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Molecular characterization of infectious bursal disease virus detected in Morogoro, Tanzania

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SUMMARY

Infectious bursal disease (IBD) virus (IBDV) is a double-stranded RNA virus that belongs to the genus *Avibirnavirus* of the family *Birnaviridae*. IBDV is a causative agent of IBD, the highly contagious viral infection of young chickens aged 3 to 6 weeks. IBD outbreaks occur frequently in both vaccinated and non-vaccinated chickens in Tanzania causing significant economic loss among poultry keepers. The control of IBD is mainly done through vaccination, which requires the understanding of molecular and biological characteristics of circulating virus strains in particular geographic location. This study was conducted to determine the genotype of IBDV recovered from confirmed IBD outbreak(s) in 2014 in Morogoro, Tanzania. The investigation was performed by reverse-transcription polymerase chain reaction (RT-PCR), sequencing and phylogeny analysis of nucleotide sequences corresponding to the VP2 hypervariable (VP2-HVR) domain of IBDV. The findings indicated 100% detection rate (n = 10) of IBDV genome from infected bursa of Fabricius samples. Phylogenetic analysis revealed that the sequenced virus belonged to the African very virulent IBDV (VV-IBDV) genotype and was genetically closely related to KZC-109 strain detected in Zambia in 2004. Taken together, our findings suggest that the African VV-IBDV detected in this study was responsible for the IBD outbreak(s) in Morogoro. Further studies are required to examine the transmission dynamics, evolutionary characteristics and antigenicity of field IBDV strains order to design the appropriate control method(s) of IBD in Tanzania and neighboring countries.

Key words: IBDV, VV-IBDV, Sequencing, Phylogeny, Tanzania

Infectious bursal disease (IBD) or Gumboro disease is a highly contagious immunosuppressive viral infection of young chickens (3–6 weeks old) causing severe economic and production losses worldwide (Müller *et al*., 2003). The disease is highly contagious affecting young chickens and characterized by destruction of lymphoid organs and in particular the bursal of fabricius where B lymphocytes mature and differentiate. The cloacal bursa is the target organ of IBDV infections; however, IBD viral replication also occurs in other lymphoid structures including the spleen, thymus, Harderian gland, and ceca tonsil. The immunosuppressive effects of IBDV infections not only enhance the chicken's susceptibility to secondary opportunistic infections such as gangrenous dermatitis, chicken anemia agent, inclusion body hepatitis, respiratory diseases, and E. coli infections among others, but frequently interfere with effective immune responses to vaccination (Jacqueline, 2010).

The disease was initially recognized in 1957 as clinical entity responsible for acute morbidity and mortality in broilers on Delmarva peninsula (Hirai *et al*., 1972). Predominant signs of illness included trembling, ruffled feathers, watery diarrhea, anorexia, depression, severe prostration, and death. In addition, hemorrhages in the thigh and leg muscles, increased mucus in the intestine, liver lobe infarction, renal damage, and enlargement of the bursa of Fabricius were lesions commonly observed at necropsy (Cosgrove, 1962). Early studies suggested that the causative agent was a nephropathogenic strain of infectious bronchitis virus due to similar gross changes observed in the kidneys (Winterfield and Hitchner, 1962; Pejkovski *at al*., 1979; Winterfield *et al.*, 1978). However, revealed that IBV immunized birds could still be infected with the infectious bursal agent and develop changes in their cloacal bursas specific for the disease. Following successful isolation of IBA in embryonated chicken eggs (Winterfield *et al.*, 1962; Hitchner, 1970), proposed that the disease be termed infectious bursal disease due to its pathognomonic bursa lesions. The immunosuppressive effects of infectious bursal disease virus (IBDV) infections were first disclosed by Allan *et al.* (1972). In 1980, a second serotype was reported McFerran *et al*. (1980).

The condition spread rapidly and was recognized throughout the US broilers and commercial egg production areas by 1965, and by 1967 the highly infections nature of infectious bursal disease virus was recognized followed by reliable developments of methods to isolates the virus in embryonated eggs and to adapt it to tissue culture. In1976, the agent was characterized as a virus belonging to a new taxonomic group. The immunosuppressive property of IBDV was first recognized in and was confirmed in 1976.The first isolate of IBDV was isolated in

Gumboro, Delaware in USA in1965 (Winterfield, 1962) and from there spread worldwide causing greater economic lossesin poultry industry (Hirai *et al*., 1972). The immune suppression that results from an IBDV infection has a major economic impact on the broiler and layer chicken industries. Often the immune suppression goes unnoticed because the disease is sub-clinical in nature. Thus, the true economic impact of IBDV as the underlying cause of opportunistic respiratory and enteric diseases and vaccination failures is difficult to estimate (OARDC, 2004).

