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Research article

Molecular typing of infectious bursal disease virus field strains in endemic settings of Africa

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Abstract

Detection and differentiation of serotype 1 infectious bursal disease (IBD) virus (IBDV) field strains are important for effective control of IBD. Restriction enzyme analyses have been reported to differentiate serotype 1 strains into specific genotypes. However, the methods previously reported are not suitable for genotyping the African very virulent IBDV (VV-IBDV) in endemic settings of Africa. In this study, an appropriate molecular typing method for IBDV field strains with restriction endonucleases was determined. Six restriction patterns were identified to categorize the serotype 1 strains into six molecular groups by using Aat I, Ban III, Bsp MI, Ssp I, Sac I and Nco I restriction endonucleases. One restriction pattern was specific for the classical IBDV strains, whereas five patterns were able to categorize the VV-IBDV strains into five molecular groups. Of the five restriction patterns for VV-IBDV, one pattern was unique to the Tanzanian VV-IBDVs, whereas four patterns were able to differentiate the African and the European/Asian VV-IBDV genotypes. The six restriction endonucleases were practically applied to differentiate the Tanzanian and Zambian field IBDV strains into specific molecular groups. The molecular typing method reported in this study could be used for epidemiological surveillance of the field IBDV strains, particularly in areas where sequencing cannot be performed.

Keywords: IBDV; molecular typing; restriction endonucleases; Africa

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Introduction

IBDV is the causative agent of a highly contagious immunosuppressive disease of young chickens (Hirai et al., 1974). IBDV shows different degrees of pathogenicity and mortality in chickens. Some strains cause only subclinical infections, whereas others cause high mortality. Until 1987, most IBDV infections were subclinical, leading to impaired growth and acquired immunodeficiency (classical IBD), and IBD was controlled by live attenuated and/or inactivated vaccines (Lasher and Shane, 1994). However, in 1987, pathotypic IBDV variants with enhanced virulence, termed very virulent IBDVs (VV-IBDVs), in Europe from an unknown origin which needs specific characterization methods. Since 1987, an increasing number of acute cases have been reported in the Netherlands, Belgium, England, Turkey, Cuba, Japan, Bangladesh, Pakistan, China, Nigeria, South Africa and recently in Tanzania (Yamaguchi et al., 1997; van den Berg et al., 2000; Zierenberg et al., 2000; Kasanga et al., 2007; Yamaguchi et al., 2007).

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VV-IBDV infection is characterized by high morbidity and mortality of up to 100% not only in young chickens, but in older birds as well (Brown et al., 1994). The VV-IBDV can establish infection in chickens with antibody levels that are protective against classical IBDV strains (Chettle et al., 1989). Consequently, the identification and differentiation of IBDV genotypes are important for effective vaccination.

Molecular typing of the field IBDVs is important for epidemiological investigation of the virus origins during disease outbreaks. Since the first outbreak of the VV-IBDV in Europe, several studies have reported differentiation of the field strains by restriction enzyme analysis of cDNA encoding the viral protein 2 (VP2) hypervariable region (VP2-HVR) of the virus genome segment A (Zierenberg et al., 2000). The restriction analysis using Ssp I, Stu I and Sac I enzymes categorizes viruses into classical virulent, classical attenuated and very virulent genotypes (Lin et al., 1993). Restriction sites for Ssp I have shown a strong correlation with virulence of IBDV. However, the emergence of variant strains in the field, which retain Ssp I sites in the VP2-HVR without showing the VV-IBDV phenotype (Banda et al., 2001, Hoque et al., 2001, Majo et al., 2002), and the discovery of Tanzanian VV-IBDVs that do not contain Ssp I sites (Kasanga et al., 2007) indicate that the use of three enzymes, Ssp I, Stu I, and Sac I, is not sufficient for categorization of viruses. In addition, little is known of the rapid typing methods of the field IBDV strains in endemic settings in Africa.

