ADDIS ABABA UNIVERSTY
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INFECTIOUS BURSAL DISEASE VIRUS: OUTBREAKS INVESTIGATION, MOLECULAR CHARACTERIZATION AND DEVELOPMENT OF LIVE ATTENUATED VACCINE USING LOCAL ISOLATES

BY

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfilment of the requirements for the Degree of Master of science in Tropical Veterinary Microbiology

JUNE 2012

DEBRE ZEIT, ETHIOPIA

ACKNOWLEDGEMENTS

First of all, I would like to thank God for his grace and immeasurable love, giving me strength and patience to bring me out his humble piece of work in to light.

I take it as an extreme privilege to express my heartfelt thanks and sincere gratitude to my advisors, Dr. Tesfaye Sisay and Dr. Shiferaw Jenberie for their noble hearted help, guidance, cooperation and encouragement which have installed in me the spirit of confidence to successfully complete this research work.

It is worth enough to acknowledge the National Veterinary Institute for the supply of collected outbreaks samples for the study and unreserved financial, laboratory chemicals and reagents and material support for covering this study project.

I respectfully acknowledge the part played by the National Agricultural Research Fund (NARF), Infectious bursal disease project and The Institute of Infection and Global Health, University of Liverpool for sequencing and constructing phylogenetic tree of IBDV isolates.

My special thanks also goes to Dr. Esayas Gelaye, Dr Hundra sori , Alebachew Belay, Dr. Teferi Degefa, Hawa Mohammed, Wubet W/Medhin, Tezazu Feleke, Berhan Demek and Tebeyen Setarge for their cooperation and generous response and support to process the samples.

I would like to express my deep gratitude to my brother Ato Beshaw Belete and Engineer Kebede who cultivated and brought me up with delight and strong moral support throughout my academic career.

It is my pleasure to thank all academic teachers for their unreserved friendly advice support during the course of the study that encouraged and helped me to achieve my objective amongst the many my special thanks also goes to Prof. Pal, Dr moses kyule for constant willingness to offer assistance to explain subject matter during the course of study.

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DEDICATION

This work is dedicated to my sister, Zenash Belete, who passed away just when I have started my MSc study and to my lovely mother and father who passed away without seeing any of my achievements.

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AGID	Agar gel immunodiffusion test
cDNA	Complementary deoxyribonucleic acid
CEF	Chicken embryo fibroblast
CPE	Cytopathic effect
CSA	Central Statistical Agency
ELISA	Enzyme linked Immunosorbent Assay
HVR	Hypervariable region
I- ELISA	Indirect Enzyme linked Immunosorbent Assay
IBD	Infectious Bursal disease
IBDV	Infectious Bursal disease virus
MDA	Maternal derived antibody
NVI	National Veterinary Institute
OIE	International Animal Health Organization
PBS	Sterile phosphate buffer saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SAN	Specific antibody negative
TCID50	Tissue culture infective dose 50
USA	United States of America
VN	Virus Neutralization
VP	Virus Protein
VP1-VP4	Virus Protein1-4
VP2	Virus Protein2
VvIBDV	Very Virulent Infectious bursal disease virus
WRL	World Referral Laboratory

ABSTRACT

This study was conducted on sample collected from different regions of the country, where infectious bursal disease (IBD) outbreaks were reported. Samples were processed both at National Veterinary Institute and University of Liverpool, United Kingdom, with the aims of molecular characterization of local isolates, analyzing genetic relatedness of IBD virus circulating in the country with currently produced vaccine and development of vaccine from local isolates. The nucleotide and deduced amino acid sequence for VP2 hypervariable region (HVR) of ten IBDV isolates collected from 2009 until 2011 was also determined by RT-PCR and sequencing, and compared with well characterised IBDV isolates worldwide. A total of 136 bursa sample were pulled into 44 samples. Bursa samples were processed and cultured onto both confluent primary CEF cell cultures as well as fully confluent established cell line (Vero). Viruses were isolated from 41 (93.2%) samples out of the 44 pulled samples. All the isolates were grown and able to demonstrate CPEs on CEF cell cultures on the first passage. Phylogenetically, Ethiopian IBDV isolates were from one of two genetic lineages: very virulent IBDV and variants of the classical attenuated vaccine strain (D78). Nucleotide identity between Ethiopian vvIBDVs range between 0% to 2.6% over VP2 HVR. Ethiopian vvIBDVs clustered phylogenetically with the African IBDV genetic lineage, independent of the Asian/European genetic. This indicates the circulating of vvIBDV among Ethiopian chickens. Classical variant strain (IBDV 06/10) was selected for adaptation trial using Vero cells. The 9th Vero passage local isolate was evaluated with the current NVI vaccine for immunogenicity and pathogenesity on chicken. The result showed that the adapted local isolate was superior to the current commercially available vaccine. The adapted trial vaccine induced significantly ($P<0.05$) higher mean antibody titre (3281.35±101.43) compared to the currently used vaccine strain (2784.12±92.34). The mean bursa weight from the unvaccinated control groups was 20.8±1.2 g while it was 3.6±0.16g and 2.8±0.07g, respectively, for commercial vaccine and 9th passaged local isolate. Although the difference in the mean bursa weight between the vaccinated groups was not statistically significant ($P>0.05$), the difference in the mean bursal weight between control and vaccinated groups was statistically significant ($P<0.05$).

Key words: Infectious bursal disease; Outbreak investigation; molecular characterization .

1. INTRODUCTION

Poultry breeding in Ethiopia has a long traditional practice which is mainly used as an immediate cash income for the rural communities with a significant role in the livelihood of the farmers. The poultry sector in Ethiopia can be characterized into three major production systems based on some selected parameters such as breed, flock size, housing, feed, health, technology, and bio-security (Dawit Alemu *et al.*, 2008). These are village or backyard, small scale and commercial poultry production systems. Backyard poultry is the predominant system in Ethiopia and accounts for nearly 99% of the poultry population consisting mainly of local chicken breeds under individual farm household management (CSA, 2004) and it is also common to find a few exotic breeds distributed through extension programs. (Dawit Alemu *et al.*, 2008).

Infectious Bursal Disease (IBD) is caused by a virus of the genus *Avibirnavirus* of the family *Birnaviridae* (van den Berg, 2000; Meihong and Vikram, 2004; OIE, 2004). 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 immune-suppression problems due to the effect of the virus on the bursa of Fabricius. Two distinct serotypes of IBDV are known to exisit Serotype 1 and 2 (De Herdt *et al.*, 2005). The incubation period is very short and clinical signs are seen in 2-3 days (Lukert and Saif, 1997) with a spiking mortality, soiled vent feathers, hemorrhage in leg muscle, swollen kidney filled with 'white Urate' deposits, whitish or watery diarrhea, anorexia, depression, ruffled feathers, swollen and hemorrhagic bursa, yellow caseous materials in the bursa (Lukert and Saif, 1991; Baxendale, 2002). IBD virus remains infectious for a very long period of time and has resistance to commonly used disinfectants (Lukert and Saif, 1997; OIE, 2004).

Infectious Bursal Disease is a newly emerging disease of chicken in Ethiopia, as described by Zeleke *et al*, (2005); the disease has been speculated to be introduced concurrent with the increased number of commercial state and private poultry farms flourishing in the country. The report of introduction and existence of IBD in the country has come recently with the report of IBD outbreak in Debre Zeit poultry farms in 20-45 old days' broiler and layer

chickens with a mortality rate of 45-50%, and seroprevalence 93.3 % (Zeleke *et al.,* 2005). Case report study from Andasa poulty farm indicated that the overall mortality of chickens due to IBD was 72.0% in young (1-70 days old) and 7% in adult (>70 days old) and a 100% seroprevalence has been recorded in non-vaccinated flocks (Woldemariam and Wossene, 2007). Recently, Hailu 2008, Unpublished) reported a 51.10% overall seroprevalence of IBD; with a 29.40% and 21.70% in Bahir Dar and Farta areas, respectively. Additionally, molecular characterization of the Ethiopian IBD virus isolates was done for the first time in 2005 from the samples collected from Kombolcha poultry multiplication center and the samples were processed by NVI researchers for virus isolation and submitted to AFSSA- France, OIE-IBD Reference Laboratory for future antigenic and genomic characterization of the isolates and identified as very virulent IBD virus (vvIBDV) (Zeleke *et al.,* 2005).

Recently a questionnaire survey was conducted by NVI researchers in three large scale commercial poultry farms found in Debre Zeit town with the objectives of identifying major poultry diseases encountered in their farms. The interviewed professionals and farm owners explained that diseases like Newcastle, Fowl pox, Fowl typhoid, Infectious Bursal Disease (Gumboro) and Mycoplasma are the common problems existed in the farms (NVI, 2010). They have regularly vaccinated chickens against the above mentioned diseases, but Gumboro is the worst and number one disease which causes high mortality in spite of regular vaccination using imported vaccines. Currently, the disease is widely distributed in all regions in the backyard chickens, commercial farms and poultry multiplication centers. Despite the fact that IBD incidences are increasing at alarming rate all over the country where commercial state and private poultry production and backyard chickens are intensified, there is no any concerted effort towards the implementation of cost effective control strategies of IBD like regular vaccination, strict importation policy and quarantine services.

Therefore the Objectives of this study are

- To investigate IBD outbreaks in different parts of Ethiopia.
- To conduct Molecular characterization of the isolated IBD Viruses from the outbreaks.
- To develop live attenuated IBD vaccine from the selected strain

2. LITERATURE REVIEW

2.1. The disease

Infectious bursal disease (IBD) is an acute contagious viral disease of young chickens. (Jackwood and Sommer, 2004). Chickens are susceptible to clinical disease at 3-6 weeks of age. Clinical disease is characterized by inflammation of the bursa of Fabricius, haemorrhages in skeletal muscles and death. Economic losses in the poultry industry result from high mortality rates due to this acute form of the disease or from a subclinical infection in chickens below 3 weeks age characterized by B-cell dependent immunodeficiency (Lukert and Saif, 1991). The latter enhances the susceptibility of chickens to other infections and depresses the response of infected chickens to vaccines against other diseases such as Newcastle disease, Marek's disease and infectious bronchitis. Because vaccination is the principal method of viral disease control in commercial poultry worldwide (van den Berg, 2000; Muller *et al.,* 2003), IBDV should be considered as one of the most important viral pathogens of the commercial poultry industry.