Infectious bursal disease (IBD) is caused by Infectious bursal disease virus (IBDV) a double-stranded dsRNA virus that has a bi-segmented genome and belongs to the genus Avibirnavirus of the family Birnivirida (Swai *et al*., 2011). Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. Two distinct serotypes of infectious bursal disease virus (IBDV) are known to exist. Serotype 1 virus causes clinical disease in chickens younger than 10 weeks. Older chickens usually show no clinical signs. Antibodies are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 antibodies are very widespread in turkeys and are sometimes found in chickens and ducks. There are no reports of clinical disease caused by infection with Serotype 2 virus. (OIE, 2008).

IBDV genome consists of two segments of double stranded RNA (dsRNA), segments A andB. The large 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 (NH2-VP2-VP4-VP3-COOH), which is cleaved by auto proteolysis to produce the viral capsid protein (VP2), the ribonucleoprotein (VP3) and the viral protease (VP4). The smaller segment B encodes VP1 and RNA-dependent RNA polymerase (RdRp) responsible for viral genome replication and RNA synthesis.

Based on serology and in vivo challenges, serotype 1 IBDV strains are classified into very virulent (VV), classical (virulent, mild/intermediate or attenuated), antigenic variant, and Australian classic genotypes. The hypervariable region (HVR), which spans amino acids from position 206 to 350 within VP2 (VP2-HVR), is known to be critical for determination of the conformational epitopes responsible for recognition of virus neutralizing antibodies in VP2. The VP2-HVR has the highest amino acid sequence variation among serotype 1 strains and the nucleotide and deduced amino acid sequences within this region are widely usedfor molecular diagnosis and genotyping of IBDVs **(**Kasanga *et al*., 2007**).**

Since its discovery in the USA in 1961, numerous IBDV isolates have been detected continually in chickens from different parts of the world (Cosgrove 1962). Reassorted viruses have recently been discovered in different parts of the world. In China, two reassortant strains which have a segment A evocative of a cell-culture-adapted vaccine virus but a VV-IBDV-like segment B, have been described (Wei *et al*., 2006). Also, the study by (Kasanga *et al*., 2012) to sequence the full-length genome of an IBDV field strain detected from Zambia and to analyze its genetic characteristics, determined the entire coding sequences of KZC-104 strain which provide the first evidence for the occurrence of reassortment of natural genome segments A and B in IBDV in Africa.

Furthermore, the study revealed that the KZC-104 genome had 96 to 99.5 % nucleotide sequence identity to that of the other very virulent strains, and it was most closely related to the KMRG-48 strain from Tanzania, T09 from Nigeria, D6948 from Netherlands and KS from Israel. Since 1987, pathotypic IBDV variants with enhanced virulence, called very virulent IBDVs (VV-IBDVs), emerged in Europe, and have spread to many places of the world. These strains were called European VV-IBDV. The first IBDV isolates from various locations in Tanzania were characterized as very virulent (vv) type in 2007.

These 'variants' were found to be widely distributed throughout Tanzania and demonstrated great similarities with isolates from western Africa and European/Asian vv IBDV variants (Swai *et al*., 2011). In Tanzania is insufficiently studied but it appears that IBDV is the most important recurring disease every year. Although IBDV represents one of the most severe poultry diseases and is responsible for marked economic losses, few studies of IBDV have been done on chickens in Tanzania, which hinders the implementation of effective disease control measures (Swai *et al*., 2011).

Infectious bursal disease (IBD) is among the most important constraints for commercial and local chicken production in Tanzania (Matovello and

Maselle, 1989). Genetic and antigenic characteristics of circulating infectious bursal disease virus (IBDV) strain have not been extensively studied in Tanzania. The vaccine used in controlling IBDV is of classical type discovered in 1960's without considering variationsdue to continuous genetic mutation of IBDV stains. Control of IBDV in Tanzania is mainly done through vaccination. However, the vaccines used do not match with the antigenic features of the prevailing viruses. Therefore, this study will focus on the seasonality of IBD virus infection and strain identification so that preventive and control programmes can be designed. The aim of the study was to establish genetic characteristics involved with specific objectives to identify IBDV genome and determine genotype of field IBD viral strain involved in IBDV infection Morogoro Municipality

