MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS RECENTLY DETECTED IN DAR ES SALAAM, TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

Infectious bursal disease virus (IBDV) causes an acute and highly contagious immunosuppressive disease in young chickens aged 3 to 6 weeks. The molecular epidemiology of IBD virus causing severe disease in chickens in Tanzania has not been consistently studied. A cross-sectional study that involved collection of bursal of Fabricius from dead chicken following IBD outbreak(s) in Dar es Salaam was conducted. The laboratory analysis of samples was performed by reverse transcription polymerase chain reaction (RT-PCR), nucleotide sequencing, sequence alignment and phylogeny analysis targeting the VP2-hypervariable region (VP2-HVR). The findings of this study revealed that one out of eight samples (12.5%; n=1) was positive for VP2-HVR by RT-PCR and sequencing. A BLAST search of generated sequence indicated 96% nucleotide identity of the field isolate (TZ/DSM/2018) to the LUSC 47-2016 strain detected in chicken from Lusaka, Zambia. The TZ/DSM/2018 virus had conserved putative virulence marker amino acids at 222(A), 242(I), 256(I), 294(I) and 299(S) positions corresponding to very virulent IBDV feature, with unique amino acids at positions 263S and 338P. On phylogeny analysis, the TZ/DSM/2018 virus clustered in the same clade with the African VV-IBDV genotype. Taken together, this study has revealed the existence of the African VV-IBDV genotype in Dar es Salaam, which is genetically different from the vaccine isolate. Further studies are required to perform the in-depth genetic and antigenic characterization of circulating IBDV strains in Tanzania, so that a rational IBD control method can be recommended in the region.

DECLARATION

I, PASKALINA BADI, do hereby declare to neither the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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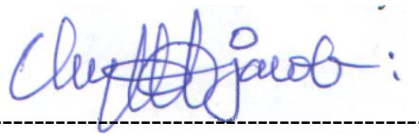
Paskalina Badi Date
(MSc. Candidate)

The above declaration is hereby confirmed by;



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Prof. Christopher J. Kasanga Date
(Supervisor)

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Prof. Robert A. Max Date
(Supervisor)

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For the completion of this work I would like to give my special thanks to those who contributed in one way or another. First and foremost, I thank the omnipresent and omnipotent almighty God, for his grace, power and immeasurable love that gave me strength, wisdom and patience which made this work a reality and without whom nothing valuable in this research would have been attained. Let his name be glorified forever and ever. With endless gratitude, I would like to express my deepest appreciation to my Supervisor Prof. Christopher J. Kasanga for his excellent advice, devotion of time, patience, support, thorough guidance, valuable knowledge, encouragement and his calm approach from the onset of this research work to its completion. I would like to present my heartfelt thanks and appreciation to my second Supervisor Prof. Robert A. Max for his unreserved help, devotion of time, constructive comments, kind cooperation, constant encouragement and for critically reviewing my research work with a keen eye which helped greatly shape this dissertation. I acknowledge the assistance of Sengiyumva Kandusi (Laboratory Manager), Hashim Matimbwa, Ramadhani Juma and Herberther Mpete (Laboratory Scientists) for mentoring me in every part of my laboratory work throughout my study. This work would have been unnecessarily hard without their guidance and constant help. I also thank Dr. Antipachius Msomi and Arafa Rupia and all staff at the Molecular Biology and Biotechnology Laboratory, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture for their assistance during my stay at the laboratory and creating a friendly atmosphere to do my laboratory work. I would like to mention Dr. Isaya Kibasa and Dr. Aron Kasindo with great appreciation and love for helping me during sample collection and all others who are not mentioned in this dissertation but offered me a helping hand. Moreover, I am very grateful for the support from Wellcome Trust through the Intermediate Fellowship

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May the Almighty God bless you all!

DEDICATION

To my lovely parents, BADI NIIMA and JULITHA YOHANI, who brought me into this world and nurtured my early childhood talents that have brought me where I am today, and to my wonderful husband CLEMENT W. MWAKABENGA, my brothers, my sisters and my friends for their prayers and encouragement throughout the long journey of the study; I appreciate their support.

