Research article

Coding sequences of both genome segments and virulence phenotype of the African very virulent infectious bursal disease virus strain KMRG-48

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Abstract

Little is known on the molecular characteristics of genome segments A and B of the African very virulent (VV) infectious bursal disease (IBD) virus (VV-IBDV) variant strains. In this study, the nucleotide sequence of genome segments A and B encoding VP5, precursor polyprotein (NH_2-VP2-VP4-VP3-COOH) and VP1 for an African very virulent IBDV, KMRG-48 strain, was determined. The VP5, precursor polyprotein and VP1 coding regions of KMRG-48 consisted of 437 nucleotides (145 deduced amino acids), 3036 nucleotides (1012 deduced amino acids) and 2637 nucleotides (879 deduced amino acids), respectively. Comparison of deduced amino acid sequences of serotype 1 IBDVs revealed 6 unique amino acid residues at positions 223 (S), 296 (F), 343 (S), 384 (I), 721 (T) and 960 (D) in precursor polyprotein. KMRG-48 also conserved 18 amino acid residues, which are found only in VV-IBDVs. Of these unique amino acids, 3 were in VP5, 4 were in VP2, 2 were in VP4, 3 were in VP3 and 6 were in VP1. Phylogenetic analysis based on nucleotide sequences of VP5, precursor polyprotein, VP2, VP4, VP3 and VP1 revealed that KMRG-48 belonged to the VV genotype. The tree topologies obtained for the nucleotide sequences of VP5, precursor polyprotein, VP2, VP4 and VP3 were the same while the tree topology for the nucleotide sequence of VP1 was quite different compared with that obtained from nucleotide sequences of VP5, precursor polyprotein, VP2, VP4 and VP3, suggesting a possibility of genetic reassortment to contribute in the emergence of VV-IBDV. Experimental infection of susceptible chickens with KMRG-48 confirmed that the virus is a VV-IBDV exhibiting VV phenotype. Taken together, these findings demonstrate that KMRG-48 is a VV-IBDV whose genome segments A and B are derived from VV-IBDV, and that the conserved unique amino acids could influence its virulence and lethality.

Keywords: Sequencing, African VV-IBDV, virulence, characterization, KMRG-48

Introduction

Infectious bursal disease (IBD) virus (IBDV) is a double-stranded RNA virus which is the causative agent of IBD, an acute viral disease of young chickens (Cosgrove, 1962). IBDV was first discovered in 1962 in the Gumboro area of Southern Delaware, USA. The virus causes destruction of lymphoid organs, especially

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the bursal of Fabricius (Saif, 1991), resulting in immunosuppression (Hirai et al., 1974; Hirai et al., 1979).

IBDV is classified in the genus *Avibirnavirus* of the family *Birnaviridae* (Dobos et al., 1979). Two distinct serotypes of IBDV (1 and 2) exist. Serotype 1 strain differs markedly in virulence, whereas all known serotype 2 viruses are naturally avirulent for chickens (Ismail et al., 1988). The virus has a bisegmented, double stranded RNA genome, approximately 3.4 kb (segment A) and 2.7 kb (segment B) in length contained within a non-enveloped icosahedral capsid (Muller et al., 1979).

Until late 1980's, most of the IBDV infections were subclinical, causing impaired growth and acquired immunodeficiency (classical IBD), and IBD was well controlled by the use of a range of live attenuated and killed commercial vaccines (Wyeth and Chettle, 1990; Wyeth et al., 1992). However, in 1987 a very virulent strain of serotype 1 IBDV (VV-IBDV) emerged in the Netherlands and Belgium (Chettle et al., 1989; van den Berg et al., 2000). The VV-IBDV, in contrast, caused high mortality not only in young chickens, but in older birds as well (van den Berg et al., 2004). The VV-IBDV has become a worldwide economically important pathogen of chicken industry. Of recent, Kasanga et al (2007) reported the existence of African VV-IBDV variants in Tanzania, which are different from the European VV-IBDV. However, the origin and molecular basis of high pathogenicity of both types, the African and European VV-IBDVs, has not yet been clearly defined.

Presently, there are few data in databases on the full-length genome segments A and B sequences of IBDVs derived from Africa. Only a few reports exist, which describe the full-length genome of IBDV isolated in Nigeria (Owoade et al., 2004). Determination of the complete genome sequence of the recently emerged African VV-IBDV variants are a prerequisite to identifying determinants for its enhanced virulence and origin. The information obtained should facilitate in the design of the rational control method of the African VV-IBDV variants, and on the production of specific diagnostic tools for discrimination of the African VV-IBDVs from other IBDV genotypes and pathotypes.