In the current study, *in silico* restriction analysis of the VP2-HVR of serotype 1 IBDVs was confirmed by enzyme digestion and six restriction enzymes were found suitable for typing the field strains into specific molecular groups. The analysis has a potential application for epidemiological surveillance and molecular typing of the field IBDVs in areas where sequencing cannot easily be performed.

Materials and Methods

Prediction of restriction sites

The prediction of restriction sites within the VP2-HVR was performed on cDNA sequences retrieved from GenBank database (http://blast.ddbj.nig.ac.jp/), using the Tanzanian KMRG-48 reference strain as a query. A total of 144 sequences were *in-silico* tested for the presence or absence of sites for 95 different restriction enzymes using GENETYX-MAC software version 14.0.0 (GENETYX Co., Tokyo, Japan). Restriction enzymes with recognition sites in the VP2-HVR gene of serotype 1 IBDVs were used for grouping of 144 sequences into specific molecular groups. Enzymes, which produced restriction patterns differentiating strains into genotypes and subtypes, were used for categorization of the sequences into specific diagnostic groups.

Confirmation of restriction sites

Eleven IBDV strains, namely KDSM-32, KMRG-40, KMRG-48, KARS-53, KMZA-78, KTBR-18, KMRG-26, KMZA-28, 040802-2, GEB-10 (Kasanga et al., 2007) and GBF-1 (Yamaguchi et al., 1996), which were available in the author's laboratory, were used to confirm the presence or absence of enzyme-specific restriction site(s). The RT-PCR products for VP2-HVR were synthesized as described previously (Maw et al., 2006), gel-purified with Nucleospin® DNA purification kit (Macherey-Nagel Inc., Easton, PA, USA), and digested with specific enzymes according to manufacturer's instructions (Toyobo Co, Osaka, Japan). Digested products were analysed in 1.5% agarose gel and visualized with ethidium bromide staining.

Molecular typing of the field strains by restriction enzyme analysis

A total of 40 IBDV positive bursa samples obtained from 20 chickens in Zambia and 20 chickens in Tanzania between 2004 and 2014 were used. RNA isolation and cDNA synthesis of the VP2-HVR gene, were performed as described previously (Kasanga et al., 2007). The RT-PCR products with specific VP2-HVR bands were precipitated with 99.5% ethanol, whereas those with non-specific VP2-HVR bands were gel-purified with Nucleospin® DNA purification kit (Macherey-Nagel Inc., Easton, PA, USA). All samples were digested separately with six restriction enzymes. Samples displaying the same restriction fragment length polymorphism (RFLP) pattern were categorized in the same molecular group.

Molecular groups and phylogenetic clustering of selected IBDV strains

To examine whether the molecular groups corresponded with the phylogenetic clustering, the VP2-HVR cDNA sequences of 42 serotype 1 strains (including 10 sequenced IBDVs from Zambia) were used for comparison in phylogenetic analysis.

Results

Prediction of restriction patterns and molecular groups

The computer-based analysis of 144 serotype 1 VP2-HVR nucleotide sequence for sites of 95 endonucleases revealed the conservation of sites for 9 enzymes, namely Aat I, Ban III, Bsp MI, Ssp I, Sac I, Nco I, Nae I, Spe I and Acc I. Of these, six enzymes, Aat I, Ban III, Bsp MI, Ssp I, Sac I, and Nco I, categorized the strains into six molecular groups based on restriction patterns. The molecular groups were named