2.2. Etiology

Infectious bursal disease virus (IBDV), the prototype member of the *Avibirnavirus* genus of the *Birnaviridae* family, is the causative agent of infectious bursal disease (IBD) and is the smallest segmented RNA virus in animals. The non-enveloped icosahedral virus particle of 60 nm diameter is single-shelled and contains a double-stranded (ds) RNA genome which consists of only two segments (Müller *et al.,* 1979; Kibenge *et al.,* 1988). A large open reading frame (ORF) of genome segment A (approximately 3.3 kbp) encodes a 110 kDa polyprotein (VP2–VP4–VP3) which is cleaved post translationally into structural proteins. VP2 and VP3, and a viral protease, VP4. VP2 forms the outer surface of the viral capsid considered to interact with cellular receptors present on chicken embryo (CE) fibroblasts as well as on lymphoid cells isolated from chicken bursa, thymus or spleen (Nieper and Müller, 1996). VP3 forms the inner surface and interacts with VP1, the putative RNA-dependent RNA polymerase (Lombardo *et al.,* 1999; Tacken *et al.,* 2000), as well as with the viral genome (Tacken *et al.,* 2002). VP4 is a viral serine-lysine protease (Birghan *et al.,* 2000; Lejal *et al.,* 2000). A small, partially overlapping ORF encodes VP5 which is not present in

the virus particle (Mundt *et al.,* 1995). It accumulates in the host plasma membrane and is considered to be involved in virus release (Lombardo *et al.,* 2000) and in inducing apoptosis (Yao and Vakharia, 2001). Genome segment B (approximately 2.9 kbp) contains one ORF encoding VP1 and considered to be a multifunctional protein with RNA-dependent RNA polymerase, methyltransferase and (putative) capping enzyme activities (Müller and Nitschke, 1987; Spies *et al.,* 1987; Spies and Müller, 1990). Two serotypes of IBDV can be differentiated by virus neutralization test (McFerran *et al.,* 1980). All pathogenic isolates are serotype 1 strains whereas serotype 2 strains neither cause disease in chickens nor protect against infections with the serotype 1 strain (Ismail *et al.,* 1988; Ismail and Saif, 1991). Serotype-specific antigenic determinants inducing neutralising antibodies are located on VP2 whereas group-specific antibodies recognize antigenic sites located on both structural proteins, VP2 and VP3 (Becht *et al.,* 1988).

2.3. Molecular Epidemiology

Since IBDV was first isolated in the town of Gumboro, Delaware, USA in 1957 (Cosgrove, 1962), it has caused enormous economic loss in poultry industries worldwide. Particularly in the late 1980s when an acute outbreak of IBDV in Japan and Europe resulted in a damaging mortality rate of 60–100% (van den Berg *et al.,* 1991; Nunoya *et al.,* 1992; Brown *et al.*, 1994). In recent years, IBDV variants have emerged including the very virulent IBDV (vvIBDV) strain that is seriously threatening poultry industries around the world. Since its emergence, vvIBDV has been isolated and identified as the pathogen responsible for acute IBD in many countries (Jackwood and Saif, 1987; Snyder *et al.,* 1992). It has been demonstrated that the antigencity and pathogenic signs of vvIBDV are similar to classical serotype I IBDV. However, antibodies, including maternal antibodies, induced by attenuated serotype I vaccine; do not provide satisfactory protection against vvIBDV (van den Berg, 2000).

The high mutation rate of RNA viruses and the high selection pressure generated by intensive vaccination of birds can lead to the emergence of viruses with new properties allowing them to persist in immune populations. Historically, these mutations have led to antigenic variation and modifications in virulence of circulating infectious bursal disease virus (IBDV) strains. It is therefore essential to identify and characterize new IBDV isolates as soon as they appear

and compare them with previously described viruses (van den Berg, 2000; van den Berg *et al.,* 2000). Three criteria can be used for the characterization of IBDV strains: antigenicity, genetic relatedness and pathogenicity. Three panels of neutralizing monoclonal antibodies (mAbs) have been used in antigen capture enzyme-linked immunosorbent assays (AC-ELISA) for antigenic characterization of serotype 1 isolates of IBDV (Fahey *et al.,* 1991; Snyder *et al.,* 1992). Neutralizing MAbs have been shown to bind to VP2 within a restricted region, called the variable domain, between amino acids 206 and 350, which is highly hydrophobic but has short hydrophilic regions at each end (Bayliss *et al.,* 1990). Sequencing of the VP2 gene of numerous different IBDV strains and escape mutants confirmed that this variable domain is responsible for antigenic variation (O¨ppling *et al.,* 1991; Schnitzler *et al.,* 1993; Vakharia *et al.,* 1994) and comparison of this region has been thought to offer the best option for IBDV typing. Therefore, the sequence of this region is now commonly used for phylogeny. Numerous reports of acute cases, together with epidemiological observations and mortality studies, have confirmed that the recently emerging very virulent (vv) viruses group together and form a virus lineage with remarkable genetic and antigenic homogeneity (Yamaguchi *et al.,* 1997; Eterradossi *et al.,* 1997b; van den Berg, 2000; Zierenberg *et al.,* 2000). VP2 sequence alignments have shown that vvIBDVs share unique amino acid residues at positions 222 (Ala), 256 (Ile), 294 (Ile) and 299 (Ser) Bayliss *et al.,* 1990), which have been suggested to indicate the genetic fingerprint of vvIBDV (Brown et al., 1994; Yamaguchi *et al.,* 1997; Cao *et al.,* 1998; Eterradossi *et al .,* 1998; Zierenberg *et al.,* 2000). The vvIBDVs induce higher mortality rates in fully susceptible chickens than classical virulent (cv) strains. Mortality rates as high as 100% in specific pathogen free (SPF) chickens, 60% in layers and 30% in broilers have been reported (van den Berg and Meulemans, 1991; van den Berg *et al.,*1991).

2.4. Immunity to IBD infection

Immune responses to IBDV-infection may vary depending on the maturity of the bird's immune system. IBDV-pathogenesis studies comparing different age groups under experimental conditions are limited (Yadin *et al.,* 1980; Nakamura *et al.,* 1992; Rautenschlein and Haase, 2005). A variety of studies have looked at T cell and cytokine responses following infection of at least 3-week-old chickens with different IBDV-strains (Rautenschlein *et al.,* 2003; Jochemsen *et al.,* 2004; Eldaghayes *et al.,* 2006). It has been well established that in

birds older than 2 weeks post hatch IBDV-infection induces T cell accumulation in the bursa of Fabricius, the main target organ of IBDV, coinciding with IBDV-replication (Tanimura and Sharma, 1997). These intrabursal T cells of IBDV-infected birds are activated (Kim *et al.*, 2000), and may play a significant role in viral clearance but also in bursal recovery (Rautenschlein *et al.,* 2002a; Rautenschlein *et al.,* 2003). Previous studies indicated that T cell activity may vary between age groups (Rautenschlein and Haase, 2005). Birds vaccinated in ovo with an intermediate strain of IBDV showed no or delayed T cell accumulation in the bursa of Fabricius in comparison to birds vaccinated at 14 days post hatch. Furthermore, in ovo vaccinated birds recovered faster from IBDV-induced bursa lesions than post hatch vaccinated chickens (Rautenschlein and Haase, 2005). Depending on the virus strain, IBDV infection may induce transient up-regulation of interferon (IFN)- γ, interleukin (IL)-2, and IL-6, while cytokines such as IL-1b and type I IFNs may be down-regulated temporarily (Rautenschlein *et al.,* 2002b; Jochemsen *et al.,* 2004; Eldaghayes *et al.,* 2006).

2.5. Pathology and pathogenesis

Chickens are highly susceptible to IBDV between 3 and 6 weeks after hatching. Experiments in which bursectomized chickens survived IBDV infection demonstrated that the bursa of Fabricius (BF) is the main target organ for IBDV. The acute phase usually lasts about 1 week, and peak clinical signs and mortality are recorded between 3 and 4 days after IBDV infection. The most common mode of IBDV infection is via the oral route. The virus is taken up from the gut and transported to other tissues by phagocytic cells, most likely resident macrophages and destroys actively dividing IgM-bearing B cells in the BF (Rodenberg *et al.,* 1994). Although B cells are the principal targets for IBDV; recent data shows that the virus also infects and replicates in macrophages. Infection with IBDV causes the production of proinflammatory mediators and cytokines in macrophages, which peaks during the early phase of active virus replication (Khatri *et al.,* 2005; Palmquist *et al.,* 2006). IBDV induces expression of the following cytokines and cytokine genes: interleukin (IL)-12, interferon (IFN)-g, IL-1b, IL-6 and CXCLi2 in bursal cells (Eldaghayes *et al.,* 2006; Rauw *et al.,* 2007), and expression of IL-1b, IL-6, IL-18 and inducible nitric oxide synthase (iNOS) in spleen cells (Palmquist *et al.,* 2006). Nitric oxide (NO), which is produced by activated macrophages, may promote cellular destruction of both virus-infected and virus-free cells (Yeh *et al.,* 2002). T-cells, which are not infected by IBDV, may modulate the pathogenesis

by limiting viral replication in the BF during the early phase of the disease at 5 days pi, by promoting bursal tissue damage and delaying tissue recovery, possibly through the release of cytokines and their concomitant cytotoxic effects (Rautenschlein *et al.*, 2002a).

2.6. Diagnosis

Clinical disease due to infection with the IBDV, also known as Gumboro disease, can usually be diagnosed by a combination of characteristic signs and post-mortem lesions. Laboratory confirmation of disease, or detection of subclinical infection witch is common in young chicken, has less apparent clinical signs IBD confirmed by demonstration of a humoral immune response in unvaccinated chickens or by detecting the presence of viral antigen or viral genome in tissues. In the absence of such tests, histological examination of bursae may be helpful (OIE, 2008).

2.6.1. Identification of the agent

Sample preparation

The bursa of Fabricius is removed aseptically from approximately five affected chickens in the early stages of the disease. The bursae are chopped using two scalpels, a small amount of peptone broth containing penicillin and streptomycin (1000 μg/ml each is added and homogenised in a tissue blender. The homogenate is centrifuged at 3000 *g* for 10 minutes. The supernatant fluid is harvested for use in the investigations. Filtration through a 0.22 μ filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titer (Meulemans *et al.*, 1977).

Identification by the agar gel immunodiffusion (AGID) test

For detection of antigen in the bursa of Fabricius by AGID, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in scissor movement, and then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue, and the freeze–thaw exudates may be used to fill the wells (Marquardt *et al.*, 1980; Kwang *et al.*, 1987).

Identification by immunofluorescence

Sections of bursa are prepared using a microtome cryostat, dried at room temperature and then fixed in cold acetone. Fluorescent-labelled IBDV-specific antisera are applied to the sections, which are then incubated at 37^0C for 1 hour in a humid atmosphere. At the end of the incubation period, they are washed for 30 minutes using phosphate buffered saline (PBS), pH 7.2, and then rinsed in distilled water. The sections are mounted using buffered glycerol, pH 7.6, and examined by UV microscopy for IBDV specific fluorescence (Meulemans *et al.*, 1977).

Identification by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA)

Different protocols have been described for the detection of serotype 1 IBDV using an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) Briefly, ELISA plates are coated with IBDV specific antibodies. Depending on the chosen AC-ELISA protocol, the capture antibody may be a mouse anti-IBDV monoclonal antibody (MAb), or a mix of such MAbs, or a chicken post-infectious anti-IBDV polyclonal serum. It has been suggested that AC-ELISAs using polyclonal antibodies may have a higher sensitivity. Samples of bursal homogenates diluted 1/10 to 1/25 (w/v) in a suitable dilution buffer are incubated in the coated wells. Unbound antigens are discarded at the end of the incubation period by washing with a suitable washing buffer (PBS, pH 7.2 + 0.2% Tween 20). The captured antigens are then revealed, as in an indirect ELISA, with a detection antibody (which must have been developed from a different animal species than the capture antibody), followed by an enzyme conjugate that binds to the detection antibody only (in some protocols the detection antibody may be directly conjugated to the enzyme), followed by the enzyme substrate. Finally, optical densities, which parallel the amount of captured IBDV antigens, are read with an ELISA reader (Marquardt *et al.*, 1980; Kwang *et al.*, 1987).