MATERIALS AND METHODS

Study area and sample size

The study was conducted in Morogoro municipality-Tanzania in which 10 broiler chickens were collected in Kihonda ward following an outbreak. The area lies within longitude 37.67^{o}E and latitude 6.82^{o}S and receives the total annual rainfall of 935mm. According to 2012 census, the population is 315,866. The area is located in the southern highlands of Tanzania 169 km west of Dares salaam, 223km East of Dodoma and 511meters high from sea level. The temperature varies depending on the season with average annual range of 17°C to 28°C.A total of ten broiler chickens three month were selected and collected from one household keeping broilers at Kihonda ward where outbreak of IBD occurred based on clinical signs of the disease. A cross sectional was adopted and study was conducted within a period of three months from September to December

Sample collection and preparation

The chickens were collected and humane euthanized in the laboratory by air embolus in which air was introduced in blood circulation via wing vein. Then, burse of fabricius were aseptically removed from 10 affected broiler chickens, chopped them using two scalpel blades and a small amount of peptone broth containing penicillin and streptomycin (1000 µg/ml each) was added following homogenizing them in tissue blender. Then the homogenate was centrifuged at 3000 ***g*** for 10 minutes and supernatant fluid was harvested for use in the investigations.

IBDV RNA extraction

The IBDV RNA was extracted from bursal of fibricius using Viral RNA Min Extraction Kit (Qiagen) followed by Reverse Transcription. The process was performed by preparation of RT master mix as shown in Table 1. Then 10µl of the prepared RT master mix was put in each epedorff tube followed by addition of 10µl of RNA sample in each tube. The overall process was conducted under suitable cold chain. The sample mixture was subjected into thermal cycler under the following conditions; cycler for RT under the following conditions; 25ºC for10 min, 37ºC for 120min, 85ºC for 5min.

Table 1. Master mix for Reverse Transcription

No.	Component	Volume (µL)
1	10×RT buffer	10.0
2	25×dNTPs	4.0
3	10×RT Random primers	10.0
4	Reverse Transcriptase	5.0
5	RNase Inhibitor	5.0
6	Nuclease free water	3.2
	Total	37.2

Polymerase chain reaction (PCR)

The PCR was done targeting the VP2 HVRs which have molecular diagnostic features using theprimers V1 forward primer (5-CCA GAG TCT ACA CCATAA-3) and V2 reverse primer (3-TAC GAA AGAGTG GCA ACA GG-5) (Kasanga *et al*., 2007). The process was done by preparing PCR Master Mix using the reagents given in the Table 2. Then 20µl of PCR Master Mix was added in each PCR tube and finally 5µl of template as RT product was added in each sample under ice condition. The sample mixture was put in the PCR machine and the reaction was carried out under the following condition; denaturation at 94^{o}C for 30 sec, annealing at 57^{o}C for 30 sec, elongation at 72°C for 30 sec followed by last extension at 72°C for 5 min.

Table 2. Master Mix for Polymerase chain reaction (PCR)

No.	Component	Volume (µl)
1	2×PCR ready mix	62.5
2	Forward primer (V1)	1
3	Reverse prime (V2)	1
4	Nuclease free water	27.7
	Total	92

Electrophoresis

electrophoresis of the PCR products was performed. That 1.2 g of agarose powder was mixed in 100mls of TBE in a conical flask and boiled using hot plates with magnetic stiller to make clear solution. Then the samples were loaded in wells 10µl electrophoresis and stained in ethidium bromide 0.5 mg/ml was used to stain the DNA. The reaction was carried out at 100V for 45min. UV transilluminator was used for visualization of DNA bands.

Purification of obtained DNA amplicons was performed based on Qiagen quick PCR purification protocol. Cycle sequencing was performed using 5x sequencing buffer, Big Dye Terminator, primer, DNA template and water as reaction reagents. The reaction was performed under the following conditions; 96ºC for 1min in one cycle, (96ºC for 10sec, 50 ºC for 5sec, 60 ºC for 4min and 4 ºC as

Data analysis

The primary sequence data were edited by BioEdit software, then aligned using CLUSTALW and subjected to BLAST searches to determine their identity with other strains. Phylogenetic analysis was performed using MEGA 7 software by using the neighbor-joining method to determine the evolutionary relationship.

Agarose gel electrophoresis of the PCR ampliconsto confirm the PCR reaction, 1.2% agarose gel the storage temperature in 25 cycle). Then, ethanol precipitation was done in the tubes, in which to each reaction 5µl 125Mm EDTA and 60µl 100% EtOH. Then the mixture in the tubes were vortexes and left in the dark for 15min at room temperature. And centrifuged for 30min. All the supernatant was removed following addition of 60µl 70% EtOH and vortexed for few seconds and centrifuged for 30 min following remove all the supernatant. Then Vacu-dry was performed for 15min in the dark following addition of 20 µl Hi-Di Formamide then loading in the sequencing machine. Sequencing of PCR product was performed at the College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture using AB 3500 Geneticanalyzer.