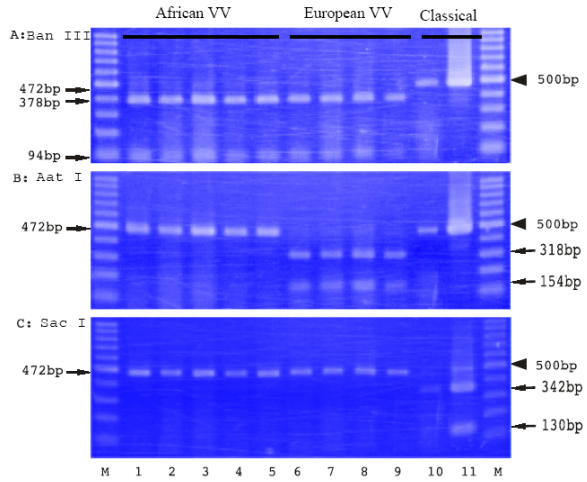

Fig. 1: Restriction enzyme analysis of the African VV variants, European VV and classical IBDV strains using Ban III, Aat I, and Sac I enzymes. A: Indicates digestion with Ban III; B: indicates digestion with Aat I; C: indicates digestion with Sac I. M: 100 base pair (bp) DNA ladder; Lane 1-5: African VV variants representing Tanzanian VV strains KDSM-32, KMRG-40, KMRG-48, KARS-53 and KMZA-78, respectively; Lane 6-9: European VV indicating Tanzanian strains KTBR-18, KMRG-26, KMZA-28 and Japanese VV isolate 040802-2, respectively; Lane 10 and 11: Classical IBDV strains GEB-10 and GBF1, respectively. Respective sizes of bands of the digested and undigested DNA fragments are indicated in bp for each electrophotograph.

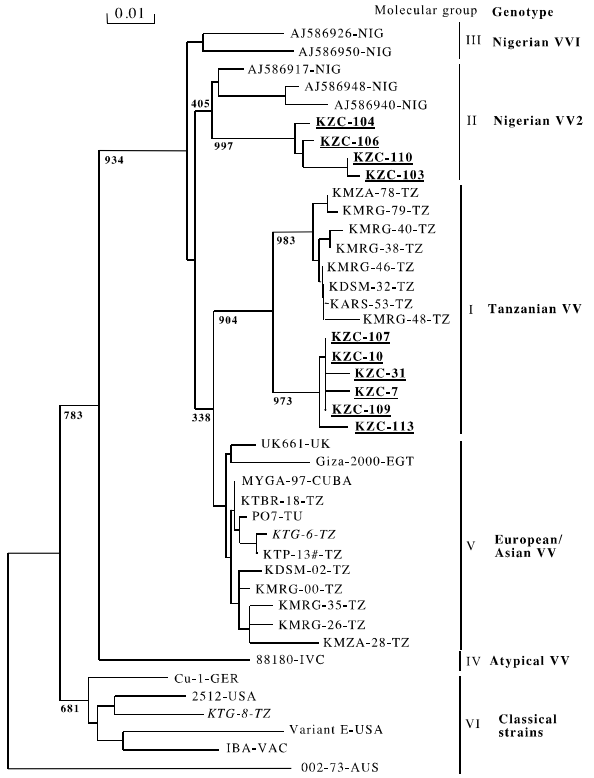

Fig. 2: Phylogenetic tree calculated by Neighbour-Joining (NJ) method based on the hypervariable domain of VP2 capsid gene of IBDV serotype 1 strains using nucleotide alignment created in Clustal W version 1.8.3. VV: Very virulent genotype based on nucleotide sequences; I-VI: molecular groups based on restriction enzyme analysis; Isolates from Zambia are in underlined boldface; Isolates detected in guinea fowls from Tanzania are in italics; Isolates detected in pigeon from Tanzania is marked with # sign; and numbers at forks represent bootstrap values. Source: Kasanga et al. (2013).

as I, II, III, IV, V, and VI, and were comprised of the Tanzanian VV, Nigerian VV1, Nigerian VV2, Very virulent atypical strain (Ivory coast 88180 VVA accession number: AJ001941), European/Asian VV, and classical strains, respectively. The specific restriction patterns corresponding to each of the molecular groups are shown in Table 1.

Molecular groups I-IV consisted of strains derived from the African continent, whereas strains in the molecular groups V and VI were derived from many parts of the world including Africa. Molecular groups I, II and III, were comprised of the African VV-IBDV strains (Kasanga et al., 2007). Restriction pattern(s) in the molecular group I was only observed in Tanzanian VV strains, whereas restriction patterns in molecular groups II and III were specifically observed in Nigerian VV-IBDVs. Restriction patterns in molecular group VI could further subdivide the classical strains into classical virulent and classical attenuated/mild/ intermediate subtypes based on the presence and absence of Aat I sites, respectively.