Identification by molecular techniques

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation (Davis *et al.,* 1990; Wu *et al.,* 1992; Khatri *et al.*, 2005). The most frequently used molecular method is the detection of IBDV genome by the reverse-transcription polymerase chain reaction (RT-PCR) (Wu *et al.,* 1992; Lin *et al.,* 1993). This method can detect the genome of IBDV, which is unable to grow in cell culture, because it is not necessary to grow the virus before amplification. RT-PCR is performed in three steps: extraction of nucleic acids from the studied sample, reverse transcription of IBDV RNA into cDNA, and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotidic primers that are short sequences complementary to the virus-specific nucleotide sequence. Different areas of the genome will be amplified depending on the location from which the primers have been selected, (Rosenberger *et al.,* 1986; Eterradossi *et al.,* 1998).

Isolation of virus in cell culture

The IBD grows well in chicken in confluent chicken embryo fibroblast (CEF) cell cultures (from a specific pathogen free source) in 25 cm2 flasks. The virus Adsorb at 37^0C for 30–60 minutes and washed twice with phosphate buffered saline solution and add maintenance medium to each flask. The cultures are incubated at 37^0C, observing daily for evidence of cytopathic effect (CPE). CPE could be observed 6 days, the medium is discarded then the cultures are frozen and thawed and inoculated into fresh cultures. This procedure may need to be repeated at least three times. Serial passage in chicken embryos reduces virulence of virus for chickens (Sharma, *et al.*, 1996). If CPE is observed, the viruses were detected in their culture media this should be tested against IBDV antiserum in tissue culture virus neutralization (VN) test. The more pathogenic IBDV strains usually cannot be adapted to grow in CEF unless the virus has first been submitted to extensive serial passage in embryos (OIE, 2004).

Isolation of virus in embryos

The sample (0.2 ml is inoculated into the yolk sac of five 6–8-day-old specific antibody negative (SAN) chicken embryos and on to the chorio-allantoic membrane route of inoculation (OIE, 2004) of five 9–11-day-old SAN chicken embryos. The chorio-allantoic route of inoculation in 9-11 days old embryos is the most sensitive route for the isolation of the virus. The embryos were examined daily for mortality by candling and those died within 24 hours of inoculation were discarded. The embryos which died in the next seven days were opened aseptically and are examined for lesions and CAM and embryos were collected and preserved at – 20^0C. Classical viruses usually kill the embryos in 3-5 days and produce lesions of vascular congestion and subcutaneous haemorrhages in the embryos (Hitchner, 1970; Lasher, 1994). Variant viruses however, do not kill the embryos but cause embryo stunting, discoloration, splenomegaly and hepatic necrosis,If lesions are observed, the virus, should then be tested against a monospecific anti-IBDV serum in an embryo-revealed virus neutralisation assay. Serotype 1 IBDV usually causes death in at least some of the embryos on primary isolation. Serotype 2 IBDV does not induce subcutaneous oedema or haemorrhages in the infected embryos, but embryos are of a smaller size with a pale yellowish discolouration. For the preparation of embryo-propagated stock virus or for subsequent passaging, embryos with lesions or embryos suspected to be infected, respectively, are harvested aseptically. Their head and limbs were discarded and the main body is minced for the preparation of virus suspension (Rosenberger, et al., 1986).

Isolation of virus in chickens

Five susceptible and five IBD-immune chickens (3–7 weeks of age) are inoculated by the eye-drop route with 0.05 ml of sample. The chickens are killed 72–80 hours after inoculation, and their bursae of Fabricius are examined. The bursae of chickens infected with virulent serotype 1 IBDV appear yellowish (sometimes haemorrhagic) and turgid, with prominent striations. Peribursal oedema is sometimes present, and plucks of caseous material are occasionally found. The presence of lesions in the bursae of susceptible chickens along with the absence of lesions in immune chickens is diagnostic of IBD. The bursae from both groups may be used as antigen in an agar gel immunodiffusion (AGID) test against known positive IBD antiserum. The extent of bursal damage may vary considerably with the pathogenicity of

the studied IBDV strain. However, as the samples submitted for virus isolation may vary in virus content, the extent of bursal damage observed in susceptible chickens at the isolation stage gives a limited indication on strain pathogenicity. The bursae of chickens infected with serotype 2 IBDV do not exhibit any gross lesions (OIE, 2008).

Agar gel immunodiffusion test

AGID test is the most useful and simple serological tests used for the detecting IBDV viral antibodies and antigen and antibodies in bursal tissue. Blood samples should be taken early in the course of the disease, and repeat samples should be taken 3 weeks later. This test has also been frequently used for primary screening of BF samples, before attempting isolation and further characterization of IBDV (Dash, *et al.,* 1991). Results are obtained after an incubation period of 48 h. Variability in results may be due to the investigator, as well as the nature of the viral strain used as an antigen

Virus neutralisation tests

Serum neutralization present the disadvantage that specialised equipment and five days incubation are required and is more laborious and expensive than the AGID test, but is more sensitive for detecting antibody and correlates better with the level of protection of the subjects tested (Weisman, *et al.*, 1978). This sensitivity is not required for routine diagnostic purposes, but may be useful for evaluating vaccine responses or for differentiating between IBDV 1 and 2 serotypes. First, 0.05 ml of virus diluted in tissue culture medium to contain 100 TCID50 (50% tissue culture infective doses) per 0.05 ml is placed in each well of a tissue-culture grade microtitre plate or the Reed and Muench. The test sera are heat-inactivated at 56°C for 30 minutes. Serial doubling dilutions of the sera are made in the diluted virus. After 30 minutes at room temperature, 0.2 ml of SPF chicken embryo fibroblast cell suspension, with a cell density allowing confluent layers to be obtained after 24 hours of incubation, is dispensed into each well. Plates are sealed and incubated at 37°C for 4–5 days, after which the minolayers are observed microscopically for typical CPE. The end-point (serum titre) is expressed as the reciprocal of the highest serum dilution that did not show CPE. To reduce test-to-test and operator-to-operator variation, a standard reference antiserum may be included with each batch of tests and the titre of the virus suspension must be

reassessed in each new experiment using a sufficient number of repeats (wells) per virus dilution (Fahey *et al.*, 1991).

Indirect Enzyme-linked immunosorbent assay

ELISA is used for the detection of antibodies to IBDV. The ELISA is the most rapid and sensitive method, and presents the fewest variations due to the viral strain used as an antigen (Roney, *et al.*, 1988). The correlation between results obtained using SN and ELISA is high, ELISA remains less sensitive, and does not detect low neutralizing titers which are sufficient to block vaccine administration. Coating the plates requires a purified, or at least semi purified, preparation of virus, necessitating special skills and techniques. Methods for preparation of reagents and application of the assay were described by Marquardt *et al.* (1980). Commercial kits are available. The test sera are diluted according to the established protocol or kit instructions and each is dispensed into the requisite number of wells. After incubation under the appropriate conditions, the sera are discarded from the plates, and the wells are washed thoroughly. Anti-chicken immunoglobulins conjugated to an enzyme are dispensed into the wells, and the plates are again incubated as appropriate. The plates are emptied and rewashed before substrate containing a chromogen that gives a colour change in the presence of the enzyme used is added to the plate. After a final incubation step, the substrate/chromogen reaction is stopped by addition of a suitable stopping solution and the colour reactions are quantified by measuring the optical density of each well. The Sample to Positive (S/P) ratio for each test sample is calculated (OIE,2008).

2.7. Socio-economic importance

Poultry play an important economic, nutritional and socio-cultural role in the livelihoods of rural households in many developing countries, including Ethiopia, where they are an integrated part of the smallholder production systems and play a significant role in alleviating poverty. Poultry production is particularly important to women, who tend to own and manage chickens. The resulting income is often used to support education of children. However, Infectious bursal disease has been known to pose potential threat to poultry industry all over the world. The economic impacts of IBDV can be attributed to both direct and indirect costs. The economic impact of this disease is related to losses due to mortality, growth retardation or

rejection of carcasses (van den Berg, 2000). A 10-year simulation study of broiler chicken farms in New Zealand suggested that the introduction of classical IBD might cause a loss of US$ 10 million per year (Christensen, 1985). The emergence of highly pathogenic vvIBDV in Europe during 1988 increased the financial impact of the disease and the outbreak of the disease resulted up to 30% mortality in affected farms. Van den Berg and Meulemans (1991) reported the losses up to 60% in 38- day old hybrid leghorn pullets with vvIBDV (849-VB) isolate. And indirect losses can be attributed to immunosuppressive effect of the virus (Allan *et al.*, 1972). This is denoted by high prevalence of viral respiratory infection and vaccine failures against other diseases but also by break of immunity in chickens vaccinated for other diseases (OIE, 2004).

2.9. IBD Vaccines and vaccination

Immunization of chickens with high quality vaccines is the primary method of control of many poultry infectious diseases; therefore IBDV is mainly controlled by vaccination (Van den Berg, 2000; Muller *et al.,* 2003). Two types of vaccine are mostly available for the control of IBD. These are live attenuated vaccines and inactivated oil-emulsion adjuvant vaccines (Thornton *et al.,* 1975). The ideal vaccine must offer the correct balance between Efficacy and innocuity and must not cause disease or bursal lesions, must not be immunosuppressive or excreted, and must confer long-lasting immunity even in birds with a high level of maternal immunity. Unfortunately, such a vaccine with all required quality does not exist (McFerran, *et al.*, 1993). Currently a live recombinant vaccine expressing IBDV antigens has also been licensed (Darteil *et al.,* 1995). To date, IBD vaccines have been made with serotype 1 IBDV only, although a serotype 2 virus has been detected in poultry. The serotype 2 virus has not been associated with disease, but its presence will stimulate antibodies. Serotype 2 antibodies do not confer protection against serotype 1 infection; neither do they interfere with the response to type 1 vaccine. Active immunization with a 'classical' serotype 1 virus or vaccine provides a good protection against the vvIBDVs (Jackwood, 1990). However; the later viruses are less susceptible to neutralization by maternally derived antibodies than 'classical' pathogenic viruses (Van den Berg *et al.,* 2000).

2.9.1. Live vaccines and method of use

Live virus vaccines are very widely used. These are made from strains of virus that have been attenuated by serial passages in Embryonated eggs. Depending on the degree of attenuation, the vaccine strains cause histological lesions of varying severity to the bursae of SPF chickens, and are classified Live as 'mild', 'intermediate', or 'intermediate plus' ('hot'), respectively (OIE,2000). Mild or intermediate vaccines are used in parent chickens to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine. They are susceptible to the effect of maternally derived antibody (MDA) so should be administered only after all MDA has waned. Application is by means of intramuscular injection, spray or in the drinking water, usually at 8 weeks of age (Skeeles *et al.,* 1979). Intermediate or intermediate plus vaccines are used to protect broiler chickens and commercial layer replacements. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with virulent IBD. As mild, intermediate or hot Although intermediate vaccines are susceptible to the presence of MDA, they are sometimes administered at 1-day old, as a coarse spray, to protect any chickens in the flock that may have no or only minimal levels of MDA. This also establishes a reservoir of vaccine virus within the flock that allows lateral transmission to other chickens when their MDA decay. Second and third applications are usually administered, especially when there is a high risk of exposure to virulent forms of the disease or when the vaccinated chicks exhibit uneven MDA levels. The timing of additional applications will depend on the antibody titres of the parent birds at the time the eggs were laid. As a guide, the second dose is usually given at 10–14 days of age when about 10% of the flock is susceptible to IBD and the third dose 7–10 days later. The route of administration is by means of spray or in the drinking water. Intramuscular injection or eye-drop is used rarely. If the vaccine is given in the drinking water, clean water with a neutral pH must be used that is free from smell or taste of chlorine or metals. (OIE, 2000).