In this study, cDNA sequences of the coding regions for both genome segments of a Tanzanian very virulent strain, KMRG-48, coding for VP5, precursor polyprotein and VP1 were determined. The nucleotide and deduced amino acid sequences of KMRG-48 strain were compared to the published sequences of any other serotype 1 and 2 strains to identify genetic determinants of its very virulent nature. By comparing the deduced amino acid sequences of VV-IBDV with other serotype 1 and 2 sequences, the unique amino acids for KMRG-

48, and putative amino acid residues involved in the virulence were identified. In addition, by constructing the phylogenetic tree based on nucleotide sequences, the phylogenetic relationship among the very virulent strains and other classical virulent strains was analyzed to determine the origin of the African VV-IBDVs. Furthermore, the virulence nature of the African VV-IBDV was demonstrated in susceptible birds after experimental infection with KMRG-48 strain.

Materials and Methods

IBDV strains

A Tanzanian VV-IBDV, KMRG-48 strain (Kasanga et al., 2007), was used in this study. The virus was isolated in the author's laboratory in Tanzania from sick bursa in broiler chicken obtained from a confirmed IBD field outbreak, which occurred in March 2002 in Morogoro region, Tanzania. Other serotype 1 and 2 strains were used for comparison purpose during the sequence and phylogenetic analysis of nucleotide and deduced amino acids of KMRG-48. Description of viruses used in this study is shown in Table 1.

Amplification and cloning of segments A and B cDNAs

The genomic RNA of segments A and B of KMRG-48 strain were copied into cDNA, amplified by polymerase chain reaction (PCR) in five and three overlapping fragments representing the entire reading frames of segments A and B, respectively. PCR fragments were constructed with sufficient overlap of adjacent fragments such that there were no primer-masked regions (Fig. 1). The first-stranded cDNA was synthesized by Random hexamer, $Pd(N)_6$ (Toyobo, Osaka, Japan), and Sixteen oligonucleotide primers were used for PCR of the VP5, precursor polyprotein and VP1 coding regions. The primers used in PCR are listed in Table 2. The amplified fragments were gel-purified using a Nucleospin DNA purification kit (Macherey-Nagel Inc., Easton PA, USA) in accordance with the manufacturer's instructions and cloned into pGEM-T-Easy vector (Promega, Tokyo, Japan). Clones containing DNA inserts were selected as previously described (Kim and Yeo, 2003).

Sequencing of cDNA clones

Because of the possibility of errors arising during PCR, five independent clones for each fragment were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977).

Sequence assembly, genetic and phylogenetic analysis

The nucleotide and deduced amino acid sequences of KMRG-48 strain were compared to the published sequences of IBDV. *In-silico* analysis of sequencing

Table 1: Description of IBDV strains used in this study

Serotype	Strains	Geographic origins	Year isolated	References	Accession numbers	
					Precursor polyprotein	VP1
1	KMRG-48	Tanzania	2002	(Kasanga et al., 2007)	AB368970	AB368971
	T09	Nigeria	1998	(Owoade et al., 2004)	AY099456	AY099457
	OKYM	Japan	1991	(Yamaguchi et al., 1997)	D49706	D49707
	UK661	UK	1989	(Brown and Skinner, 1996)	X92760	X92761
	Cu-1	Germany	1976[A]	(Nick et al., 1976)	X16107	AF362772
	CEF94	Netherlands	1999[A]	(Boot et al., 1999)	AF194428	AF194429
	Variant E	USA	1999[A]	(Akin et al., 1999)	AF133904	AF133905
	002-73	Australia	1974	(Firth, 1974)	X03993	M19336
2	OH	USA	1996[A]	(Kibenge et al., 1996)	U30818	U30819