It was found that (a) Ssp I was necessary to differentiate molecular group I from II and IV, (b) Aat I was necessary to differentiate molecular groups I and II, from IV and V, (c) Sac I was necessary to differentiate molecular group VI from other molecular groups, I-V, (d) Nco I was necessary to differentiate molecular group III from I and V, and (e) Bsp MI was necessary to differentiate molecular group III from IV. It was further observed that the use of Ban III during molecular typing depended on the presence or the absence of sites for the other 5 restriction enzymes.

Restriction analysis by restriction enzyme digestion

The cDNAs of 5 African VV, 4 European/Asian VV and 2 classical strains available in the author's laboratory were used for actual enzyme digestion with six restriction enzymes and revealed the same patterns as that of the predicted restriction analysis (Table 1). The summary of the actual enzyme digestion for each strain is shown in Table 2, whereas the photographs displaying sizes of DNA fragments digested with three representative enzymes are shown in Fig. 1.

As expected, all five African VV (Tanzanian VV) and four European VV were digested with Ban III, while classical strains were not digested. Aat I digested

all four European VV, whereas Tanzanian VV and classical strains were not digested. Sac I digested classical strains, whereas Tanzanian VV and European VV were not digested (Fig. 1). Ssp I digested three European VV strains and did not digest all Tanzanian VV, classical strains and one of the European VV. All tested strains were not digested with Nco I, whereas Bsp MI digested all strains except 2 classical strains and one Tanzanian VV strain (figure not shown).

Molecular typing of the field strains

Forty IBDV positive samples (RT-PCR positive for VP2-HVR) detected in chickens from Africa (20 in Zambia and 20 in Tanzania) were analyzed by restriction fragment length polymorphism (RFLP) using six enzymes, Aat I, Ban III, Bsp MI, Ssp I, Sac I and Nco I. Out of twenty samples in Tanzania, 5 samples were typed into molecular group VI (classical attenuated/mild/intermediate), 3 samples into molecular group VI (classical virulent), and 12 samples into molecular group I (Tanzanian VV type) (Table 3a). On the other hand, 1 sample in Zambia was typed into molecular group VI (classical attenuated/mild/ intermediate), 13 samples into molecular group I (Tanzanian VV), and 5 samples into molecular group II (Nigerian VV1) (Table 3b.). All IBDV field strains in molecular group I in Tanzania were detected in indigenous local chickens, whereas strains in molecular group I in Zambia were detected in both indigenous local chickens and broiler chickens. On the other hand, field strains in molecular group II were detected in broiler chickens, whereas molecular group VI strains were detected in broiler chickens in Tanzania and Zambia (Table 3a and b).

Relationship between molecular groups and phylogenetic clustering

The cDNA sequences of 10 field strains detected in Zambia were 434 bp in size, and had no deletion(s) or insertion(s). All sequences of representative strains in molecular group I (KZC-107, KZC-10, KZC-31, KZC-7, KZC-109 and KZC-113) retained sites for restriction enzymes Ban III and Bsp MI, and none for Sac I, Aat I, Ssp I and Nco I. Similarly, sequences of strains in

Table 1: Restriction patterns for rapid typing of the field serotype 1 IBDV strains for epidemiological surveillance

IBDV serotype 1 molecular group	Restriction enzymes					
	Sac I	Aat I	Ban III	Ssp I	Bsp MI	Nco I
I: Tanzanian VV	-	-	+	-	+	-
II: Nigerian VV1	-	-	+	+	+	+/-
III: Nigerian VV2	-	+	-	+	+	+
IV: IVC 88180 (VVA)	-	+	-	+	-	+
V: European VV	-	+	+/-	+/-	+	-
VI: Classical strains (CV)	+	+/-	-	-	-	-