2.9.2. Inactivated vaccines and method of use

Inactivated IBD vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeding hens that have previously been primed by live vaccine or by natural

exposure to field virus during rearing (Cullen *et al.,* 1976). The usual programme is to administer the live vaccine at about 8 weeks of age. This is followed by the inactivated vaccine at 16–20 weeks of age. Occasionally, inactivated vaccines may be used in programmes combining inactivated and live vaccines, in young valuable birds with high MDA levels reared in areas with high risk of exposure to virulent IBDV. The preferred routes are intramuscular into the leg muscle. A multidose syringe may be used. All equipment should be cleaned and sterilized between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. Only healthy birds, known to be sensitized by previous exposure to IBDV, should be vaccinated. Used in this way the vaccine should produce such a good antibody response that chickens hatched from those parents will have passive protection against IBD for up to about 30 days of age (Wyeth *et al.,* 1979). This covers the period of greatest susceptibility to the disease and prevents bursal damage at the time when this could cause immunosuppression. However, if there is a threat of exposure to infection with very virulent IBDV, live vaccines should be applied as described above. The precise level and duration of immunity conferred by inactivated IBD vaccines will depend mainly on the concentration of antigen present per dose. (OIE,2004).

2.10. Control and eradication of IBD: global challenge

IBD viruses show poor cross-immunity amongst strains, making the selection of the appropriate vaccine strain the cornerstone for disease control. Furthermore, live attenuated vaccines are commonly included in vaccination programmers, making it necessary to differentiate them from field strains. Antigenic variant strains are not neutralized by classical vaccine strains and, therefore, the determination of the antigenic subtype a virus involved in an outbreak is necessary to optimize vaccine programmes (Lukert and Saif, 1997). Determination of the subtype by genotyping, together with longitudinal studies, helps in designing the vaccination program that best fits the needs of a particular farm.

Vaccines developed against the Gumboro disease were effective for about 25 years. In 1987, a very virulent variant of the virus (vvIBDV) appeared and spread in many countries causing severe economic losses. The traditional vaccines were not effective because the new strain varied antigenically, was more virulent and penetrated through maternal antibodies that had provided protection in the past (Lukert *et al.,* 1982; Brown *et al.,* 1994). The new live

vaccines that were developed were intermediately rather than fully attenuated in order to stimulate a strong enough immune response that would confer protection against the very virulent strain. However, these new intermediate live vaccines may cause immunosuppression and interfere with immunization with other vaccines like NDV and Marek's disease (Faragher, 1974). Another efficient way of combating vvIBDV is to use inactivated vaccines, which have to possess high titers of virus in order to be effective. One of the most effective inactivated vaccines, especially in highly affected areas, is produced from bursa-derived antigen. However, its production requires infection with vvIBDV and the sacrifice of a large number of chicks in order to obtain the antigen although it seems that a live recombinant vaccine expressing IBDV VP2 antigens licensed has supposed to solve these problems associated with conventional vaccines, technological limitations impaired its wider application (Darteil *et al.,* 1995).

2.11. Distribution

IBDV has a worldwide distribution, occurring in all major poultry producing areas. According to Office International des Epizooties (2004) it was estimated that IBD has considerable Scio-economic importance at the international level as the disease is present in >95% of the member countries and the occurrence of acute clinical cases (vvIBDV) was reported in 80% of the countries. In some important poultry producing countries like the USA, Australia and New Zealand, no cases of vvIBD have been reported. However, many European countries have reported the isolation of vvIBDV, Di Fabio *et al.*, (1999) reported that these strains spread to South Asia, Asia, and Middle East and now distributed world wide causing economical loss due to the mortality and immune-suppression effect of the disease.

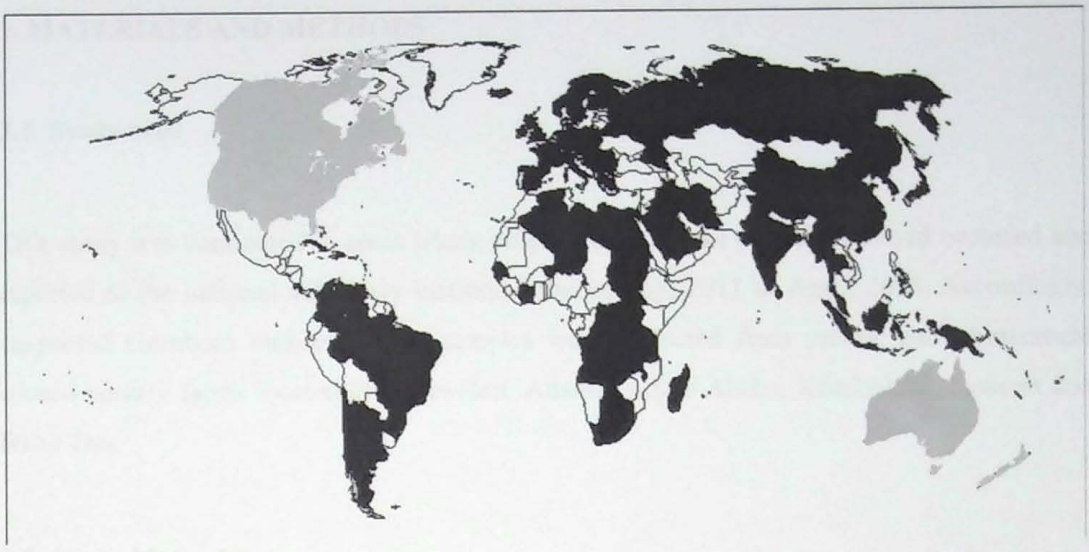


Figure 1. Worldwide geographical distribution of the very virulent forms of IBDV, Black-colour where acute case have been reported; grey- colour where no acute forms have been reported; white- countries with no report (updated from Eterradossi, Technical Report,1995).

2. 12. Current status of IBD in Ethiopia

Few studies on the prevalence of the disease have been done on chickens in some parts of the country by different researchers in backyard production system and in commercial farms. In both production systems seroprevalence of IBD were reported. The most probable reason for the existence of this disease may be via importing high producing exotic birds from abroad. The sero-prevalence of Infectious Bursal Disease by using I-ELISA was reported to be 83.33% in south west and west Shoa regions in backyard local chickens (Degefu *et al.*, 2010). Gumboro disease was first reported in 2002 in Ethiopia at a privately owned commercial poultry farm in which 45-50% mortality rate was documented (Zeleke *et al.*, 2005; Solomon and Abebe 2007). In addition to this report seropositivity was reported of 98.9% by Agar Gel Immuno-deffusion test in Amhara region (Andasa farm). In all cases the situation of the disease at small scale commercial flocks, and back yard poultry farms indicate the disease is widely distributed in the county and call for detail prevalence and epidemiological investigation.

3. MATERIALS AND METHODS

3.1. Study area

This study was conducted in areas where suspected outbreaks of Gumboro had occurred and reported to the national veterinary institute between Sep, 2011 to April, 2012. Accordingly, suspected Gumboro outbreak bursa samples were collected from private and government owned poultry farms located at Debre-Zeit, Adama, Addis Ababa, Kombolcha, Nekemt and Bahir Dar.

3.2. Study Methodology

Suspected outbreaks of Gumboro were investigated and bursa sample were collected during the study period. In addition to this, bursa samples collected from different outbreaks and stored at National Veterinary Institute virology laboratory since September 2009 were included in this study.

3.3. Study animals

The study was conducted in chickens that had experienced outbreaks of Gumboro, Chickens reared under semi-intensive and intensive production and management system were included in the study.

3.4. Sample collection and transportation

Bursa of fabricious was collected aseptically from IBD suspected clinically sick chickens after killing. Bursa samples were placed in sterile universal bottle and the samples were transported to the National Veterinary Institute virology laboratory using transport medium (glycerol, phosphate buffer saline and antibiotics) or under cold condition. In the laboratory samples were processed immediately or kept at -20 ^{0}C awaiting processing (OIE, 2004).

3.5. Laboratory investigation

3.5.1. Virus isolation

Bursa samples were first chopped into small pieces using two sterile scalpel blades, and further ground in to smaller pieces using mortar and pestle. Three to five bursa samples collected from the same outbreak area were pooled and processed to increase the chance of virus isolation. A 10% suspension of bursa samples were prepared in sterile phosphate buffer saline (PBS) supplemented with penicillin and streptomycin (1000 μg/ml each). The suspension was transferred into sterile tube and centrifuged at 3000g for 10 minutes. Supernatants were harvested and filtered using a 0.22μm Millipore filters. The filtrate was used to inoculate confluent primary chicken fibroblast cell as well as Vero cell cultures in maintenance GMEM containing 2% bovine fetal calf serum and incubated at 37^0C. Cultures were observed microscopically for up to 1 week to check for the presence of cytopathic effect (CPE) characteristic of IBDV. Those that had not showed CPE were blindly passaged up to the third passage to let the wild viruses get adapted the Vero cell cultures. Samples negative for CPE after the third passage were considered negative. Those that were positive for CPE were further analyzed by molecular techniques.

3.5.2. RNA extraction and reverse transcription

This molecular analysis of samples were conducted both at molecular biology laboratory of the National Veterinary Institute and The Institute of Infection and Global Health, University of Liverpool, Leahurst Campus, CH64 7TE, United Kingdom. Bursa suspensions as well as CPE positive culture supernatants were sent to University of Liverpool using FTA cards. RNA was eluted from FTA card by placing a section of the filter card (approximately 0.5 cm x 0.5cm) in 300μl of Elution buffer (Qiagen), vortexing and incubating on ice for approximately 15 minutes. Subsequently, 140μl was further processed using the QIAGEN Viral RNA extraction kit as outlined by the manufacturer. Similarly, at The National Veterinary Institute the total RNA was extracted directly from bursa suspension and culture supernatants by using RNeasy mini kit (Qiagen) based on the manufacturer protocols. Briefly, RNA was extracted using Qiagen RNeasy mini spin column kit. Accordingly, 460μl lysis buffer RLT was added to a 1.5 ml eppendorf tube containing 460 μl of infected cultured Vero

cell suspension. Four hundred and sixty µl 70% ethanol was applied to precipitate nucleic acids released from disrupted host/Vero/ cells due to lysis buffer RLT followed by homogenization. The homogenized suspension was transferred to RNeasy spin column and centrifuged where viral nucleic acids released bound to the silica membrane and the fluid part passed through the membrane down to the collection tube and the flow through discarded. The nucleic acids bound to the membrane were washed by using 700 µl wash buffer RW1 followed by addition of 500 µl RPE buffer and centrifuged to dry the membrane and the flow through discarded. Finally, the nucleic acids bound to the silica membrane were eluted into eppendorf tube using 50µl RNase free water and the eluted RNA was used for cDNA synthesis.

Complementary DNA was generated from RNA using the reverse transcriptase RevertAid™ (Fermentas). RNA was first incubated at 95°C for 3 minutes and placed on ice for at least 3 minutes in the presence of the gene specific primer L2 (5'-GATCCTGTTGCCACTCTTTC-3'), which binds nucleotides 1194 to 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 minutes and the reverse transcriptase inactivated at 70°C for 10 minutes.