RESULTS

PCR results

Diagnosis of IBDV was done using PCR employing diagnostic primers V1/V2 (5-CCA GAG TCT ACA CCATAA-3) / (3-TAC GAA AGAGTG GCA ACA GG-5). Following amplification of IBDV DNA, single major amplicons of approximately 472 bp was generated in all ten samples (Figure 1).

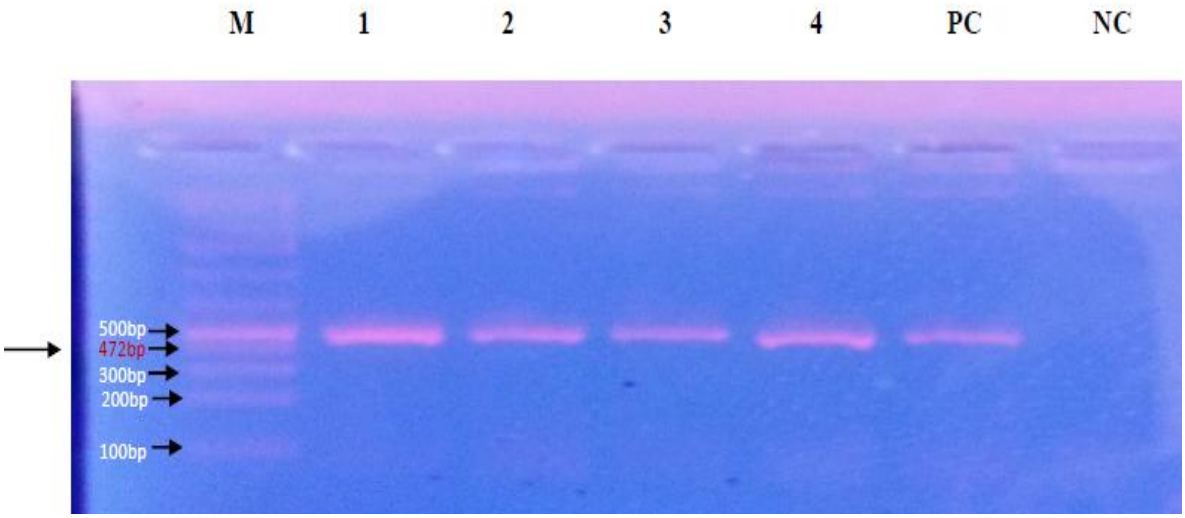

Figure 1. An Agarose gel after performing IBDV PCR using V1/V2 primers. Diagnostic PCR for the diagnosis of IBDV produced a PCR product of approximately 472 bp shown by arrow. All samples were positive for IBDV. Key; M = marker, number 1to4 = samples, PC= positive control, NC= negative control.

Sequencing results

Two PCR products samples 4& 5 out of ten were sequenced and both shows similar results. The strain obtained after sequencing was named M-/MRG/04/2014/C. Obtained sequence was compared to sequences of IBDV available in GenBank for genetic relatedness, in Table 3.

Phylogenic analysis

The data obtained in GenBank above was used to construct phylogenetic tree using neighbor-joining method to determine the evolutionary relationship (Figure 2).

Table 3. Percentage nucleotide identity of T-MRG/0414/C to IBDV in GenBank

% Identity	Strain	Year	Country	Genotype
94	KZC- 109	2004	Zambia	VV- A type
93	KMRG 38	2005	Tanzania	VV- EU type
93	TI/TW	2000	Taiwan	VV- EU type
93	Br/99/BN	1999	Brazil	VV- EU type
92	IBDV/NG2010	2010	Nigeria	VV- EU type
91	HuB-I	2007	China	VV- EU type