(+) = Positive for digestion with specific restriction enzyme;(-) = Negative for digestion with specific restriction enzyme; VV = very virulent; VVA= Atypical very virulent [4]; CV= Classical strains; (+/-): Imply that some strains contain sites for specific restriction site and some do not, but the patterns obtained are discrete for each molecular group based on data available in GenBank; I, II, III, IV, V, VI: Molecular groups corresponding to specific restriction patterns; Bold: Restriction pattern specific for Tanzanian very virulent strains; Fragment sizes of digested DNA sequences with specific enzymes at respective recognition sites are as follows:-Sac I: 340bp, 132bp (recognition site: GAG/CTC); Aat I: 316bp, 156bp (recognition site: AGG/CCT); Ban III: 376bp, 96bp (recognition site: ATC/GAT); Ssp I: 272bp, 200bp (recognition site: AAT/ATT); Bsp MI: 422bp, 50bp (recognition site: ACCTGC/GCAGGT); Nco I: 404bp, 68bp (recognition site: CCA/TGG). Note: All strains in molecular group 1 lack sites for Nco I, whereas all strains in molecular group III contains sites for Nco I at the same position, and hence group I and III can be differentiated with Nco I enzyme digestion apart from digestion with other five enzymes; All strains in group III, which contain sites for Aat I are also Ban III (+), Ssp I (-) and Nco I (+), whereas group III strains, which do not contain sites for Aat I are also Ban III (+), Ssp I (+) and Nco I (+); On the other hand, group II strains which are Ban III (+) are usually Nco I (-), and hence group II and group III strains can be differentiated based on restriction patterns described above.

Table 2: Restriction enzyme digestion of the tested IBDV strains

Virus strains	Sac I	Aat I	Ban III	Ssp I	Bsp MI	Nco I	Molecular group
KDSM-32	-	-	+	-	+	-	I
KMRG-40	-	-	+	-	+	-	I
KMRG-48	-	-	+	-	-	-	I
KARS-53	-	-	+	-	+	-	I
KMZA-78	-	-	+	-	+	-	I
KTBR-18	-	+	+	+	+	-	V
KMRG-26	-	+	+	+	+	-	V
KMZA-28	-	+	+	+	+	-	V
040802-2	-	+	+	-	+	-	V
GEB-10	+	-	-	-	-	-	VI
GBF-1	+	-	-	-	-	-	VI

+ = Digested with specific restriction enzyme; - = Not digested with specific restriction enzyme

Table 3: Restriction enzyme analysis of the positive field IBDV samples from Tanzania and Zambia

(a) IBDV isolates from Tanzania

Chicken source	Sample ID	Restriction enzyme						Molecular group of detected IBDV
		Sac I	Aat I	Ban III	Ssp I	Bsp MI	Nco I	
Broiler	48, 51, 52, 35, 38	+	-	-	-	-	-	VI: Classical attenuated
	46, 64, 65	+	+	-	-	-	-	VI: Classical virulent
Local	43, 55, 58, 59, 60, 62, 68, 83, 29,51,33, 67	-	-	+	-	+	-	I: Tanzanian VV

(b) IBDV isolates from Zambia

Chicken source	Sample ID	Restriction enzyme						Molecular group of detected IBDV
		Sac I	Aat I	Ban III	Ssp I	Bsp MI	Nco I	
Broiler	108	+	-	-	-	-	-	VI: Classical attenuated
	101, 102, 105, 107, 57, 69	-	-	+	-	+	-	I: Tanzanian VV
	103, 104, 106, 110, 111	-	-	+	+	+	-	II: Nigerian VV1
Local	7, 10, 31, 113, 8, 25, 12, 14	-	-	+	-	+	-	I: Tanzanian VV

(+): Positive for specific enzyme; (-): Negative for specific enzyme; I-VI: Designated molecular groups
Bold: Restriction pattern corresponding to Tanzanian very virulent strains

molecular group II (KZC-104, KZC-106, KZC-110 and KZC-103) contained sites for Ban III, Ssp I and Bsp MI, and none for Sac I, Aat I and Nco I. These observations corresponded with that of restriction patterns displayed after actual enzyme digestion in RFLP analysis (Table 3a and b).