3.5.3. PCR amplification and sequencing

RT-PCR were performed in the molecular biology laboratory of the NVI following three steps: extraction of nucleic acids using the commercially available RNA extraction kit, reverse transcription (RT) of IBD virus RNA into cDNA using reverse transcriptase, and amplification of the resulting cDNA by PCR using Taq DNA polymerase and specific designed primers based on the sequence of the virus. The parameters for each PCR cycle were set according to the supplier's recommendations as explained below. Analysis of the PCR products were carried out by 1.5 % (w/v) Agarose electrophoresis gel containing ethidium bromide. Each PCR experiment conducted in the presence of negative and positive control reactions. The different produced viral band pattern produced were determined using 1Kb ladder (marker) that was loaded on a separate lane (OIE, 2004; Thierry *et al.*, 2004).

Polymerase chain reaction (PCR) amplification of products intended for sequencing was carried out using a high fidelity DNA polymerase, *Pfu* DNA polymerase (Fermentas) at University of Liverpool. A typical 25µl 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 to 1213 and 658 to 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 minutes, 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minutes and 1 cycle at 72°C for 7 minutes. The amplified 604 base pair product contained the VP2 hypervariable region (HVR) coding sequence. Amplicons were extracted from agarose gel using QIAGEN Gel extraction kit; with the concentration of extracted DNA was determined spectrophotometrically using a NanoDrop Spectrophometer 1000 (Thermo Scientific). Purified amplicons were sequenced using both L2 and U2 primers by a commercial sequence provider (Macrogen) using the BigDye terminator cycling (Applied Biosystems) condition and analysed by the automated sequencer ABI 3730XL.

3.5.4. Sequence and phylogenetic analysis

A total of 20 isolates were sent to Liverpool University using FTA cards for further characterization by sequencing the VP2 gene. Sequences were analysed using Geneious (Drummond *et al.,* 2011). The deduced amino acid sequence (amino acid position 191 to 350) of Ethiopian IBDV isolates sequenced in this study were in alignment to the VP2 HVR 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 to 1190), spanning the HVR at nucleotide position 845 to 1126 (Bayliss *et al.,* 1990).

3.5.5. Adaptation and attenuation trial of local isolates for vaccine seed

The results obtained from sequencing of viruses were used to select local isolates intended for adaptation and attenuation. Because of the high risk of reversion and residual virulence, the classical virulent IBD virus was selected and used to infect healthy, semi confluent monolayer of Vero cells (Ahasan *et al.,* 2002). A 0.2 ml of virus inoculum that had passed through 0.22

µm pore size filters (Whatman Int. Ltd., England) was used to infect Vero cell cultures. The flasks were incubated at 37 °C in the presence of 5% CO2 in CO2 incubator. The culture fluid was harvested each time by three freeze-thaw cycles and clarified by centrifugation at 5000 rpm for 5 min (Peilin *et al.,* 1997). The IBD virus was passaged serially for adaptation and attenuation. The virus was identified on Vero cells through characteristic cytopathic effects (CPEs). For confirmation, culture supernatant from each passage was subjected to RT-PCR.

3.5.6. Indirect Enzyme Linked Immuno-Sorbent Assay (I-ELISA)

An IBD antibody test kit (IDEXX, FlockChek) was used according to manufacturer's instruction (Annex 1) to screen chickens from maternally derived antibody against IBD before assigned into treatment groups and also to determine antibody titre after vaccination.

3.5.7. Pathogenicity testing

Sixty day-old broiler chicks free from maternal antibodies against IBD virus, as confirmed by FlockChek I-ELISA Kit were assigned randomly into three treatment groups of 20 birds each. At 15 days old, the chicks of group one and two were vaccinated of the currently produced NVI IBD vaccine and the 9^{th} Vero cell passaged classical virulent virus (10^5 mean tissue culture infectious dose TCID50/bird), respectively. The control birds (group 3) were inoculated similarly with 0.2 ml normal saline. Fifteen days post vaccination, blood samples were collected from wing vein using 3ml disposable syringe for serum antibody analysis and all the three groups were challenged with 0.2 ml vvIBDV 10^2 mean embryo infectious dose ID50/bird (0.1 ml intraocular and 0.1 ml intranasal). Chicks were examined three times a day for clinical signs. At days 7 post-challenge, all birds were slaughtered and gross pathological lesions were observed and bursa were weighed (Abdel *et al.,* 2001).

3.6. Data analysis

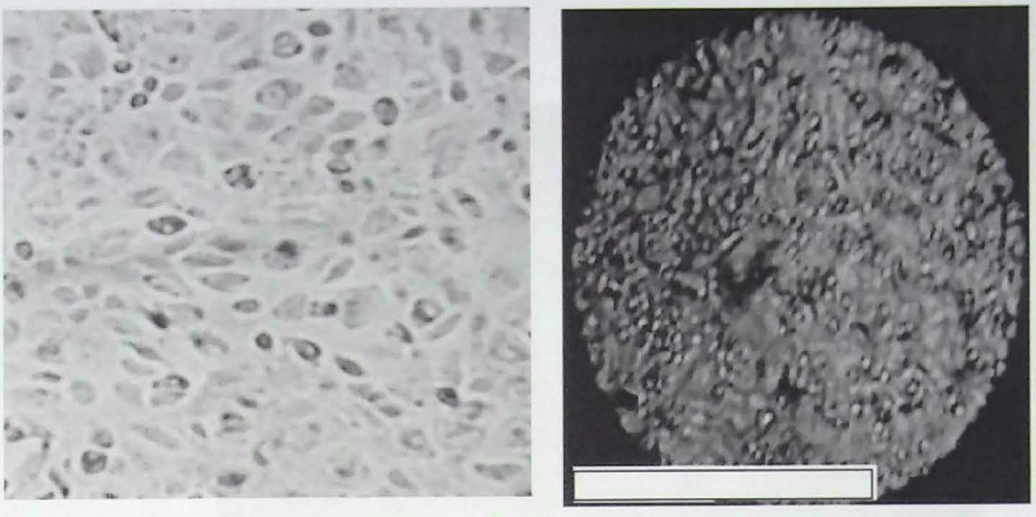
Data collected were inserted into MS excel spread sheets and SPSS version 15 was used to analyse the data. Descriptive statistics and ANOVA were used to analyze the variation in mean antibody titer and bursa weight among treatment groups. 5% absolute precision was used throughout this study. Nucleotide alignment was performed using ClustalW, within

Mega 5.1 (Tamura *et al.,* 2011), with the phylogenetic analysis inferred using the neighbor-joining method (Eterradossi *et al.*, 1999).

4. RESULTS

4.1. IBD virus isolation

A total of 136 bursa samples were collected from different outbreaks of the disease and from NVI virology laboratory. Bursa samples were processed and cultured onto both semi-confluent primary CEF cell cultures as well as fully confluent established cell line (Vero). Confluent primary CEF cultures were prepared from SPF eggs incubated for nine days. In this study healthy and confluent monolayer of Vero cells were formed following 48 hours of growth in GMEM growth medium containing10% bovine fetal calf serum. Since it is believed that in most cases a single type of virus circulates in one outbreak, 3-5 bursa samples obtained from the same outbreak were pooled and processed together to improve the chance of virus recovery and improve the sensitivity of the virus isolation assay. Out of 44 pooled samples, viruses were isolated from 41 (93.2%) preparations. All the isolates were grown and able to demonstrate CPEs on CEF cell cultures on the first passage. Similarly, 32 (78%) of the isolates including the vaccinal strain of the National Veterinary Institute used as a positive control in this study were adapted and grown on Vero cell cultures on the first passage. The rest, 9 (22%) isolates showed visible CPE on Vero cell cultures on the third passage. Virus growth on cell cultures was evidenced by clear and consistent CPEs up to the ninth passage. The type of CPEs observed includes aggregation and rounding of cells, vacuole formation and finally granulation (figure 2).



A B

Figure. 2. (A) Confluent monolayer of Vero-cells grown in GMEM growth medium 48 hours post-culturing (100×); (B) Cytopathic effects (CPEs) of IBD virus on Vero-cell line at third passage 72 hours post-infection (100×).

4.2. Detection of viral RNA by RT-PCR

The two-round amplification reaction was used to amplify a 647 bp fragment, from nucleotide position 737 to 1228 (Bayliss *et al.,* 1990) of the hypervariable region of the VP2 gene and allowed us to reach a definite conclusion of the causative agent of the outbreak. RT-PCR was used for detection of IBDV directly from bursal samples and cell culture passaged material. Forty one pooled bursal samples along with NVI vaccine strain were tested for IBDV genome. All the bursal samples suspension as well as the NVI vaccine strain showed the presence of 647 bp PCR product (figure 3). Similar results were also obtained when PCR was carried out on the cell culture supernatants. The bursal sample, which was negative for virus isolation was also negative in RT-PCR. No amplification was detected in the uninfected cell culture.

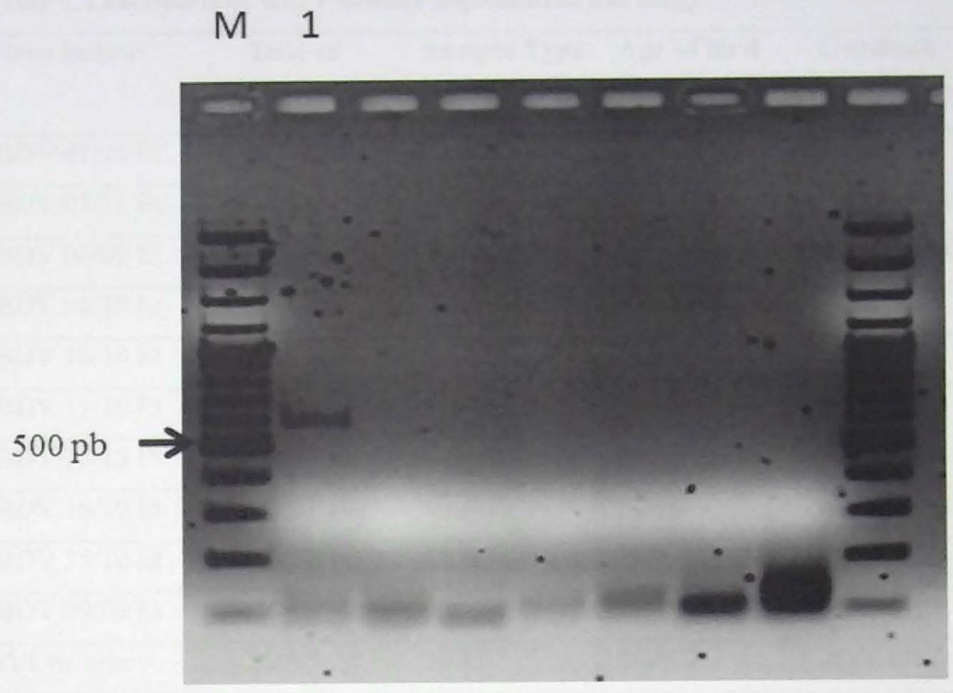


Figure 3. Gel picture of RT-PCR tested samples, where positive result on lane 1 showing band size of 647 bp PCR product and lane M is 100bp marker.