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LIST OF ABBREVIATIONS

AGP	Agar Gel Precipitation test
BLAST	Basic Local Alignment Search Tool
cDNA	complementary deoxyribonucleic acids
CEF	Chicken Embryo Fibroblast
cvIBDV	Classical virulent Infectious Bursal Disease Virus
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acids
dNTPs	deoxynucleotides
dsRNA	double stranded ribonucleic acids
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
HVR	Hyper-variable region
IBA vaccine	Infectious Bursal Agent vaccine
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
MEGA	Molecular Evolutionary Genetics Analysis
Min	Minutes
NCBI	National Centre for Biotechnology Information
NJ	Neighbor Joining
ORF1	Open Reading Frame 1
ORF2	Open Reading Frame 2
PCR	Polymerase chain reaction
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acids

RNase	Ribonuclease
RT-PCR	Reverse transcriptase polymerase chain reaction
Sec	Second
VN	Virus Neutralization test
VP1	Viral Protein 1
VP2	Viral Protein 2
VP2-HVR	Viral protein 2 hypervariable region
VP3	Viral Protein 3
VP4	Viral Protein 4
VP5	Viral Protein 5
VV-1	Very Virulent type 1
VV-2	Very Virulent type 2
VV-3	Very Virulent type 3
VV-IBDV	Very Virulent Infectious Bursal Disease Virus

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Infectious bursal disease virus (IBDV) is the etiological agent of Gumboro disease infecting young chicken aged 3 to 6 weeks (Muller *et al.,* 2003). The disease has as well been observed in chickens older than 6 weeks and up to 20 weeks of age (Okoye *et al.,* 1981). IBD is also known as "Gumboro disease" due to the geographical location of the first recorded outbreak, which occurred in and around Gumboro, Delaware, USA (Muller *et al*., 2003). The occurrences of infectious bursal disease in poultry greatly contribute to huge economic losses (Kibenge *et al.,* 1988). Thus IBDV is one of the most important viral pathogens of commercial poultry (Kibenge *et al.,* 1988).

There are two known existing IBDV serotypes, 1 and 2 (Kibenge *et al.,* 1988). Among the two serotypes, only serotype 1 viruses are pathogenic to chickens and are classified as classical virulent, antigenic virulent and very virulent (Kasanga *et al*., 2007). Serotype II viruses are non-pathogenic to chickens, but mostly isolated from other birds like turkey and guinea fowl without manifesting clinical signs (Kasanga *et al*., 2007). The only avian species known to be susceptible to clinical disease and characteristics lesion caused by IBDV are chickens (Sharma *et al*., 2000). The target cells for IBDV serotype I are the actively proliferating B-lymphocytes of lymphoid cells at a certain stage of cellular differentiation in the bursal of Fabricius (Burkhardt and Muller, 1987). The virus infects the surface of IgM-bearing B-lymphocytes in the bursa of Fabricius leading to immunosuppression of chicken (Sharma *et al*., 2000). The immunosuppression caused by IBDV not only increases chicken`s susceptibility to other secondary infection like Newcastle disease, Marek`s disease and infectious bronchitis but also interfere with

effective immune response to vaccination which is the primary method for controlling IBD (Kibenge *et al.,* 1988; Hirai *et al.,* 1974). IBD viral replication highly takes place in the bursa of Fabricius and the viral antigen can abundantly be detected in the bursal follicles and in other peripheral lymphoid organs such cecal lymphoid tonsils, thymus and spleen (Sharma *et al*., 2000).

The IBDV was first discovered in the USA in 1961, where numerous IBDV isolates were detected continually in chickens from different parts of the world (Cosgrove, 1962). Although it is 56 years ago since discovery, the disease continues to pose a threat to the commercial poultry industry worldwide. In 1987, the first cases of IBD caused by very virulent strains was discovered in Benelux countries which also spread throughout Europe and caused high mortalities up to 70% for laying birds, 25% for broilers and 100% for Specific pathogen free birds (Brown and Skinner, 1996; Van den Berg *et al*., 1991). Tanzania experienced the first outbreaks of the disease in 1988, which affected broiler flocks in the Eastern Zone (Dar-es Salaam and Kibaha) (Kapaga *et al*., 1989). In 2007, the field IBDV isolates were characterized and found that both African and European/Asian VV-IBDVs existed in Tanzania (Kasanga *et al*., 2007). The pathotypes that exist in Tanzania are African VV-IBDV (VV1 &VV2) and European VV-IBDV (VV3) (Kasanga *et al*., 2007).

