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Genetic characterisation of infectious bursal disease virus isolates in Ethiopia

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ABSTRACT

The objective of the investigation was to characterise infectious bursal disease viruses (IBDV) circulating in commercial and breeding poultry farms in Ethiopia between 2009 and 2011. The nucleotide and deduced amino acid sequence for VP2 hypervariable region of ten IBDVs were determined by RT-PCR, sequenced and compared to well characterised IBDV isolates worldwide. IBDV genetic material was amplified directly from bursa or cell passaged material. Phylogenetically, Ethiopian IBDVs represented two genetic lineages: very virulent (vv) IBDVs or variants of the classical attenuated vaccine strain (D78). The nucleotide identity between Ethiopian vvIBDVs ranged between 0% and 2.6%. Ethiopian vvIBDVs are clustered phylogenetically with the African IBDV genetic lineage, independent of the Asian/European lineage. This report demonstrates the circulation of vvIBDV in commercial and breeding poultry farms in Ethiopia.

1. Introduction

Infectious bursal disease virus (IBDV) belongs to the *Birnaviridae* family and has a non-enveloped, icosahedral capsid. The genome of virus is double-stranded RNA and bi-segmented (Eterradossi and Saif, 2008). Infectious bursal disease virus replicates in differentiating lymphocytes of the Bursa of Fabricius, causing the immunosuppressive and often fatal condition called infectious bursal disease (IBD) or Gumboro. IBDV consists of two serotypes, 1 and 2. Serotype 1 viruses are infectious for chickens, differing in their pathogenicity and are classified as avirulent, classical, variant and very virulent (vv) strains (Muller et al., 2003; Sapats and Ignjatovic, 2000).

Variant and vvIBDV strains have been isolated from disease outbreaks despite the presence of high levels of maternal antibody to classic strains of IBDV (Jackwood and Saif, 1987). The use of an appropriate vaccine is vital for effective protection and hence differentiation and identification of local IBDV isolates is crucial for selection of appropriate vaccine strain. Amplification of IBD virus protein 2 (VP2) gene and linking genetic variation found in this region with antigenic variation has been the major focus for strain identification in recent years (Bayliss et al., 1990; Brown et al., 1994; Wu et al., 2007). The IBDV VP2 hypervariable region (HVR) is commonly used to differentiate IBDV strains (Jackwood, 2004).

The first report of IBD in Ethiopia was in 2005 involving 20–45 day old broiler and layer chickens from commercial farms (Zeleke et al., 2005). Subsequently, IBD has become a priority problem in commercial and backyard poultry production system despite regular vaccination practices (in some cases) using attenuated IBDV D78 vaccine and improved biosecurity measures. This study was therefore initiated to determine the molecular characteristics of IBDV responsible for wide spread mortality and morbidity in Ethiopia.

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2. Materials and methods

2.1. Study design

In this study, over 25 outbreaks of IBD were investigated by the National Veterinary Institute, Ethiopia, between 2009 and 2011. Outbreaks occurred in both commercial and breeding poultry farms under private and government ownership. This study involved the collection of clinical and epidemiological data, and the post-mortem examination of sick birds.

2.2. Bursa sample collection and virus isolation

For virus detection, bursa samples were aseptically collected from suspected cases, placed into individual sterile universal bottles and transported under cold chain to the virology laboratory, National Veterinary Institute, Ethiopia. Bursa samples were chopped into small pieces using a sterile scalpel blade, and minced using a mortar and pestle. A 10% suspension of each bursa sample was prepared in sterile phosphate buffer saline supplemented with penicillin and streptomycin (1000 μg/ml each). The suspension was transferred into sterile centrifuge tube and centrifuged at 3000 × *g* for 10 min. The supernatant was harvested and filtered using 0.22 μ milipore filters.

Samples of the resulting suspension were added to FTA card (Whatman) (to capture the viral RNA for molecular analysis) and inoculated onto confluent primary chicken fibroblast cell cultures for virus isolation. Cultures were maintained in GMEM containing 2% bovine calf serum and incubated at 37 °C. Cultures were observed microscopically for up to seven days for the presence of cytopathic effect (CPE) characteristic of IBDV. After seven days, samples with no CPE were blindly passed further three times following two cycles of freeze–thawing. Samples showing no CPE after the third passage were considered negative. Supernatant fluid from CPE positive cultures were also added onto FTA cards for molecular analysis. A total of ten IBDV specimens were available for genetic analysis, seven from original bursa material and three from virus isolated in cell culture.