4.3. Nucleotide and deduced amino acid sequence

The RNA genome of 20 different isolates out of the 41 isolates were selected and sent to the Institute of Infection and Global Health, University of Liverpool, Leahurst Campus, CH64 7TE, United Kingdom using FTA cards for further molecular analysis, nucleotide and deduced amino acid sequence. Samples were selected based on their severity of clinical signs, geographical area and vaccination history of the affected flocks. The NVI vaccine strain was also sent anonymously to compare with local isolates. Due to the poor stability nature of the RNA genome, only 10 of the genomes were recovered from the FTA cards and sequenced accordingly. The Genbank accession number codes of the 10 isolates genome and the vaccine strain is shown below in (Table 1).

Table 1. Description of IBDV isolates sequenced in this study

Virus isolate	Date of collection	Sample Type	Age of bird (days)	GenBank Accession No.
IBDV 01/10 Et	12/02/10	Bursa	7	JQ684021
IBDV 03/11 Et	14/07/11	Bursa	31	See JQ68401
IBDV 04/09 Et	07/09/09	Bursa	30	See JQ68401
IBDV 06/10 Et	28/04/10	Bursa	31	JQ684020
IBDV 10/10 Et	09/12/10	Bursa	85	JQ68401
IBDV 11/10 Et	8/09/10	Bursa	29	JQ684016
IBDV 17/10 Et	04/05/11	CL(p10)	N/A	JQ684017
IBDV 16/10 Et	27/07/10	CL(p7)	N/A	See JQ684018
IBDV 15/10 Et	31/12/10	CL(p1)	N/A	JQ684018
IBDV 09/09 Et	24/08/09	Bursa	30	JQ684019
NVI vaccine	N/A	N/A	N/A	JQ684022

N/A- Not Available

CL-Cell lysate (passage number)

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%. Infectious bursal disease virus isolates IBDV 15/10 and IBDV 16/10 showed 100% identity over the region sequenced and were genetically related to IBDV 11/10, IBDV 6/10, IBDV 17/10 and IBDV 01/10 (nucleotide identity 99.8%). Infectious bursal disease isolates IBDV 03/11, IBDV 04/09 and IBDV 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 (IBDV 23/11) ranged between 90.2% and 94.6% in nucleotide identify.

The deduced amino acid sequence of the hypervariable region was determined for each of the isolates and compared to well characterised classical virulent IBDV isolates (F52/70), classical attenuated IBDV isolates (IBDV 78) and vvIBDV isolates (vvIBDV UK 661) (figure 3). Four Ethiopian IBDV isolates (IBDV 03/11, IBDV 04/09, IBDV 09/09 and IBDV 10/10) contain the genetic signature of vvIBDVs, specifically, A222, I256, I294, S299 (Brown et al., 1994), however, lacked L324 and 321V, characteristic of antigenically atypical vvIBDV

(Eterradossi *et al.,* 1998). Six of the IBDV isolates (IBDV 12/10, IBDV 15/10, IBDV 11/10, IBDV 06/10, IBDV 17/10 and IBDV 16/10) have an amino acid sequence which contained amino acids linked to propagation in cell culture (N279 and T284) (Mundt, 1999).

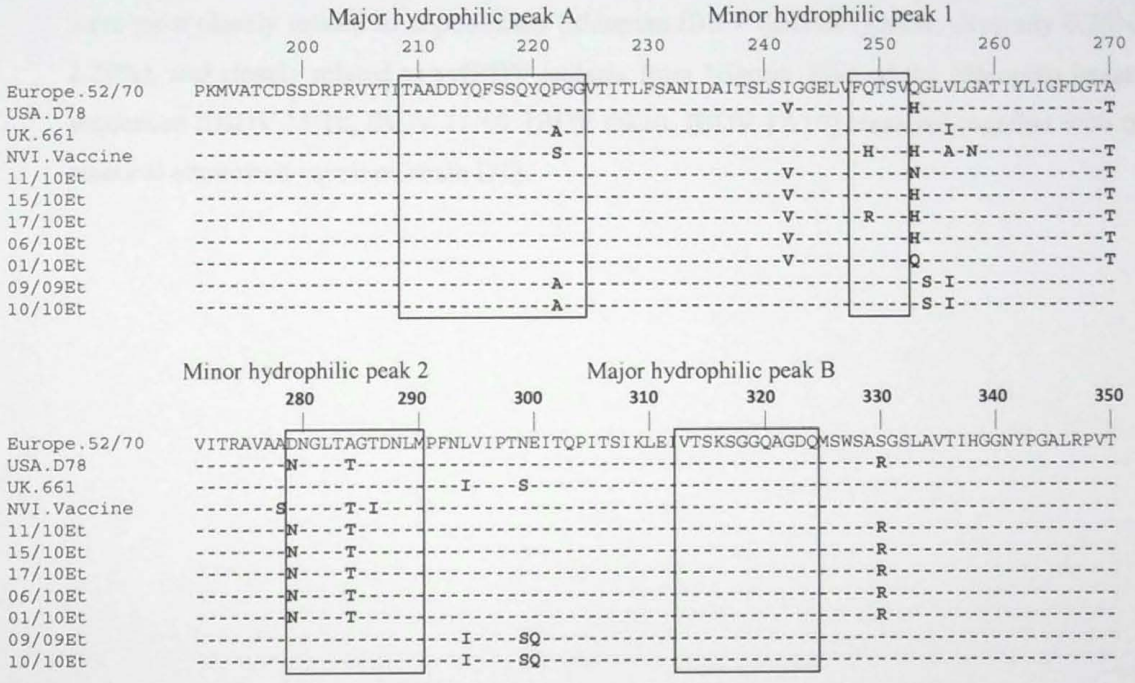


Figure 4. Deduced amino acid alignment of VP2 hypervariable region of Ethiopian IBDV isolates sequenced in this study (Table 1); classical IBDV (Europe.F52/70, Y14958); classical attenuation IBDV (USA.D78, EU162087); vvIBDV (UK.661, Z25480) and an Ethiopian produced IBDV vaccine (NVI Vaccine).

4.4. Phlyogenetic analysis

The VP2 HVR nucleotide sequence of Ethiopian IBDV isolates IBDV10/10, IBDV 09/09, IBDV 15/10, IBDV 06/10; IBDV 17/10 and IBDV 11/10 were used in a nucleotide database search BLAST [http://blast.ncbi.nlm.nih.gov/Blast.cgi] to identify IBDV isolates with highest identity. For simplicity, Ethiopian IBDVs with 100% nucleotide identity to another Ethiopian IBDV were removed from the analysis. The phenotypic relationship between IBDV isolates

showing high nucleotide identity to the Ethiopian IBDVs sequenced in this study, in addition to several well characterised vvIBDVs and Classical IBDVs (inc. variant strains and attenuated vaccine strains) was inferred using the Neighbor-joining method (figure 5). The Ethiopian IBDV isolates sequenced in this study represent two distinct genetic lineages (1) vvIBDV and (2) Classical IBDV strains. Ethiopian isolates IBDV 10/10 and IBDV 09/09 were most closely related to unpublished Ethiopian IBDV isolates (genetic diversity 0.76% - 2.29%), and closely related to vvIBDV isolates from Nigeria. Five of the Ethiopian isolates sequenced (IBDV 15/10, IBDV 11/10, IBDV 06/10, IBDV 17/10) clustered together with the classical attenuated vaccines strain D78.

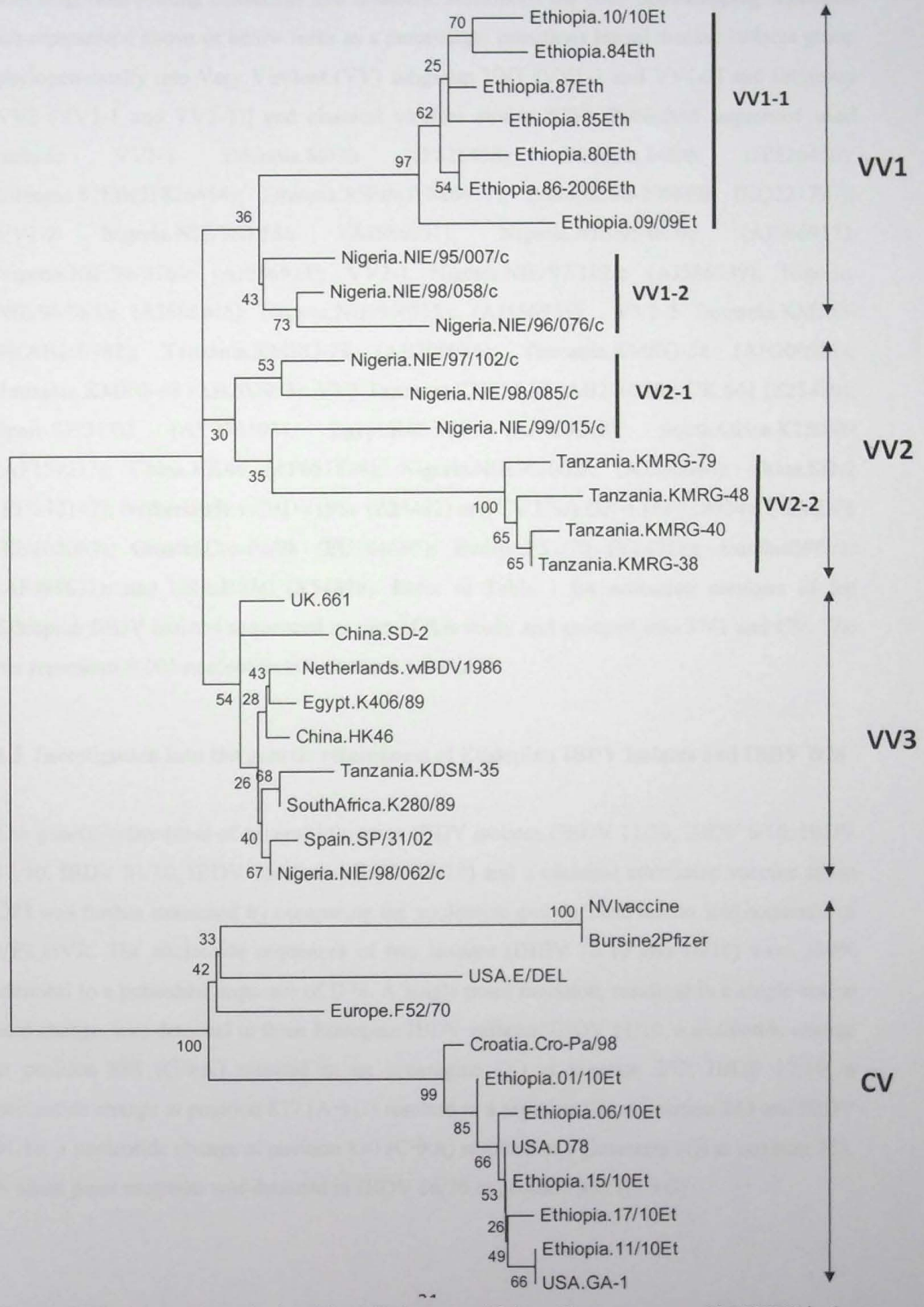


Figure 5. Phylogenetic analysis of the VP2 hypervariable coding sequence of 38 IBDV isolates

