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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

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
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 and B. 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 used for 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
Molecular characterization of infectious bursal disease virus

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 variations due 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°E and latitude 6.82°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 months 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 fabricius 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 epedorf 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; 25°C for 10 min, 37°C for 120 min, 85°C for 5 min.

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10×RT buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>25×dNTPs</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>10×RT Random primers</td>
<td>10.0</td>
</tr>
<tr>
<td>4</td>
<td>Reverse Transcriptase</td>
<td>5.0</td>
</tr>
<tr>
<td>5</td>
<td>RNase Inhibitor</td>
<td>5.0</td>
</tr>
<tr>
<td>6</td>
<td>Nuclease free water</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>37.2</td>
</tr>
</tbody>
</table>

Table 1. Master mix for Reverse Transcription

Polymerase chain reaction (PCR)

The PCR was done targeting the VP2 HVRs which have molecular diagnostic features using the primers 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°C for 30 sec, annealing at 57°C for 30 sec, elongation at 72°C for 30 sec followed by last extension at 72°C for 5 min.

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2×PCR ready mix</td>
<td>62.5</td>
</tr>
<tr>
<td>2</td>
<td>Forward primer (V1)</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Reverse prime (V2)</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Nuclease free water</td>
<td>27.7</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>92</td>
</tr>
</tbody>
</table>

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

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 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 Vacudry 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).

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

<table>
<thead>
<tr>
<th>% Identity</th>
<th>Strain</th>
<th>Year</th>
<th>Country</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>KZC-109</td>
<td>2004</td>
<td>Zambia</td>
<td>VV- A type</td>
</tr>
<tr>
<td>93</td>
<td>KMRG 38</td>
<td>2005</td>
<td>Tanzania</td>
<td>VV- EU type</td>
</tr>
<tr>
<td>93</td>
<td>TI/TW</td>
<td>2000</td>
<td>Taiwan</td>
<td>VV- EU type</td>
</tr>
<tr>
<td>93</td>
<td>Br/99/BN</td>
<td>1999</td>
<td>Brazil</td>
<td>VV- EU type</td>
</tr>
<tr>
<td>92</td>
<td>IBDV/NG2010</td>
<td>2010</td>
<td>Nigeria</td>
<td>VV- EU type</td>
</tr>
<tr>
<td>91</td>
<td>HuB-I</td>
<td>2007</td>
<td>China</td>
<td>VV- EU type</td>
</tr>
</tbody>
</table>
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 in 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 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 in 2014 in Morogoro region of Tanzania identical to Zambia (KZC-109) which is VV African type. The disease is still a threat in the poultry farming in the region due to continuous genetic mutation of IBDV strains and the vaccines used do not match with the antigenic features of the prevailing viruses. It is recommended that further studies are required to investigate; Evolutionary characteristics of the A – VV-IBDV Factors 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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