2.3. RNA extraction and reverse transcription

RNA was eluted from FTA card by placing a section of the filter card (approximately 0.5 cm × 0.5 cm) in 300 μl of Elution buffer (Qiagen), vortexing and incubating on ice for approximately 15 min. Subsequently, 140 μl was further processed using the QIAGEN Viral RNA extraction kit as outlined by the manufacturer. Complementary DNA was generated from RNA using the reverse transcriptase RevertAid™ (Fermentas). RNA was first incubated at 95 °C for 3 min and placed on ice for at least 3 min in the presence of the gene specific primer L2 (5′-GATCCTGTTGCCACTCTTTC-3′), which binds nucleotides 1194–1213 of the positive strand of IBDV segment A (Bayliss et al., 1990), and 20% DMSO (Martin et al., 2007). RNA was reverse transcribed in a final volume of 20 μl containing reaction buffer (Fermentas), 1 mM of each dNTP (Thermo Scientific), 20 U RiboLock™ RNase Inhibitor and 200 units RevertAid™ Reverse transcriptase. Reverse transcription reactions were performed at 42 °C for 60 min and the reverse transcriptase inactivated at 70 °C for 10 min.

2.4. PCR amplification and sequencing

Polymerase chain reaction (PCR) amplification of products intended for sequencing was carried out using a high fidelity DNA polymerase, *Pfu* DNA polymerase (Fermentas). A typical 25 μl reaction contained *Pfu* Buffer with $MgSO_4$ (Fermentas), 0.2 mM dNTPs (Thermo Scientific), 200 nM of each primers L2 (5′-GATCCTGTTGCCACTCTTTC-3′) and U2 (5′-GGTATGTGAGGCTTGGTGAC-3′) which binds nucleotide position 1194–1213 and 658–677 of IBDV segment A, respectively (Bayliss et al., 1990), 2.5 units (U) *Pfu*DNA polymerase (Fermentas) and 2 μl of cDNA template. PCR reactions were carried out for 1 cycle at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and 1 cycle at 72 °C for 7 min. The amplified 604 base pair product contained the VP2 hypervariable region coding sequence. Amplicons were separated from reaction components using the QIAGEN Gel extraction kit, with the concentration of DNA determined spectrophotometrically using a Nano Drop Spectrophometer 1000 (Thermo Scientific). Purified amplicons were sequenced using both L2 and U2 primers by a commercial sequence provider (Macrogen) using the Big Dye terminator cycling (Applied Biosystems) condition and analysed by the automated sequencer ABI 3730XL.

2.5. Sequence and Phylogenetic analysis

Sequences were analyses using Geneious (Drummond et al., 2011). The deduced amino acid sequence (amino acid position 191–350) of Ethiopian IBDV isolates sequenced in this study were alignment to the VP2 amino acid 239–332 (Bayliss et al., 1990) of well characterised IBDVs. Phylogenetic analysis was based on an alignment of partial IBDV VP2 sequences (nucleotide position 804–1190), spanning the HVR at nucleotide position 845–1126 (Bayliss et al., 1990). Nucleotide alignment was performed using ClustalW, within Mega 5.1 (Tamura et al., 2011), with the phylogenetic analysis inferred using the neighbour-joining method. One thousand bootstrapping replicates were used to estimate the robustness of tree branches.

3. Results

3.1. Nucleotide and deduced amino acid sequence

The nucleotide sequence of the VP2 HVR was determined for 10 Ethiopian IBDV isolates from cDNA transcripts. Nucleotide identity between the 10 isolates ranged between 90.2% and 100%. Isolates IBDV 15/10 and IBDV 16/10 showed 100% identity over the region sequenced and were genetically related to IBDV 11/10, 6/10, 17/10 and 01/10 (nucleotide identity 99.8%). Isolates IBDV 03/11, 04/09 and 10/10 showed 100% nucleotide identity over the region sequenced and were genetically related to IBDV 09/09 (97.7%). Nucleotide identity between the Ethiopian produced IBDV vaccine and Ethiopian IBDV isolates ranged between 90.2% and 94.6%.

The deduced amino acid sequence of the hypervariable region was determined for each of the isolates and compared to well characterise classical virulent IBDV isolates (F52/70), classical attenuated IBDV isolates (IBDV 78) and vvIBDV isolates (vvIBDV UK 661) (Fig. 1). Four Ethiopian IBDV isolates (IBDV 03/11, 04/09, 09/09 and 10/10) contain the genetic signature of vvIBDVs, specifically, A222, I256, I294, S299 (Brown et al., 1994), however, lacked L324 and V321, characteristic of antigenically atypical vvIBDV (Eterradossi et al., 1998). Six of the IBDV isolates (IBDV 12/10, 15/10, 11/10, 06/10, 17/10 and 16/10) contain amino acid sequences linked to propagation in cell culture (N279 and T284) (Lim et al., 1999; Mundt, 1999). Interestingly, heterogeneous variation was also detected in these six isolates at amino acid 253 (H/Q/N), a residue shown to be involved in both cell tropism and virulence (Boot et al., 2000; Brandt et al., 2001; Qi et al., 2009; Van Loon et al., 2002).