The neighbour-joining consensus tree is shown. Results of the 1000 bootstrapping replicates are represented above or below forks as a percentage. Infectious bursal disease isolates group phylogenetically into Very Virulent (VV) subgroup VV1 (VV1-1 and VV1-2) and subgroup VV2 (VV2-1 and VV2-2)] and classical virulent strains (CV). Published sequenced used include: VV1-1 Ethiopia.80Eth (JF826458); Ethiopia.84Eth (JF826456); Ethiopia.87Eth(JF826454); Ethiopia.85Eth(JF826457); Ethiopia.86-2006Eth (HQ231797); VV1-2 Nigeria.NIE/98/058/c (AJ586951); Nigeria.NIE/95/007/c (AJ586917); Nigeria.NIE/96/076/c (AJ586924); VV2-1 Nigeria.NIE/97/102/c (AJ586939); Nigeria. NIE/98/085/c (AJ586945); Nigeria.NIE/99/015/c (AJ586955); VV2-2 Tanzania.KMRG-40(AB200982); Tanzania.KMRG-79 (AB200986); Tanzania.KMRG-38 (AB200981); Tanzania.KMRG-48 (AB200983); VV3 Tanzania.KDSM-35 (AB200980); UK.661 (Z25480); Spain.SP/31/02 (AY770593); Egypt.K406/89 (AF159218); SouthAfrica.K280/89 (AF159217); China.HK46 (AF051838); Nigeria.NIE/98/062/c (AJ586946); China.SD-2 (EU042147); Netherlands.vvIBDV1986 (Z25482) and CV USA.GA-1 (EF418034); USA.D78 (EU162087); Croatia.Cro-Pa/98 (EU184689); Europe.F52/70 (Y14958); Bursine2Pfizer (AF498631); and USA.E/Del (X54858). Refer to Table 1 for accession numbers of the Ethiopian IBDV isolates sequenced as part of this study and grouped into VV1 and CV. The bar represents 0.005 nucleotide substitutions per site.

4.5. Investigation into the genetic relatedness of Ethiopian IBDV isolates and IBDV D78

The genetic relatedness of several Ethiopian IBDV isolates (IBDV 11/10, IBDV 6/10, IBDV 17/10, IBDV 01/10, IBDV 16/10 and IBDV 15/10) and a classical attenuated vaccine strain D78 was further examined by comparing the nucleotide and deduced amino acid sequence of VP2 HVR. The nucleotide sequences of two isolates (IBDV 15/10 and 16/10) were 100% identical to a published sequence of D78. A single point mutation, resulting in a single amino acid change, was detected in three Ethiopian IBDV isolates: IBDV 11/10, a nucleotide change at position 888 (C→A) resulted in an asparagine (N) at position 253; IBDV 17/10, a nucleotide change at position 877 (A→G) resulted in a arginine (R) at position 249 and IBDV 01/10, a nucleotide change at position 890 (C→A) resulted in a glutamine (Q) at position 253. A silent point mutation was detected in IBDV 06/10 at position 965 (A→G).

4.6. Adaptation trial of local isolates for vaccine seed

From the above sequencing result, classical variant strain (IBDV 06/10) was selected for adaptation trial. The basis for the selection was its genetic closeness to NVI vaccine strain. Although this isolate had a silent point mutation, it had caused severe outbreak in one of the big poultry farm at Debre-Zeit. This isolate was easily adapted and produce CPE in Vero cell cultures beginning from the first passage. It had also passed serially to the ninth passage. The CPEs were clear and consistent up to ninth passage and observed at 72 hour post inoculation. A clear band was produced in RT-PCR, which confirmed the presence of IBDV in culture supernatant harvested.

4.7. Attenuation and pathogenecity testing

There were no any clinical signs of IBD manifested by two of the vaccinated groups of chicken at the end of the observation time. This finding indicated that the classical variant strain that had reached the 9^{th} passage lost its virulence and safe for chickens. Sera samples collected at day 15 had mean antibody titre of 2784.12±92.34 and 3281.35±101.43 for group 1 and group 2, respectively. Higher antibody titre was observed in chickens that were vaccinated with the 9^{th} passaged classical variant strain and this mean antibody titre difference between the two groups was statistically significant ($P<0.05$). Similarly, sera samples collected and tested from the unvaccinated control groups had remained unchanged (negative).

Chickens in all treatment groups had survived the challenge trial. However, mild clinical signs including watery diarrhoea and reduce appetite were observed in the unvaccinated groups. Seven days post challenge, all chickens were slaughtered and post mortem examination was conducted. Severely oedematous, haemorrhagic and enlarged bursa and haemorrhages on thigh and pectoral muscles were observed in unvaccinated control groups. No pathological lesions were observed from the other two groups. Immediately following slaughter, bursal tissues were carefully removed and weighed. The mean bursa weight from the unvaccinated control groups was 20.8±1.2 g while group one and two had 3.6±0.16g and 2.8±0.07g, respectively. Although difference in the mean bursa weigh between the vaccinated groups was observed, this difference was not statistically significant ($P>0.05$). However, the

difference in the mean bursal weight between control and vaccinated groups was statistically significant ($P<0.05$).

5. DISCUSSION

Adapting IBDV strains to replicate in cell culture has historically been used to attenuate these viruses (Wang, *et al.*, 1997). A number of continuous cell lines of mammalian origin are reported to be susceptible to IBDV. The use of these cell lines has several advantages over the use of primary cell culture of avian origin. Continuous cell lines are easier to handle and maintain and free from vertically transmitted extraneous viruses of avian origin (Jackwood *et al.*,1987; Hassan *et al.*, 1996). Among continuous cell lines, Vero-cell line has been used for the growth and replication of IBD virus (Kibenge *et al.*, 1988; Peilin *et al.*, 1997; Ahasan *et al.*, 2002). Vero cells, an approved substrate for virus vaccine production was also used in the present study in attempt to adapt and attenuate indigenous IBD virus and to prepare Vero-cell derived vaccine. In this study IBDVs were successfully adapted and grown on Vero cell cultures. In agreement with Rasool and Hussain, (2006) all viruses used in this study were able to produce CPF after the third passage and the CPEs were clear and consistent up to ninth passage and observed at 72 hours. As described by (Hitcher, 1970; Rosenberger *et al.*, 1986) the traditional isolation method for IBD virus using the ECF cell of 9 to 11-day-old chicken embryos is no longer reliable, as some variant strains of virus cause no embryo mortality. In addition most field isolates cannot be readily adapted to grow in primary chicken cell cultures (McFerran *et al.*, 1980; Lee *et al.*, 1986). These primary cell cultures produce low yield of virus, may be contaminated with some extraneous virus of avian origin and have limited growth properties (Lukert *et al.*, 1975).

Due to the high resistance of IBDV to environmental exposure and its wide distribution, hygienic measures alone, while essential, are often insufficient. Active immunization with a 'classical serotype 1 vaccine provides a good protection against the vvIBDVs (Lutticken, 1997), however the latter viruses are less susceptible to neutralization by maternally derived antibodies than 'classical' pathogenic viruses (Van den Berg *et al.*, 1991). Reversion of IBDV has been described by Raue *et al.*, (2004). Using site-directed mutagenesis, they partially attenuated a vvIBDV strain. Following inoculation of commercial chickens, the mutant strain reverted to the wild-type phenotype during replication in the bursa of fabricius. They concluded that several rounds of IBDV replication provided the opportunity for the mutant virus to revert to the very virulent phenotype (Jackwood *et al.*, 2008).

Infectious bursal disease viruses belonging to the vvIBDV genotype are circulating in Ethiopia. The amino acid characteristic of vvIBDVs (222A, 256I, 294I and 299S (Brown *et al.,* 1994) were detected in the vvIBDV analysed. Phylogenetic analyses of these and other unpublished Ethiopian IBDV sequences demonstrate the clustering of Ethiopian vvIBD viruses within the tentatively named African VV1 lineage (Kasanga *et al.,* 2007), independent of the Asian/European lineage (VV3). Although Ethiopian and Nigerian vvIBDVs share a common ancestor, VP2 HVR sequences group into two distinct clusters (tentatively named VV1-1 and VV1-2) within VV1. Sequence divergence from a single origin within multiple countries is consistent with the proposed evolution of the two distinct clusters in the African VV2 lineage, specifically, VV2-1 (Nigerian vvIBDVs) and VV2-2 (Tanzania vvIBDVs) (Kasanga *et al.,* 2007). Investigations into the molecular epidemiology of vvIBDV in other African countries (such as Uganda, Sudan and Kenya) where their VP2 HVR sequence data is not publically available, may provide further phylogenetic insight into the origin and molecular epidemiology of vvIBDVs.

Ethiopian vvIBDV isolates show considerable genetic homogeneity (0.0% - 2.6%), with phylogenetic analysis suggesting a single origin. This is in contrast to the sequence heterogeneity detected among vvIBDV isolates from other African countries. In Nigeria, suggested to be the origin of vvIBDV, vvIBDVs show a high level of genetic heterogeneity (5.7%) and two distinct genetic clusters (specifically VV1 and VV2-1) (Owoade *et al.,* 2004), while there is evidence of vvIBDV from the Asian/European lineages circulating in both Tanzania (Kasanga *et al.,* 2007) and Nigeria (Zierenberg *et al.,* 2000). Three of the four vvIBDV isolates sequenced as part of this study had 100% nucleotide identity in HRV VP2, despite being isolated over two years. Genetic stability over time within the HRV VP2 has been detected in Nigeria (Owoade *et al.,* 2004) and Italy (Martin *et al.,* 2007), with low immune pressure or presence of a non replicative, static virus source (Owoade *et al.,* 2004).
Six of the 10 Ethiopian IBDV isolates sequenced as part of this study were phylogenetically related to classical attenuated vaccine IBDV strains (D78). These Ethiopian IBDV isolates contained amino acid markers of cell culture passage 279N and 284T (Mundt, 1999), despite the sequence being determined directly from bursal viral RNA in two out of the five isolates (IBDV 11/10 and IBDV 06/10). In contrast to the attenuated vaccine strain D78, these viruses were isolated from the bursas of chickens showing significant gross pathology. Virulent genetic variants of the classical attenuated vaccine strain (IBDV D78), such as GA-1, have

2007), virulent classical viruses, genetically related to current attenuated classical vaccine strains, are responsible for outbreaks. Therefore, ensuring appropriate vaccine usage should be adequately addressed before attenuated vvIBDVs are used for vaccine production.

In this study we have reported IBDV with the genetic markers of typical vvIBDVs circulating in Ethiopia. Ethiopian vvIBDVs cluster phylogenetically with vvIBDVs from Tanzania and Nigeria in an African genetic lineage, independent of the Asian/European genetic lineage. Virulent IBDVs lacking the genetic markers of vvIBDV, however, genetically related to the attenuated classical vaccine strains, D78 were also detected. Information from this study could be used to guide IBDV vaccine selection in Ethiopia and further supports an independent vvIBDV African lineage.

In this study most of the field viruses (78%) could grow and produce CPE on Vero cell at first passage. All these viruses have a genome of classical variant strains which is closely related to the vaccinal D78 strain. In agreement with Rasool and Hussain, (2006), however, 22% of the isolates had produced CPE after the third passage on Vero cell. Sequence analysis of these viruses indicated that these viruses had a genome sequence typical of vvIBDV. It has been well documented that vvIBDV, cannot directly adapt to tissue culture (Lim *et al.,* 1999).