[A]: Year reported

Table 2: Oligonucleotides used for construction of the full-length cDNA clones

Primer	Position	Segment	Orientation	Sequence
A3F	3 - 25	A	Sense (+)	TGA TGG TTA GTA GAG ATC AGA C
A2	859 - 882	A	Antisense (-)	GCT TGT TTG AAA CAC GAG TTC TCC
A3	587 - 608	A	Sense (+)	ACA AAA TCG GGA ACG TCC TAG T
A4	1393 - 1414	A	Antisense (-)	CCT CCA TGA AGT ACT CGC GAA A
A5	1200 - 1222	A	Sense (+)	GGC AAC AGG ATC TGT CGT TAC GG
A6	2066 - 2087	A	Antisense (-)	CCG CTG CTT CCC ACA ATA GGA G
A7	1582 - 1601	A	Sense (+)	GCA CAG GCT GCT TCA GGA AC
A8	2882 - 2904	A	Antisense (-)	AGC TGC CCT TAG GAT TTG TCC TT
A9	2363 - 2382	A	Sense (+)	ATG CAG GAC GCC AGT ACG AC
A10	3143 - 3167	A	Antisense (-)	CAC TCA AGG TCC TCA TCA GAG ACA G
BF1	1 - 22	B	Sense (+)	CCT CTT CTT GAT GAT TCT ACC A
BR1	1021 - 1040	B	Antisense (-)	GAC CAT ATG TTA CGG GTC TT
BF2	1027 - 1049	B	Sense (+)	CGT AAC ATA TGG TCA GCT CCA TC
BR2	1869 - 1893	B	Antisense (-)	GTA CCT GAC AAC TTT GTA TGC TTG
BF3	1801 - 1824	B	Sense (+)	TTA TTT TGC TCT GCT GCG TAT CCC
BR3	2674 - 2696	B	Antisense (-)	AGT GTC CTC TTC TGG AGT GGT TC

data was accomplished using GENETYX software version 14.0.1 (Software Development Co., Tokyo, Japan).

Prior to the phylogenetic analysis, nucleic acid sequences were aligned using CLUSTAL W (Thompson et al., 1997). 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).

Evaluation of virulence and pathogenicity of the African VV-IBDV

To assess phenotypic characteristics of the African VV-IBDVs, the Tanzanian VV-IBDV, KMRG-48 strain, was used for experimental infection of susceptible chickens. The virus-infected bursa were homogenized as 40% (w/v) suspension in sterile saline (pH = 7.04) and subsequently clarified by centrifugation for 10 minutes at 2000 x g. The resulted supernatant was used as the virus stock.

Experimental chickens used in this study were seronegative by ELISA test against serotype 1 IBDV prior to infection with KMRG-48 virus. A group of fifteen, four-week-old, exotic broiler and Tanzanian indigenous local chickens each, both types produced and kept as specific pathogen free (SPF) chickens, were orally infected with 0.1ml of $10^{4.5}$ 50% embryo lethal dose (ELD_{50}) of KMRG-48 virus. Fifteen age-matched birds of each breed (exotic broiler and Tanzanian indigenous local chickens) served as uninfected controls. The infected birds were monitored for 3 weeks. On day 3, one bird from each group was randomly selected, killed and examined for evidence of IBDV pathogenicity. IBDV infection was confirmed by virus isolation from homogenized bursa and by histopathology.

Results

Sequence analyses of segment A (VP5, and precursor polyprotein (NH$_2$-VP2-VP4-VP3-COOH))

The sequences were derived from five independent clones for each cDNA fragment. No differences were observed between the clones. The full-length genome segment A consisted of 3,074 nucleotides (nt), of which 437 nt (145 deduced amino acids) were found on the small ORF coding for VP5, whereas 3,036 nt (1,012 deduced amino acids) were located on the large ORF encoding for the precursor polyprotein. The sequence reported here spans the hypervariable domain (VP2-HVR) that was previously reported for KMRG-48

(Kasanga et al., 2007), and the relevant sequence is identical to that reported previously, even though both were derived from cDNA amplified independently using different primers. On BLAST search, the full-length genome sequence showed 98% (2,996/3,049) closest nucleotide homology to the VV-IBDV strain, KS, which was originally isolated in Israel.

Sequence analyses of VP5

The deduced amino acid sequence of the VP5 of the KMRG-48 strain was aligned with other VP5 sequences available in GenBank. Comparison of the VP5 of KMRG-48 with that of any other serotype 1 strains indicated the minimum amino acid divergence of 0.7% between KMRG-48 and either OKYM, UK661, or T09. The maximum amino acid substitution observed between KMRG-48 and any other classical serotype 1 strain (002-73) was 17 (11.72%). There was no any unique amino acid residue found only in KMRG-48 strain when compared with other serotype 1 strains. The KMRG-48 strain conserved the same amino acid substitutions with that of very virulent strains (OKYM, UK661 and T09) at positions 45 (R), 125 (P) and 133 (W), as compared to the deduced amino acids of the other classical serotype 1 strains (figure not shown).