Phylogenetic analysis of 10 sequenced strains and selected serotype 1 IBDVs showed a close phylogenetic relationship between the molecular groups created based on RFLP patterns and the clustering of strains in the phylogenetic tree (Fig. 2). The sequenced strains were clustered in the same groups with the strains that were categorized into specific groups based on the actual enzyme digestion.

Discussion

The detection and differentiation of different serotype 1 IBDV subtypes in the field are important for effective control of IBD by vaccination. A suitable method for typing of the field IBDV strains with appropriate restriction endonucleases was established. The predicted restriction patterns of some serotype 1 strains were confirmed experimentally. The identified patterns enabled categorization of the serotype 1 IBDVs into specific molecular groups. This permitted further the typing and differentiation of the newly emerged viruses.

The restriction enzyme analysis of the VP2-HVR gene revealed that six restriction enzymes were suitable for categorization of the serotype 1 IBDVs into six molecular groups (Table 1). Of these six molecular groups, five belonged to the very virulent genotype, whereas one group consisted of the classical strains, and belonged to the classical genotype. Other researchers (Lin et al., 1993, Zierenberg et al., 2000) proposed Bsp MI and Ssp I sites to characterize the VV-IBDVs. The current findings demonstrate the existence of VV-IBDVs in the field, which lack Bsp MI and Ssp I restriction sites (Fig. 1 and Table 2). Thus, it is important not to use only Ssp I and/or Bsp MI digestion for rapid epidemiological identification of the VV-IBDVs. Additional restriction enzymes should be used to identify and categorize the viruses into specific molecular groups and genotypes. The author recommends that the restriction patterns determined in this study, which involves actual digestion with six restriction enzymes namely, Aat I, Ban III, Bsp MI, Ssp I, Sac I and Nco I, could be used for rapid identification of the serotype 1 viruses during epidemiological surveillance of the field strains.

Recently, the author discovered the existence of the African VV-IBDV variants in Tanzania, which are genetically different from the European/Asian VV-IBDVs (Kasanga et al., 2007). Restriction enzyme analysis conducted in the current study revealed a unique restriction pattern for the Tanzanian VV strains, which were categorized into molecular group I (Table 1). These findings imply that the unique pattern displayed by strains in the molecular group I can be used for the rapid identification and monitoring the spread of the Tanzanian VV strains to other countries.

The restriction patterns established herein were practically employed in genotyping the field strains detected in Tanzania and Zambia, and enabled the identification of serotype 1 viruses in molecular groups I and VI in Tanzania, and viruses in molecular groups I, II and VI in Zambia (Table 3a and b). Although the actual differentiation of the field strains may need sequencing data, the ability of the six restriction enzymes used in this study to categorize the IBDV field strains into specific molecular groups implies that the method has a practical use in identification and differentiation of the virus population circulating in a certain geographical area, at a given period of time.

The molecular groups of IBD viruses determined in this study (Table 1) corresponded with the virus

genotypes that were categorized based on the nucleotide sequences grouping specific molecular groups into the same clusters with specific genotypes in the phylogenetic tree (Fig. 2). The sequenced isolates detected in Tanzania (Kasanga et al., 2007) and Zambia (Kasanga et al., 2013), which were categorized in molecular group I (Table 3a and b), conserved the unique amino acids that could not be retained by other serotype 1 strains (data not shown). These observations indicate that restriction enzyme analysis using six restriction endonucleases and six restriction patterns established in this study can be useful for genotyping the serotype 1 IBDVs in endemic areas where sequencing may not be easily performed. The rapid identification and genotyping of the field viruses provides necessary information for selection of the vaccine candidates that could be used in preparation of appropriate vaccine for control of IBD in particular geographic areas where the disease is endemic and different IBDV pathotypes exist.

Conclusion

In this study, a practical molecular typing method of serotype 1 IBDVs by using restriction enzyme analysis was determined. This method may be useful for typing the field strains during epidemiological surveillance, which could be needed for rapid identification and characterization of the prevailing strains and can contribute to the development of appropriate and effective control method(s) of IBD in endemic settings.

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