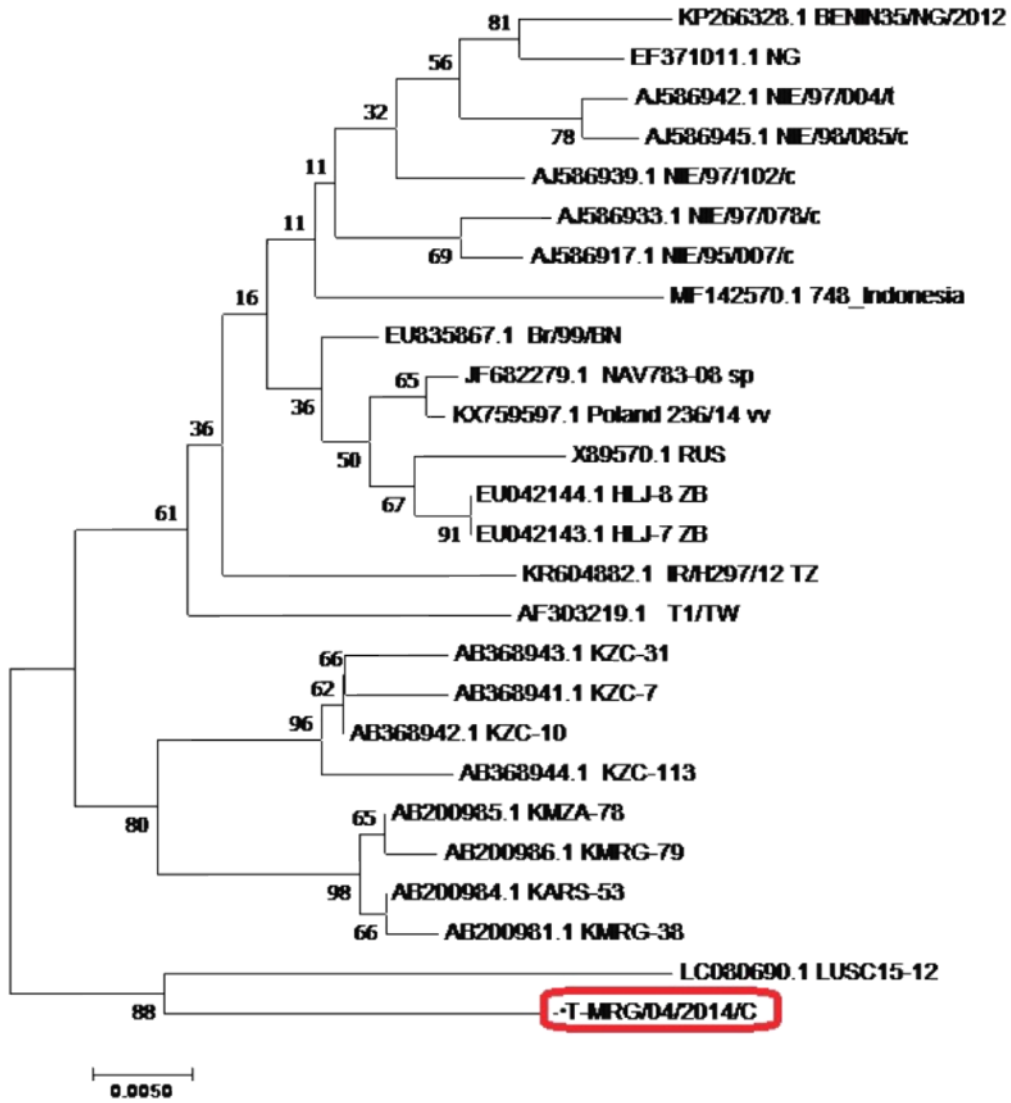

Figure 2. Phylogenetic tree of T-MRG/04/14/C indicating genetic relatedness of IBDV serotype 1

DISCUSSION

The results obtained from the present study confirmed that IBD caused massive death of broiler chickens in Kihonda ward Morogoro. This was confirmed after performing PCR using diagnostic primers V1/V2 that target a conserved region of VP2 HVRs which have molecular diagnostic features. Analysis of M-/MRG/04/2014/C sequences obtained showed that the virus isolate was 94% related to other previously reported outbreaks virulent European and classical type.

Based on the results of this study, it can be concluded that from the results obtained in this study the IBDV strain caused the massive death of young chickens in outbreak occurred in2014 in Morogoro region of Tanzaniais identical to Zambia (KZC-109) which is VV African type. The disease is still a threat in the poultry farming in the region dueto continuous genetic mutation of IBDV stains Zambia(KZC-109) which is VV African type, 93% related to KMRG 38(VV EU type) Tanzania, T1/TW (VV EU type) Taiwan, 92% IBDV 71/NG/2010(VV EU type) Nigeria, 91% HuB-1(VV EU type) China This IBDV isolate belongs to genotype I and also clusters together with the latter outbreaks isolates in the phylogenetic analysis. This shows that M-/MRG/04/2014/C strain is antigenically close related to very virulent African type which are different from very

and the vaccines used do not match with the antigenic features of the prevailing viruses. It is recommended that **f**urther studies are required to investigate**;** Evolutionary characteristics of the A – VV-IBDVFactors responsible for its maintenance and spread as well as development of rational vaccine for control of the African type VV-IBDV.

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