Sequence analyses of NH$_2$-VP2-VP4-VP3-COOH

The deduced amino acid sequence of the precursor polyprotein of the KMRG-48 strain was aligned with other polyprotein sequences available in GenBank. Comparison of the precursor polyprotein of the KMRG-48 strain with that of any other serotype 1 strains demonstrated 6 unique amino acid residues, which were found only in the KMRG-48 strain at positions 223 (S), 296 (F), 343 (S), 384 (I), 721 (T) and 960 (D). Among the 6 unique amino acid differences, 4 (223, 296, 343 and 384) were in VP2, 1 was in VP4, and 1 was in VP3. The KMRG-48 strain conserved the same amino acid substitutions with very virulent strains (OKYM, UK661 and T09) at positions 222 (A), 242 (I), 256 (I), 279 (D), 294 (I), 299 (S), 451 (L), 685 (S), 715 (S), 751 (D), 990 (V) and 1005 (A), as compared to the deduced amino acids of the other classical serotype 1 strains. The maximum amino acid substitution of precursor polyprotein observed between KMRG-48 and any other classical serotype 1 and antigenic variant strain (variant E) was 42 (4.15%). The minimum amino acid divergence within precursor polyprotein was observed between strains KMRG-48 and T09 (1.09%). The minimum amino acid divergence within the coding regions of VP2, VP4 and VP3 between KMRG-48 and any other classical serotype 1 IBDV included 0.60% (OKYM, UK661 and T09), 0.20% (OKYM and T09) and 0.30% (UK661 and T09), respectively. The amino acid divergences between the African very virulent

KMRG-48 and European very virulent UK661 strain within precursor polyprotein, VP2, VP4 and VP3 were 0.60%, 0.30% and 0.30%, respectively. On the other hand, the amino acid divergences between the African very virulent KMRG-48 and Japanese very virulent OKYM strain within precursor polyprotein, VP2, VP4 and VP3 were 0.60%, 0.20% and 0.30%, respectively.

Sequence analyses of segment B (VP1)

The sequences were derived from four to five independent clones for each cDNA fragment, and no differences were observed between the clones. The sequenced full-length genome segment B of the very virulent KMRG-48 strain consisted of 2,651 nt. On BLAST search, the full-length genome sequence showed 98% (2,619/2,651) closest nucleotide homology to the VV strain D6948, which was isolated in the Netherlands in 1989 (Boot et al., 2001). The VP1 coding region of KMRG-48 consisted of 2,637 nt (879 deduced amino acids).

The deduced amino acid sequence of the VP1 of the KMRG-48 strain was aligned with other VP1 sequences available in GenBank database. Eight unique amino acid residues were found only in the VP1 of the KMRG-48 strain when compared to non-very virulent strains of Cu-1, CEF94, Variant E, 002-73 and OH (figure not shown). Of these 8 unique amino acid residues, 7 at positions 146 (D), 147 (N), 242 (E), 390 (M), 562 (P), 687 (P) and 695 (R) were commonly found in the VP1 sequences of very virulent strains of OKYM, UK661 and T09. Among the very virulent strains, the unique amino acid at position 393 (D) in KMRG-48 was commonly found in VP1 sequence of UK661 but not in the VP1 sequences of OKYM and T09 with amino acid residues of 393 (V) and 393 (E), respectively. All 8 unique amino acid residues were located in the external regions of proposed RNA dependent RNA polymerase (RdRp) motifs and GTP binding sites (Shwed et al., 2002).

The amino acid sequence of VP1 from KMRG-48 strain showed divergences of 0.60%, 0.91% and 1.48% when compared with VP1 sequences from very virulent strains of T09, UK661 and OKYM, respectively. Conversely, the amino acid divergence between very virulent KMRG-48 and classical serotype 1 strains ranged from 1.93% to 5.12% when compared with VP1 sequences from CEF94 and 002-73, respectively.

