 2007 and this study	AB200976	AB368955
KMRG-00	Tanzania	2001	Kasanga et al., 2007 and this study	AB200975	AB368956
KMZA-28	Tanzania	2004	Kasanga et al., 2007 and this study	AB200979	AB368957
KZC-107	Zambia	2004	This study	AB368948	AB368958
KZC-103	Zambia	2004	This study	AB368945	AB368959
KZC-110	Zambia	2004	This study	AB368950	AB368960
KZC-104	Zambia	2004	This study	AB368946	AB368961
T09	Nigeria	1998	Owoade *et al*., 2004	AY099456	AY099457

Table 2: Unique amino acid variations in VP1 (corresponding to 332 amino acids in the N-terminus) and hypervariable region (VP2-HVR) for differentiation of IBDV strains

IBDV strain or genotype	Part of VP1 (N-terminus)					VP2-HVR							
	61	145	147	219	242	242	254	256	279	284	294	299	300
Classical attenuated	V	N	G	D	D	V	G	V	N	T	L	N	E
Classical virulent	•	•	•	•	•	•	•	•	D	A	•	•	•
European/Asian VV	I	T	N	•	E	I	•	I	D	A	I	S	•
African VV-1	I	T	N	•	E	I	S	I	D	A	I	S	A
African VV-2	I	T	N	•	E	I	S	I	D	A	I	S	Q
KZC-104*	•	•	•	•	•	I	S	I	D	A	I	S	Q
ZJ2000*, TL2004*	I	T	N	•	E	•	•	•	•	•	•	•	•
02015.1	•	T	S	•	•	I	•	I	D	A	I	S	•
KDSM-02, KMRG-00, KMZA-28	I	S	•	E	•	I	•	I	D	A	I	S	•
Gx	I	T	•	•	•	I	•	I	D	A	I	S	•
SF95	•	•	D	•	•	I	•	I	D	A	I	S	•

*: Reassortant strains; Underlined: Unrelated type of strains. Different amino acids from attenuated type are shown, whereas positions with similar amino acid substitutions to that of attenuated type are marked with dots. VV: very virulent.

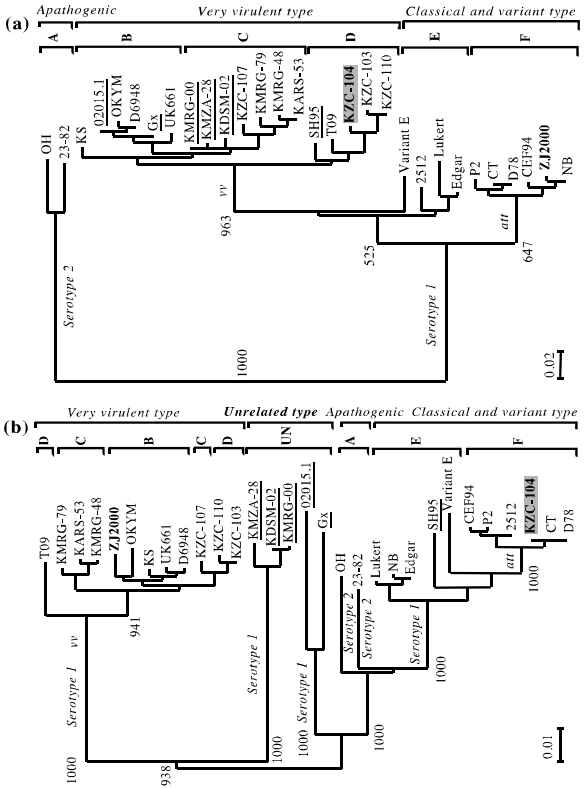

Fig. 1: Phylogenetic trees based on the nucleotide sequence encoding (a) VP2-HVR and (b) VP1 corresponding to 332 amino acids of the N-terminus of serotype 1 and 2 representative strains. Trees were calculated by the neighbor-joining (NJ) method with nucleotide alignment created in Clustal W version 1.8.3. The numbers at forks indicate the bootstrap values (1,000 replicates) in important junctions of the tree. vv: very virulent; att: classical attenuated strains; A: serotype 2 strains, which are non-pathogenic to chickens; B: European/Asian very virulent strains; C: African very virulent type 1 (VV-1) corresponding to the Tanzanian very virulent genotype; D: African very virulent type 2 (VV-2); E: Classical strains including virulent, mild and antigenic variants; F: Classical attenuated strains; UN: Unrelated serotype 1 virus strains; Bold with dark background: reassortant virus isolated in chickens from Zambia; Bold: reassortant virus isolated in chickens from China, and Underlined: Unrelated type of viruses.