The virus has high rate of mutation between amino acid residues 206 and 350 in the hypervariable region (HVR) within the VP2 protein where it contributes to antigenic variations (Kasanga *et al*., 2007). Serotype I viruses have highest amino acid sequence variation that are important for the binding and the escape of IBDV from neutralizing monoclonal antibodies (Vakharia *et al*., 1994). Antigenic variations among viruses usually cause vaccination failures, when antigenic structure among field and vaccine

strain no longer coincide (Berg, 2000; Cao *et al.,* 1998; Jackwood and Saif, 1987). Antigenic variants and very virulent IBDV strains have been held responsible for causing disease in vaccinated broiler flocks or progeny from vaccinated parents (Muller *et al.*, 1992). Molecular epidemiologic studies suggested that IBDV has continued to have mutation of amino acids in VP2-HVR that are contributing to antigenic drift in the virus ((Jackwood and Sommer-Wagner, 2011). The mutation allows the virus to circumvent immunity induced by variant IBDV vaccines, which lead to failure of current vaccines to neutralize virus. The purpose of the study was therefore to determine any mutation that occurred since 2007 to date, leading to genetic variation.

1.2 Problem Statement and Justification of the Study

Genetic variation of circulating IBDV in Tanzania was not clearly known as there were no consistent studies done since 2007. Previous molecular epidemiological studies suggest that IBDV undergo subsequent mutation within VP2-HVR that is contributing to antigenic drift in the virus (Jackwood and Sommer-Wagner, 2011). Antigenic variation is therefore, a possible cause of vaccination failure (Adamu *et al.*, 2013).

Several studies have been conducted on IBDV outbreak and molecular characterization (Mekuriaw *et al.*, 2017; Ndashe *et al.*, 2016; Jenberie *et al.*, 2014; Mutinda *et al.*, 2013). The study conducted by Mutinda *et al.* (2013) in Kenya in flocks of layers, broilers and in indigenous chickens suggested that all types of chickens were infected by IBDV. Also the study done by Ndashe *et al.* (2016) in vaccinated broiler flocks in Lusaka, Zambia revealed the existence of very virulent IBDV and belonged to African very virulent type. Another study conducted in Ethiopia by Mekuriaw *et al.* (2017) demonstrated the incidence of IBDV from clinically diseased chickens reared under different production system which is in agreement with the previous studies reported the existence of IBDV

in commercial chickens. In addition, the other study carried out in Ethiopia by Jenberie *et al.* (2014) demonstrated the existence of very virulent IBDV in commercial and poultry breeding farms and clustered phylogenetically with the African IBDV genetic lineage. In Tanzania the last study was conducted in 2007 where the field IBDV isolates were characterized, and the results showed that both African and European/Asian VV-IBDVs exist in Tanzania (Kasanga *et al*., 2007). The origins of both types remain unclear (Kasanga *et al*., 2007). Many IBD outbreaks are increasingly being reported by farmers from different parts of Tanzania. The proposed study therefore provided clear information about the genetic variation and genetic relatedness of recently circulating IBDV strains to that of vaccine candidate strains.

1.3 Research Questions

1. What are the genotypes of IBDV strains circulating in Tanzania?
2. What is the genetic relatedness of circulating IBDV strains to that of vaccine candidate?

1.4 Objectives of the Study

1.4.1 Overall objective

Determination of IBDV genotypes recently circulating in Tanzania with the main focus of developing the appropriate control method of IBD in the country

1.4.2 Specific objectives

1. To determine the genotype of IBDV strains recently circulating in Tanzania
2. To examine the genetic relatedness of recently circulating IBDV strains to that of vaccine candidate strains

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Classification and Physical Structure of IBDV

Infectious bursal disease virus (IBDV) belongs to genus Avibirnavirus of the family Birnaviridae (Dobos *et al.,* 1979). The virus is non-enveloped, single icosahedral capsid and its size ranges from 55 to 65 nm in diameter (Bottcher *et al*., 1997; Hirai and Shimakura, 1974). The IBD virus has major capsid protein namely VP2 (on capsid surface) and VP3 (inside the capsid) (Fig. 1). The VP3 which is inside the capsid has group-specific antigenic sites and minor virus-neutralizing epitopes (Hudson *et al.,* 1986; Fahey *et al.,* 1985). The VP2 which is located on the capsid surface contains conformation-dependent epitopes that are responsible for eliciting neutralizing antibodies (Becht *et al.,* 1988; Azad *et al.,* 1987).