Phylogenetic analyses

The phylogenetic trees were constructed by the NJ method to examine evolutionary relationship of very virulent strains, classical serotype 1 IBDV and serotype 2 strain, using 9 nucleotide sequences (Table 2), coding for VP5, precursor polyprotein, VP2, VP4, VP3 and VP1. The phylogenetic trees are shown in Fig. 2 (A-G).
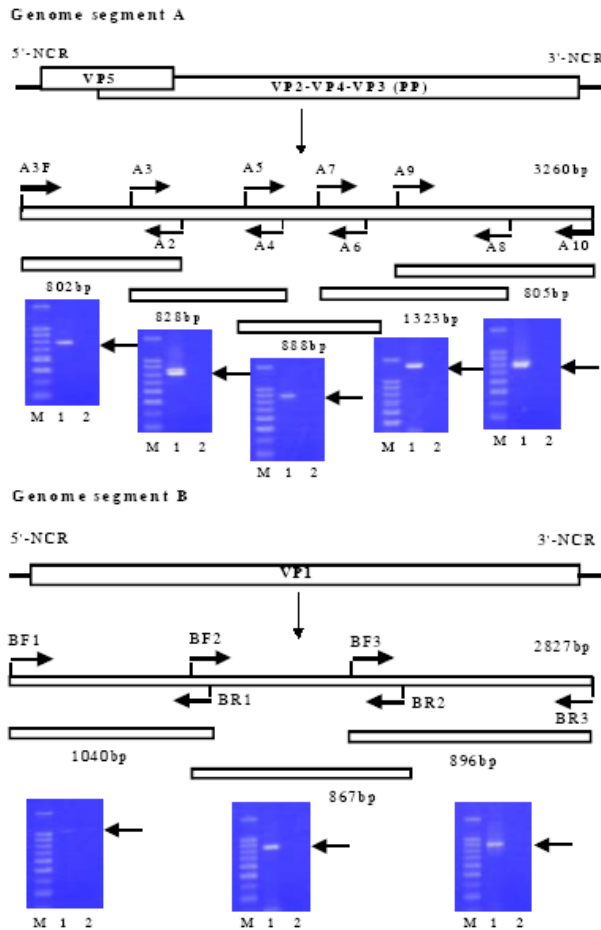
Fig. 1: Schematic representation of the locations of oligonucleotide primers, and the corresponding sizes of amplified DNA fragments used for cloning and sequencing of the full-length genome segments A and B of VV KMRG-48 strain. Arrows indicate position and orientation of oligonucleotides. Arrows adjacent to the electrophotograph indicate specific DNA fragment amplified during PCR using specific pair of primers. M: 100bp DNA ladder; 1: KMRG-48 strain, and 2: Distilled water (negative control).

The overall branching of trees constructed for the nucleotide sequences coding for VP5, precursor polyprotein, VP2, VP4 and VP3 were similar to each other (Fig. 2A, B, C, D and E). The nine strains used in this study were split into four distinct branches comprised of 1: very virulent strains KMRG-48, OKYM, UK661 and T09; 2: classical strains Cu-1, CEF94, and Variant E; 3: Australian classic strain 002-73; and 4: serotype 2, OH strain. The bootstrap values as determined by 1,000 replicates for very virulent strains were extremely high and ranged from 931 in Fig. 2A to 1,000 in Fig. 2E. There were slight differences on the branching patterns of very virulent strains in Fig. 2A, B, C, D and E, displaying genetic differences in evolutionary pathways. The branch consisting of very virulent strains was closely related to

the classical serotype 1 IBDVs. Each branch of Australian classic serotype 1 strain 002-73 and serotype 2, OH strain, was distinct from the branch of classical serotype 1 strains.

The phylogenetic tree constructed from the nucleotide sequences coding for VP1 (Fig. 2F, and G) showed that the VV-IBDVs formed one group, which was distinct from any other groups. The tree topology was quite different from that obtained from the nucleotide sequences of VP5, a precursor polyprotein, VP2, VP4 and VP3. The Tanzanian VV-IBDV strain KMRG-48 clustered with other VV strains of T09, OKYM and UK661, while the classic Australian strain 002-73, serotype 2 strain OH, classical serotype 1 strains (Cu-1 and CEF94), and Variant E strain formed another lineage. The bootstrap values (1,000) showed very high divergences for very virulent strains, classic Australian strain 002-73, serotype 2 strain OH, classical serotype 1 strains Cu-1 and CEF94, and Variant E strain in Fig. 2F. The branches of nine strains comprising the tree in Fig. 2F, were at least 3, which included: 1: VV-IBDV, KMRG-48, T09, OKYM and UK661; 2: Australian classic strain 002-73, and 3: serotype 2 strain OH, classical serotype 1 strains (Cu-1 and CEF94), and Variant E strain. Serotype 2 strain OH and classical serotype 1 strains (Cu-1 and CEF94), and Variant E strain formed one cluster regardless of their serotype, year of isolation or geographic location. When the tree topology of Fig. 2F was compared by including infectious pancreatic necrosis virus strains NV1-015 (GenBank accession: AY379741) and Jasper (GenBank accession: M58756), a similar trend of branching was obtained (Fig. 2G).