sequences available. Comparison of VP2-HVR and part of VP1 of serotype 1 strains revealed 8 amino acid positions in VP2-HVR (242, 254, 256, 279, 284, 294, 299 and 300) and 5 amino acid positions, in part, of VP1 (61, 145, 147, 219 and 242), which amino acid substitutions were different among IBDV genotypes of very virulent and classical strains, reassortant strains and unrelated type of strains (Table 2). All classical attenuated, classical virulent and mild, and variant strains conserved amino acid residues at positions 61 (V), 145 (N), 147 (G) and 242 (D) of part of VP1, which were different from that of very virulent strains (European/Asian and African VV types), which instead

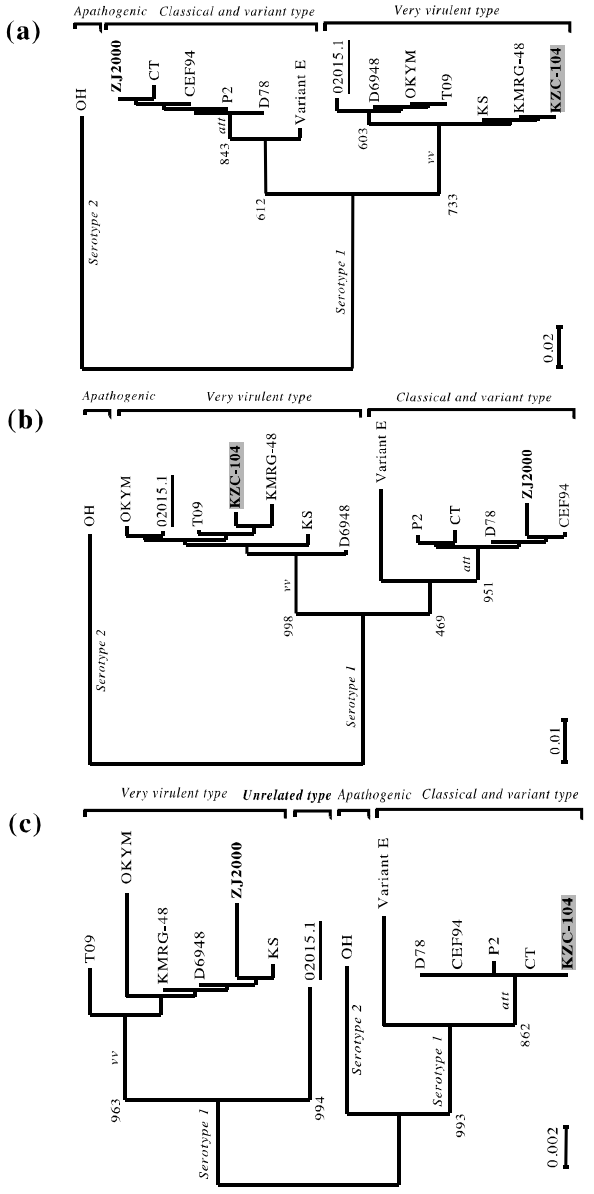

Fig. 2: Phylogenetic trees based on the amino acids sequence for (a) VP1, (b) precursor polyprotein and (c) VP5 of serotype 1 and 2 strains. Trees were generated by the neighbor-joining (NJ) method with amino acids alignment created in the parallel editor option of Genetyx-mac software version 14.0.0. The numbers at forks indicate the bootstrap values (1,000 replicates) in important junctions of the tree. vv: very virulent; att: classical attenuated strains; Bold with dark background: reassortant virus isolated in chickens from Zambia; Bold: reassortant virus isolated in chickens from China, and Underlined: Unrelated type of virus. Source: Kasanga et al., 2013b.