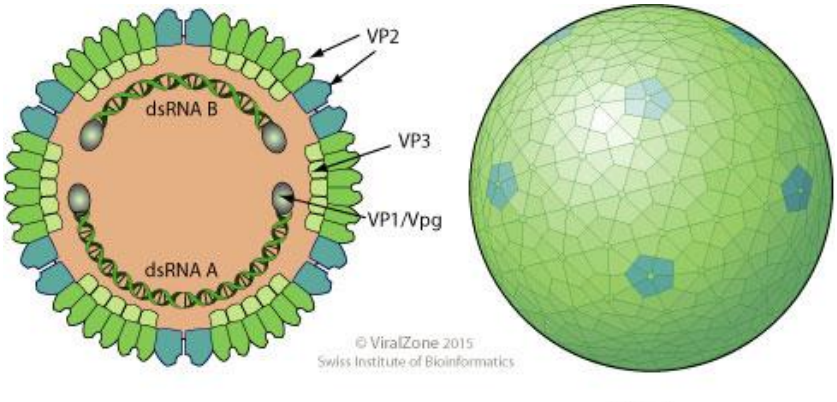

Figure 1: IBDV physical structure
Source; Viral Zone (Swiss Institute of Bioinformatics, 2015)

2.2 Genome Structure of IBDV

The IBDV genome is made up of two RNA segments, designated A and B (Fig. 2). The large segment A (3.4 kb) contains two partially overlapping open reading frames

(ORFs), ORF1 and ORF2 (Bottcher *et al.*, 1997; Ozel and Gelderblom, 1985). The small ORF1 of segment A encodes a non-structural protein VP5 (17–21 kDa), which is not essential for viral replication in cell culture, but it may be important in virus release and dissemination (Lombardo *et al.*, 2000; Yao *et al.*, 1998; Bottcher *et al.*, 1997; Mundt *et al.*, 1995). The large ORF2 of segment A is monocistronic and encodes a precursor polyprotein (NH2-VP2-VP4-VP3-COOH), which is cleaved by autoproteolysis to produce viral capsid protein VP2 (48 kDa), ribonucleoprotein VP3 (32–35 kDa) and the viral protease VP4 (24 kDa) (Da Costa *et al*., 2002; Lejal *et al*., 2000). The VP4 protein is involved in the self-cleavage of the VP2 (Muller, 1986). The hyper-variable region (HVR) within VP2, between amino acid residues 206 and 350, has the highest amino acid sequence variation among serotype 1 strains, and the nucleotide and deduced amino acid sequences of this region are widely used for molecular diagnosis and genotyping of IBDVs (Kasanga *et al.*, 2007; Jackwood and Sommer, 1999). The small segment B (2.8 kb) on the other hand, contains one ORF encoding VP1, an RNA-dependent RNA polymerase (RdRp) responsible for viral genome replication and RNA synthesis (24 kDa) (Shwed *et al.*, 2002; Bottcher *et al.*, 1997).

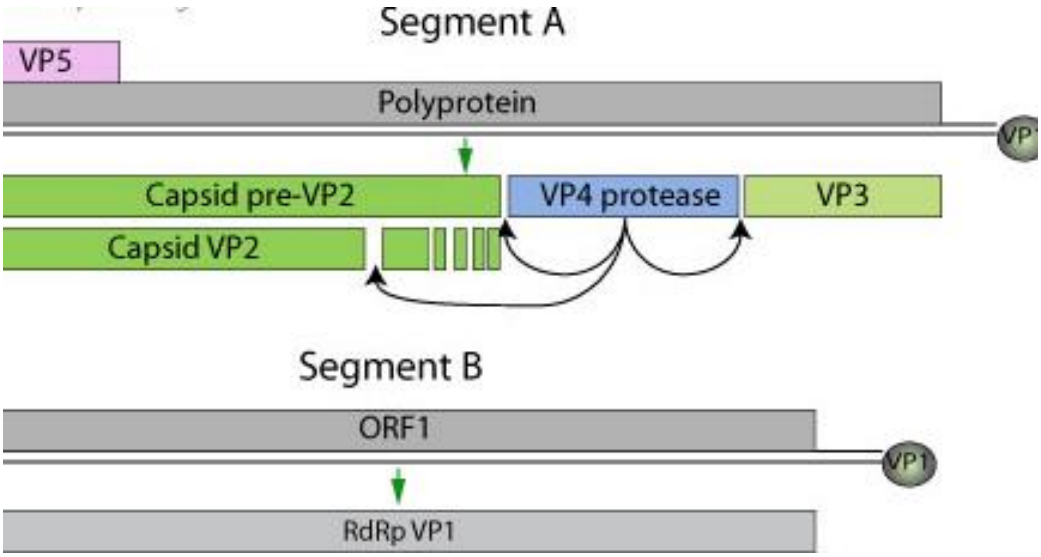

Figure 2: IBDV genome structure
Source: Viral Zone (Swiss Institute of Bioinformatics, 2015)