Pathogenicity of KMRG-48 strain

The first clinical signs of IBD in SPF chickens inoculated with strain KMRG-48 appeared about 36 hr post inoculation (PI) in broilers, and at about 48 hr PI in indigenous chickens (Table 3). Clinical signs typical of IBD such as acute onset of depression, disinclination to move, pecking at vents and elevated water consumption, were observed in both broilers and indigenous chickens, with a higher severity in broilers. Mortality in broilers (40%) was higher than that of indigenous chickens (13%). Virus isolation and gross-histopathological changes typical of VV-IBDV infection, such as rapid and profound bursa atrophy and lymphocyte depletion, were observed in infected broilers and indigenous chickens. However, among chickens that died on the same day PI, lymphocyte depletion and bursa atrophy were more severe in broilers than in indigenous chickens. No IBD clinical manifestations or histopathological lesions were observed in, and no virus was isolated from, the uninfected control chickens.
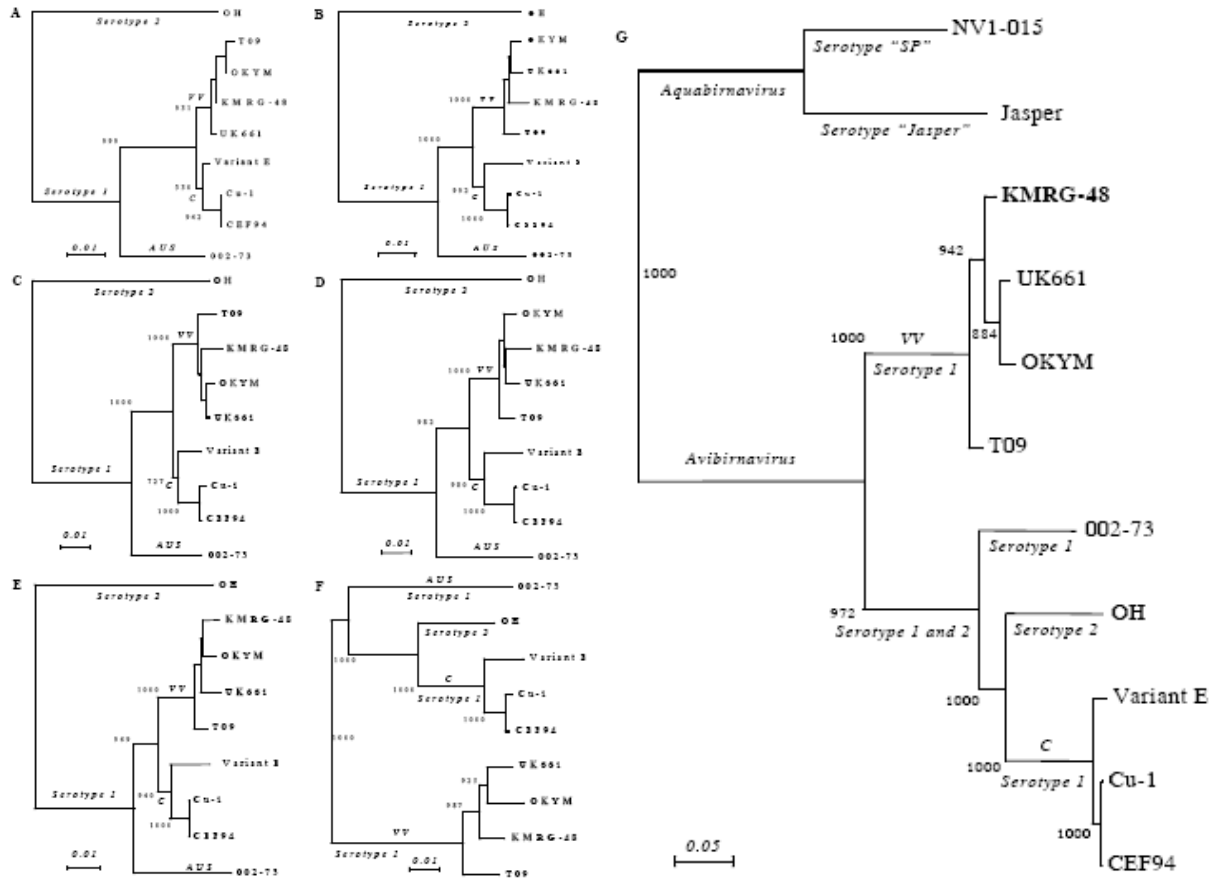
Fig. 2: Phylogenetic trees based on the nucleotide sequence encoding (A) VP5, (B) precursor polyprotein, (C) VP2, (D) VP4, (E) VP3 and (F) VP1 of very virulent strains KMRG-48, OKYM, UK661 and T09, non-very virulent serotype 1 strains Cu-1, CEF94, Variant E and 002-73, and serotype 2 strain OH. Figure 2 (G) represents topological comparison of VP1 nucleotide sequences of strains in (F) by including aquabirnaviruses, NV1-015 (Santi et al., 2004) and Jasper (Duncan et al., 1991) as an out-group. 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; C: classic strains including virulent, attenuated and antigenic variants; AUS: Australian classic strain.