conserved amino acid residues 61(I), 145(T), 147(N) and 242(E). Strain KZC-104 conserved amino acid residues at positions 61(V), 145(N), 147(G) and 242(D) of part of VP1 similar to that of classical attenuated strains, while amino acid residues of the VP2-HVR

were the same as that of the African VV-2 (Table 2). On the other hand, strains ZJ2000 and TL2004 conserved amino acid residues 61(I), 145(T), 147(N) and 242(E) in part of VP1, which were similar to that of very virulent strains, while amino acid residues of the VP2-HVR were the same as that of classical attenuated strains. Strains 02015.1, KDSM-02, KMRG-00, KMZA-28, Gx and SF95 conserved amino acid substitutions in part of VP1 that were not related to either classical attenuated, classical virulent and mild, variant type, or very virulent strains, whereas amino acid substitutions of these strains were the same as that of very virulent strains in VP2-HVR (Table 2). The African VV strains, which instead conserved amino acid substitutions at positions 254 (S) and 300 (A/Q), were different from the European/Asian VV strains that conserved amino acid residues at positions 254 (G) and 300 (E) in VP2-HVR. In addition, the African VV-1 conserved amino acid residue 300 (A), which was different from that of the African VV-2, which instead conserved amino acid residue 300 (Q) in VP2-HVR. Classical attenuated strains conserved amino acid residues 279 (N) and 284 (T) contrary to other strains, which conserved amino acid residues 279 (D) and 284 (A) in VP2-HVR (Table 2). The nucleotide and amino acid sequences of part of VP1 of 02015.1, KDSM-02, KMRG-00, KMZA-28, Gx and SF95 strains seems to be different from that of very virulent, classical attenuated, classical virulent, variant strains and serotype 2 strains (Table 2 and Fig. 1). These observations imply that the segment B of 02015.1, KDSM-02, KMRG-00, KMZA-28, Gx and SF95 strains could be evolved from certain recombination mechanisms and/or genetic mutations in the sequenced part of VP1. In addition, these strains conserved at least one amino acid substitution at positions 61, 145, 147, 219 and 242 in VP1 spanning the one-third of segment B to the 5'-end, which were critical for the evolution of genome segment B.

Phylogenetic analyses based on VP2-HVR, part of VP1, VP5, polyprotein and VP1 of selected IBDV strains

Phylogenetic analyses were performed to determine the genetic and antigenic relationships of IBDV and examine the evolutionary pathways of the field viruses. These analyses were important as they could provide information required for the development of appropriate vaccine against circulating viruses in particular geographic location, time and space. The phylogenetic trees of 30 IBDV strains based on VP2-HVR nucleotide sequences were split into major clusters of a pathogenic type comprised of serotype 2 strains, very virulent type sub-clustered into European/Asian VV, African VV-1 and African VV-2, and classical and variant type sub-clustered into classical and variant strains, and classical attenuated strains (Fig. 1a). KZC-104 was clustered with African VV-2 strains. Phylogenetic tree based on the nucleotide sequences of part of VP1 was different from that constructed from the VP2-HVR sequences of the same strains (Fig. 1b). The strains were split into a pathogenic serotype 2 strains, very virulent type, classical and variant type, and unrelated type of strains. Within classical and variant type, two sub-clusters of classical attenuated and classical strains (virulent and mild) including variant E strain were evident. Strikingly, part of VP1 of KZC-104 formed a cluster with all classical attenuated strains, although VP2-HVR of KZC-104 was clustered with African VV-2 strains. In addition, part of VP1 of five strains, KMZA-28, KDSM-02, KMRG-00, 02015.1 and Gx, which were clustered with very virulent strains in phylogenetic tree based on VP2-HVR, formed a group which was not related to either serotype 2, classical strains, variant E or very virulent strains (Fig. 1b). The part of VP1 of Strain SF95, which clustered with very virulent on phylogenetic tree created based on VP2-HVR, formed a cluster with classical strains but different from that of classical attenuated strains. Furthermore, strain ZJ2000, which was grouped with classical attenuated strains in the tree created based on VP2-HVR, formed a cluster with very virulent strains on the tree constructed based on the nucleotides of part of VP1. The phylogenetic analysis based on VP2-HVR and part of VP1 revealed genetic differences, which inferred the evolutionary changes in segment B of KMZA-28, KDSM-02, KMRG-00, 02015.1 and Gx viruses.

The phylogenetic analysis based on VP5, polyprotein and VP1 was used for comparison to the analysis performed basing on the partial segments A (VP2-HVR) and B (part of VP1) sequences. The phylogenetic trees of 14 selected IBDV strains constructed based on deduced amino acid sequences of VP5, polyprotein and VP1 revealed a similar pattern to that of phylogenetic trees constructed based on nucleotide sequences of VP2-HVR and part of VP1 corresponding to 332 amino acids of the N-terminus (Fig. 2). The similarity in clustering patterns of sequences in figures 1 and 2 revealed that VP2-HVR and part of VP1 could represent some segments A and B genetic nature responsible for virus evolution, respectively.