In agreement with Rasool and Hussain, (2006) the pathogenicity of very virulent IBD virus (vvIBDV) isolated from field outbreaks reduced considerably after serial passages in Vero cells. In this study the 9th Vero cell passaged classical variant virus that was inoculated into susceptible chickens did not produce any clinical sign. The passage-9 virus showed mean bursa weight comparable to or even less than the vaccinal strain used by The National Veterinary Institute. The birds vaccinated with passage-9 virus showed no gross lesions in bursae following challenge with vvIBDV.Indicating that it had conferred protective immunity against the virus challenge.

In this study the ninth passage virus was evaluated as live attenuated vaccine. After vaccination with this Vero cell attenuated isolate, no apparent untoward reaction was observed. In agreement with Rasool and Hussain, (2006) the results of mean bursa weight 7 days post-challenge were significantly lower in vaccinated groups than non vaccinated group. In the present study the local classical variant virus attenuated via Vero cell passage had induced better antibody titer and reduced mean bursa weight than the vaccinal strain used by

The National Veterinary Institute. Although the mean bursa weight was not statistically ($p>0.05$) significant between the vaccinated groups following challenge, the difference in antibody titer was statistically significant ($p<0.05$).

6. CONCLUSIONS AND RECOMMENDATIONS

IBD is important health problem of poultry production all over the world. Currently the strategy used to control infectious bursal disease is mainly through vaccination using classical low pathogen IBDV. However, the possibility of antigenic mutation which can be one of the causes of less protection in population jeopardizes the efficacy of the available vaccines. Therefore, it is highly recommended to develop indigenous vaccine from local isolates and make efficacy trial to demonstrate the effectiveness.

It has been identified that infectious bursal disease viruses belonging to the vvIBDV genotype are circulating in Ethiopia. Phylogenetic analyses of these and other unpublished Ethiopian IBDV sequences demonstrate the clustering of Ethiopian vvIBD viruses within the tentatively named African VV1 lineage independent of the Asian/European lineage. Therefore, the commercially available vaccines produced from European strains have reduced protection to the locally circulating African strains. The findings of this research are also suggested that local vvIBDV was successfully adapted to grow in Vero-cell line and completely attenuated after nine serial passages. The live attenuated vaccine prepared from Vero-cell attenuated IBDV was more efficacious, non-pathogenic and highly immunogenic as compared to commercially available live cell culture vaccine. In future, we hope that this vaccine will help in the implementation of better disease control programme against IBD in the country. The following recommendations are therefore forward based on the findings:

- Local isolates that are selected very wisely and attenuated in Vero cell should be used to confer better protection against the disease
- The efficacy of vaccines made from standard strains should regularly be checked against locally circulating vvIBDV.
- Apart from giving better protection, the currently used method can be used to replace the current ECF cell based vaccine production into Vero cell vaccine production.
- Molecular characterization of outbreak IBDV should be conducted on regularly basis to generate information regarding field viruses, thereby device appropriate control strategies.

- It is assumed that indigenous local chickens are slightly resistance for the virus future researches should target isolation and characterization of the virus from indigenous local chickens.

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8. ANNEXES

Annex 1. Procedure of IBD Indirect enzyme linked immunesorbent assay

Preparation of conjugate solution

The horseradish peroxidase conjugated anti-chicken IgG is supplied in HRP stablzer. Dilute 100 µl stock conjugate in 10 ml Dilution Buffer (1:100 dilution) mix well. This 10 ml preparation will supply sufficient conjugate for one 96 well ELISA plate.

Procedure preparation of serum dilution plate

A. Add 300 µl Dilution Buffer to each well of an uncoated 96 well microtitre plate .This plate is referred to as the serum dilution plate.

B. Add 6 µl unknown serum per well start with well A4 and end with well H9 (moving left to right, row by row of wells).

C. Add 6 µl of negative control serum to well A2 ,H10 and H12.

D. Aspirate and remove any liquid in dilution plate well A1, A3 and H11

E. Allow all diluted serums to equilibrate in dilution buffer for 5 minutes before transferring to an IBD antigen Coated ELISA plate.

F. Diluted serum should be tested within 24 hours.

ELISA Test Procedure

A. Remove IBD antigen coated plate from the protective bag and label according to dilution plate identification

B. Add 50 µ Dilution Buffer to all wells on the test plate

C. Add 50 µl diluted IBD positive control serum to all wells on A1, A3 and H11. Discard pipette tip.

D. Use 8-12 pipette to transfer 50 µl per well of each of the Diluted serum samples and negative control serum samples from the serum dilution plate to the corresponding wells of the IBD coated test plate. Discard tips after each row of sample is transferred.

E. Incubate the plate for 30 minutes at room temperature.

F. Fill each well with approximately 300 µl of washing solution and allow the wash solution to soak for 3 minutes then discarding the content. Repeat the wash procedure 2 more times.

G. Dispense 100 µl diluted conjugate in each assay well

H. Incubate the plate for 30 minutes at room temperature

I. Wash as in step F above

J. Dispense 100 µl substrate solutions in to each test well

K. Incubate the plate for 30 minutes at room temperature

L. Add 100 µl Diluted stop solution to each test well

M. Allow bubbles to dissipate before reading the plate.

N. Read the plate using ELISA plate reader set AT 405-410 nm .

Annex 2. Preparation of chicken embryo fibroblast (CEF) cell culture

Use 9-11 day old embryos. The technique described here is for 3-5 embryos.

1. Spray eggs with disinfectant place in hood. Using sterile technique, open shell and remove embryo with blunt ended curved forceps. Place all media and typsin in 37°c water bath
2. Place embryos in petri dish and cut off heads. Removal of limbs and viscera is optional.
3. Transfer bodies to new petri dish or beaker containing PBS .In the beaker, the bodies can be fragmented by carefully chopping them with sterile Scissors.
4. Wash with PBS 3-4 times to remove red blood cells.
5. Pour tissue fragments into trypsinization flask containing magnetic stirring bar. add about 50 ml pre-warmed (37_ ^{0}C) Trypsin solution (0.25%) to the flask and put on stir plate at slow speed into 37_ ^{0}C incubator for 10-15 minutes. Pour off supernatant into centrifuge tube with calf serum. Add 50 ml trypsin solution and stir slowly in 37_ ^{0}C incubator for 8 minutes. (Total trypsinization time: 30-35 minutes at 37_ ^{0}C.) This may be repeated 1 more time for a total of 3 trypsinizations.

6. Centrifuge 10 min. at 1000 rpm. Note the amount of pelleted cells obtained. Pour off trypsin solution and resuspend cells in 3-5 ml GMEM or . The cells may be**Counted****Annex 3:**

Procedure for RT-PCR for IBDVV

Protocol 1: Extraction of Virus RNA using RNeasy Spin-Columns

1. Put 500 µl of your sample into a 1.5 ml tube and centrifuge at 5000 rpm for 15 sec. remove all supernatant by aspiration and add 460 µl volume of Lysis buffer RLT (containing 1% 2-mercaptoethanol) to your sample and mix by vortexing.
2. Add 460 µl 70% ethanol and mix by vortexing.
3. Apply to RNeasy spin column (700 µl maximum loading volume). Spin in a microfuge for 15 sec at 10,000 rpm. Discard flow through and reuse collection tube. Repeat with remaining volume.
4. Wash with 700 µl wash buffer RW1. Centrifuge as before.
5. Wash with 500 µl wash buffer RPE. Centrifuge as above.

6. Repeat wash with 500 µl wash buffer RPE. Centrifuge at max speed for 2 min to dry membrane.

7. Elute RNA with 50 µl DEPC-H_2 O into a new clean collection tube. Spin in a microfuge for 60 sec at 10,000 rpm.

8. Store in clean tube at -20°C. Label the tube clearly with "RNA", the virus name, today's date and your name.

Protocol 2: Reverse Transcription of Virus RNA (cDNA synthesis)

1. In a sterile 0.5 ml eppendorf tubes prepare the following mixture (depending on the number of RNAs to be reverse transcribed).

Reagent	**No. of RNA preps**				
	1	2	3	4	5
10 mM dNTPs	1*	2	3	4	5
DEPC-H $_2$O	3	6	9	12	15
Oligo dT (25 pmol/µl)	1	2	3	4	5
Mix-1 (Total)	5	10	15	20	25
10× RT Buffer	2	4	6	8	10
0.1M DTT	2	4	6	8	10
25mM $MgCl_2$	4	8	12	16	20
RNase Out (3.3 U/µl)	1	2	3	4	5
Mix-2 (Total)	9	18	27	36	45

* amount in µl

2. Add 5 µl of the Mix-1 to 5 µl of each RNA template on PCR tubes giving at total volume of 10 µl.

3. Spin briefly in microfuge. Then incubate at 65°C for 3 mins and chilled on ice for 3 mins

4. Add 9 µl of Mix-2 to each tubes then Heat at 42°C for 2 mins and then add 1.5 µl Supermix III and incubate at 42°C for 50 mins and then at 85°C for 5mins.(either in a waterbath or in the thermocycler).

5. Chilled on ice for 2 mins. Then add 1 μl RNase-H on each tube and centrifuge at 13,000 rpm for 15 secs and finally incubate at 37°C for 20 mins.

5. Store at -20°C. Label the tube with "RT product", the virus name, today's date and your name.

Protocol 3: PCR Amplification of Reverse Transcribed RNA (cDNA)

1. Into a sterile 0.5 ml tube add the following. Take care to use sterile tips and avoid cross contamination. Use new tips for each reagent and template.

Reagent	Concentration	Amount (μl)
25 mM MgCl 2	1.5 mM	1.5
10 mM dNTPs	200 μM	1
10× buffer	1x	5
Taq DNA polymerase (5 U/μl)	2.5 U	0.5
DEPC-H O_2		35
Primer 1	10 pmol	1.5
Primer 2	10 pmol	1.5
Master Mix		46

2. Add 15 μl Master Mix and 5 μl template in PCR tubes and spin on microfuge

3. Run on Thermocycler as follows:

Temperature	Time	No. of cycles
95°C	3 min	1
95°C	30 secs	
60°C	30 secs	
72°C	1 min	35
72°C	7 min	1

4. Take 5-10 μl of PCR product and check it on an agarose gel.

Protocol 4: Agarose Gel Electrophoresis of PCR Products

Note: Take care ethidium bromide is harmful; gloves should be worn at all times.

1. Prepare 120 ml of 1.5% agarose in 1x TAE buffer.
2. Either heat in microwave for ~2 min on full power or place in beaker of boiling water until melted.
3. Allow to cool to about 45°C and add 5-10 μl ethidium bromide (stock=5 mg/μl) per 10 ml, giving a final concentration of 0.5 μg/ml.
4. Pour gel and insert well former (comb). Allow to set on a flat surface for about 15 min.
5. Pour buffer 1x TAE (containing 0.5 μg/ml ethidium bromide, i.e. 1 μl of 5 mg/μl stock to every 10 ml of buffer) into tank and remove comb from gel.
6. Prepare samples in tubes, a multiwell plate or on parafilm. 2 μl loading buffer 5-10 μl PCR product
7. Prepare molecular weight marker. 8-10 μl molecular weight marker GeneRuler™ 100bp loading buffer
8. Load samples into the wells formed in the gel. It is often useful to load the molecular weight markers in both the first and last lanes.
9. Electrophoresis at 100 volts for 1 hour or 10 volts overnight.
10. The DNA band was visualized by UV illumination, documented and the size was determined against the DNA molecular weight marker and serotype was identified. Use UV-safety spectacles.