Discussion

The nucleotide sequences of the Tanzanian VV-IBDV segment A and B coding for VP5, precursor polyprotein and VP1 were determined. Comparison of deduced amino acid sequences of VV-IBDV, classical serotype 1 and serotype 2 strains allowed the identification of unique amino acid residues in KMRG-48 and VV-IBDVs, which are responsible for strain virulence. Phylogenetic analysis clarified further the relationship of very virulent and classical IBDV strains. Experimental infection of susceptible chickens with the virus allowed the confirmation of KMRG-48 as a very virulent strain.

The comparison of deduced amino acid sequences of VP5 and precursor polyprotein of KMRG-48 with that of any other classical serotype 1 strains revealed 3 and 12 unique amino acids, respectively, which were commonly conserved in very virulent strains. The Tanzanian KMRG-48 strain conserved the same amino acid substitutions with VV-IBDVs. These observations suggest that the VP5 and polyprotein of KMRG-48 are genetically related to that of VV genotype, and that the unique amino acids in both VP5 and precursor polyprotein may be important in determining the very virulent phenotype.

Six unique amino acid residues in the precursor polyprotein of KMRG-48 strain, at positions 223 (S), 296 (F), 343 (S), 384 (I), 721 (T) and 960 (D), which are not found in any other serotype 1 strains were identified. The amino acid residues at positions 223 (S), 296 (F) and 343 (S) were located in the VP2-HVR, whereas amino acid at position 721 (T) was found in the putative VP4 upstream to the VP4-VP3 cleavage site. VP2-HVR is known to be important for the antigenicity of IBDV, and contains neutralizing epitopes responsible for virus neutralization (Eterradossi et al., 1997&1998). The amino acid changes at position 223 have been reported to be

critical for the antigenicity of IBDV (Schnitzler et al., 1993). Thus, the amino acid differences observed in the VP2-HVR imply that KMRG-48 is antigenically different from other serotype 1 strains.

The amino acid substitutions at flanking sequences of VP4-VP3 cleavage sites have been reported to influence the efficiency of processing and assembly of the virus particle (Sanchez and Rodriguez, 1999). The observation of the unique amino acid at position 721 (T) upstream to the VP4-VP3 cleavage site could contribute to the viral pathogenicity of KMRG-48 and influence the biological characteristics of KMRG-48 as compared to other VV-IBDVs.

The amino acids from position 997 to 1012 in the C-terminus of VP3 are known to be essential for interaction of VP3 to VP1 (Maraver et al., 2003a &b), and can influence virus replication and capsid formation (Shwed et al., 2002). The substitution of deduced amino acid at position 1005 (A→T) in VV-IBDVsas compared to non-VV-IBDVs could contribute to differences in VP1 binding activity and virus replication, and hence contribute to the phenotype differences between very virulent and non-very virulent classical serotype 1 strains.

Comparison of deduced amino acid sequences of KMRG-48, OKYM, UK661 and T09 in VP2, VP4 and VP3 showed low variation of 0.60%, 0.20% and 0.30%, respectively. These observations indicate that the segment A of VV-IBDVs could be originated from a common ancestor and undergo independent evolution in different ecological systems. Furthermore, of the three proteins, VP2, VP4 and VP3, variation were higher in VP2 as previously reported by other researchers (Le Nouen et al., 2006; Le Nouen et al., 2005; Yamaguchi et al., 1997), suggesting that amino acid variations in VP2, which spans the VP2-HVR, could best represent the genome segment A of VV-IBDV.

Comparison of deduced amino acid sequences of VP1 indicated that KMRG-48 strain conserved the same unique amino acids with that of VV-IBDVs. This observation implies that the VP1 of KMRG-48 could be related to that of VV strains, and that the genome segment B of KMRG-48 is derived from VV-IBDV strain.

Phylogenetic analysis based on trees constructed from nucleotide sequences encoding VP5, precursor polyprotein, VP2, VP4, VP3 and VP1 (Fig. 2 A-G) indicated that the Tanzanian KMRG-48 strain clustered with VV-IBDV strains T09, OKYM and UK661. The phylogenetic data clearly confirmed that the Tanzanian KMRG-48 strain belonged to the very virulent genotype.