Discussion

Sequence analyses are important as they provide information and insights on the genetic diversity, antigenic variation and evolutionary characteristics of viruses. The evolution of the virus is normally brought about by cumulative mutations that lead to genetic and antigenic variants, which through natural selection they

lead to emergent and/or re-emergent strains. Understanding the basis for evolutionary characteristics and/or pathways and antigenic variation of field viruses could help veterinarians and researchers to design and develop novel vaccines meant for controlling new strains, which cannot be controlled by conventional vaccines.

Double-stranded RNA viruses have a high rate of mutation, recombination, and reassortment (Wei et al., 2006). In this study, parts of genome segments A and B of the African-origin IBDV strains were sequenced. Comparison of deduced amino acids and phylogenetic analyses were conducted and evidence of genomic reassortment between segments A and B of IBDV in some of the strain was revealed.

Comparison of deduced amino acid sequences of the VP2-HVR and part of VP1 of ten African-origin IBDVs with that of other strains showed that one of the African strains, KZC-104, consisted of amino acid residues related to that of the very virulent strains in VP2-HVR, while amino acids in part of VP1 were similar to that of classical attenuated strains (Table 2). Previous study revealed that KZC-104 was a natural reassortant strain with genome segments A and B originated from very virulent and classical attenuated IBDV strains, respectively (Kasanga et al., 2013b). Phylogenetic analysis based on the nucleotide sequences also revealed clustering of KZC-104 with very virulent strains in the VP2-HVR, and clustering with classical attenuated strains in the tree constructed based on part of VP1. These findings suggest that the VP2-HVR on segment A and part of VP1 spanning the first one-third of the N-terminus are the representatives of genome segments A and B of IBDV and could be used for investigation of genome reassortment and/or recombination mutations.

Other findings indicated that strains 02015.1, KDSM-02, KMRG-00, KMZA-28, Gx and SF95 retained unique amino acid substitutions in part of VP1 that were not related to classical attenuated, classical virulent, variant E strain or very virulent strains, while amino acid substitutions of these strains in VP2-HVR were the same as that conserved in the very virulent strains (Table 2). Non-homologous recombination, evidenced by segregation of branches in phylogenetic trees, have been documented to occur during synthesis of the RNA strands in flock house virus (Li and Ball, 1993) and dengue virus (Holmes et al., 1999; Huson, 1998). In these viruses, the recombination mechanism resulted in the creation of new virus genomic strands different from the parent strands. Although it is impossible to predict the effect of the unique amino acid without knowing the tertiary (3-dimension) structure of the virus, and function of individual proteins, the amino acid substitutions at positions 61, 145, 147, 219 and 242 may be critical for determination of the evolutionary pathway(s) of genome segment B of 02015.1, KDSM-02, KMRG-00, KMZA-28, Gx and SF95 strains. Further studies are required to define the role of each deduced amino acid for the evolution of the virus and antigenicity of pathogenic strains.

Furthermore, detailed sequence and phylogenetic analyses based on the nucleotides of the VP2-HVR and part of the VP1 also revealed clustering of 02015.1, KDSM-02, KMRG-00, KMZA-28, Gx and SF95 strains in the very virulent genotype, and in a separate cluster unrelated to other strains, respectively (Fig. 1a & b). The clustering pattern of the strains in the phylogenetic tree when using partial sequences were similar to the clustering pattern obtained after using the full-length genome segments A and B sequences (Fig. 2). These findings raise the possibility that VP2-HVR and part of the VP1 corresponding to 332 amino acids of the N-terminus, are the best representative of genome segments A and B of IBDV, respectively. Further in-depth experiments are required to elucidate the involvement of VP2-HVR (in segment A) and part of VP1 (in segment B) in the evolution of reassortant IBDV strains under field condition(s).

Conclusion

In this study, it was demonstrated that (i) the segment B of 02015.1, KDSM-02, KMRG-00, KMZA-28, Gx and SF95 IBDV strains evolved from certain recombination mechanisms and/or genetic mutations in the sequenced part of VP1, (ii) and that these strains conserved at least one amino acid substitution at positions 61, 145, 147, 219 and 242. Furthermore, it was established that VP2-HVR and the N-terminus of VP1 (encoding 332 amino acids to the 5' end) represent IBDV genome segments A and B respectively, and could potentially be used for investigation of recombination mutation(s) and genome reassortment of the virus.

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