In the phylogenetic tree constructed from nucleotide sequences encoding VP5, precursor polyprotein, VP2, VP4 and VP3 (Fig. 2 A-E) indicated that the very virulent genotype was clearly segregated from genotypes of classical serotype 1 strains,

Australian classic 002-73 and serotype 2 strain OH. Recent studies conducted on the evolution of Varicella-zoster virus (VZV) revealed that recombination during the genesis of isolates results in partial segregation of genotypes and branching inconsistency in gene trees (Norberg et al., 2006). The findings of the current study of phylogenetic tree based on precursor polyprotein were consistent with a previous study by Yamaguchi et al. (1997). This suggests that genetic recombination did not play a role in the emergence of KMRG-48 and other VV-IBDVs.

In the phylogenetic tree constructed from nucleotide sequences encoding VP2 (Fig. 2C), the Tanzanian KMRG-48 was genetically closely related to the T09 strain than UK661 and OKYM within the cluster of VV strains. The close phylogenetic relationship of the Tanzanian KMRG-48 strain to the T09 strain, which was isolated from turkey in Nigeria in the western Africa, than UK661 isolated in Europe and OKYM isolated in Japan, suggests that KMRG-48 was derived from Africa. This observation supports the previous findings (Kasanga et al., 2007), which categorized KMRG-48 strain into the African VV-IBDV based on sequence analysis of the hypervariable domain.

Phylogenetic trees constructed from nucleotide sequences of VP1 also showed that the VV-IBDVs formed one group. The cluster of VV-IBDV was distinct from that of Australian classic 002-73, Serotype 2, classical serotype 1 (Cu-1 and CEF94), and Variant E. There was inconsistency branching when using the coding sequences of precursor polyprotein and VP1 (Fig. 2B and F) for the same strains. The inconsistency branching was clearly shown after using *aquabirnavirus* as an out-group for the topology of a tree constructed using coding sequences of VP1 (Fig. 2G). These inconsistency branching patterns could probably reflect the possibilities of reassortment events in the genesis of some IBDV strains.

Experimental infection of susceptible indigenous and exotic broiler chickens with strain KMRG-48 virus revealed IBD clinical manifestations typically found in VV-IBDV natural infections (Table 3). The high mortality (up to 40%) with profound bursa atrophy and severe lymphocyte depletion in experimentally infected chickens observed in this study conform to the pathogenicity of some previously reported VV variant strains (Lasher and Shane, 1994). These findings, together with the unique amino acid substitutions, and phylogenetic analyses, confirmed that the Tanzanian KMRG-48 strain belongs to the very virulent phenotype. However, the observed differences in clinical manifestation and mortality between chicken ecotypes could suggest that susceptibility and patho-genicity differences do exist among birds following infection with the same dose and type of VV-IBDV.

Table 3: Virus pathogenicity in four-week old SPF chickens infected with KMRG-48 IBDV strain

Observed pathogenicity index	Virus-challenged chickens		Non-challenged chickens	
	Tanzanian indigenous	Exotic broiler	Tanzanian indigenous	Exotic broiler
Incubation period	48hrs	36hrs	NA	NA
Clinical manifestations (morbidity)	9/15 (60%)	15/15 (100%)	0 (0%)	0 (0%)
Mortality	2/15 (13%)	6/15 (40%)	0 (0%)	0 (0%)
Virus isolation from bursa on 3, 5, 7, 9 days PI	+	+	-	-
Disease manifestation average score	1	2	0	0
Gross-histopathological lesions average score	1	2	0	0
Remission of clinical signs	19 days pi	13 days pi	NA	NA

NA: Not applicable; Pi: post inoculation/infection; (+): positive (virus isolated); (-): negative (no virus isolated)
Disease manifestation score: [0: no clinical signs (normal); 1: moderate (ruffled feathers, reduced motility); 2: severe (droopy wings and head, recumbence, trembling, white diarrhea) and 3: dead]
Gross-histopathological lesions score: [0: normal (no gross hemorrhage, normal bursa size, normal lymphocytes count and architecture); 1: moderate (slight gross hemorrhage, slight enlargement of bursa, moderate bursa lymphocyte depletion, normal architecture) and 2: severe (gross hemorrhage on thighs and keel muscles, bursa hypertrophy, bursa lymphocyte depletion and necrosis, disrupted lymphocyte architecture)]. Incubation period: Indicates the minimum time lapse between virus inoculation and development of IBD clinical signs in the most susceptible chicken between challenged groups.

Conclusion

The full-length genome segments A and B of a Tanzanian KMRG-48 strain were determined. Sequence, phylogenetic analysis and experimental infection of susceptible chicken with KMRG-48 virus confirmed its designation as a very virulent strain. A possibility of genome reassortment involvement in the evolution and emergence of new virus strains was speculated. Further investigation on natural genome reassortment of IBDV in Africa is recommended to define evolutionary pathways of emerging strains in endemic settings of Africa.

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