2.3 Infectious Bursal Disease (IBD)

Infectious bursal disease (also known as Gumboro disease) is an acute, highly contagious and immunosuppressive viral disease of young chickens (Mekuriaw *et al.,* 2017; Liu *et al.,* 2013; Berg, 2000; Cao *et al.,* 1998; Muller *et al.,* 1992). The disease remains one of the economically most important diseases threatening the poultry industry worldwide (Ndashe *et al.,* 2016). Chickens infected with IBDV between 3 to 6 weeks of age develop clinical form of disease characterized by sudden onset and short incubation period which result in death but those infected at less than 3 weeks of age usually have subclinical form of disease and lack clinical signs (Mutinda *et al.,* 2013). Both clinical and subclinical infection with IBDV may cause immunosuppression (Sharma *et al.,* 2000). The disease has also been observed in chickens older than 6 weeks; even in up to 20 weeks old chickens (Mutinda *et al*., 2013; Okoye *et al*., 1981). Irrespective of when the infection occurs the disease causes immunosuppression which makes the birds vulnerable to a variety of secondary infections. As a result, the infected chickens develop a poor immune response to vaccination against other pathogens (Mazariegos *et al.,* 1990). The predominant clinical signs of IBD are inappetence, ruffled feathers, watery diarrhea, depressions, anorexia, severe prostration and finally death (Mutinda *et al*., 2013). On postmortem examination typical IBD lesions are commonly observed; including enlarged bursal of Fabricius, hemorrhage in the thigh and leg muscles, renal damage, increased mucus in the intestine, swollen of the spleen, caecal tonsils and thymus (Mutinda *et al*., 2013; Cosgrove, 1962).

2.4 Diagnosis of IBD

Infectious bursal disease (Gumboro disease) caused by infectious bursal disease virus can be diagnosed by direct and indirect methods. Direct method is performed by directly determining the antigenic part of IBDV and virus isolation.

2.4.1 Direct IBD diagnosis

Traditional methods for diagnosis of IBDV is directly performed by serology using the enzyme-linked immunosorbent assay (ELISA) test, agar gel precipitation (AGP) test, virus neutralization test (VN), immunoperoxidase staining, direct immunofluorescence test and direct electron microscopic examination of feaces and bursal of Fabricius. Molecular diagnostic methods based on RT-PCR are available for the detection of IBDV and differentiation of IBDV subtypes (Ching Wu *et al.*, 2007). These methods have the advantages of being more rapid, more sensitive and less laborious than ELISA or VN assays. The conventional RT-PCR which amplifies VP2 hyper-variable region, in combination with DNA sequencing of the PCR product, can be a very effective tool for detecting and differentiating IBDV subtypes (Ching Wu *et al.*, 2007).

IBDV isolation

The isolation of IBD virus can be done by inoculation of tissue or organ specimen infected with IBDV into antibody-free embryonated chicken eggs (Muller *et al.*, 2003). The cultures of chicken embryo fibroblast (CEF) inoculated with 0.5 ml of the bursal specimen is allowed to adsorb to the cells for 1 hour at 37°C. Finally, the cultures are re-fed with 1.5 ml maintenance medium, incubated at 37°C in a roller apparatus and examined daily for evidence of a cytopathic effect (Allan *et al.*, 1984). At intervals of 7 days, the cells are disrupted by freezing and thawing and the resulting lysate is passed in fresh cell cultures. Cell culture harvests are examined by electron microscopy for the presence of virus (Allan *et al.*, 1984).

2.4.2 Indirect diagnosis of IBD

2.4.2.1 Symptomatology and lesions

Hypervirulent IBDV infections are characterized by severe clinical signs and high mortality. Severe outbreaks are characterized by sudden onset of depression in

susceptible flocks (Berg *et al.,* 2000). Animals in the acute phase of the disease are prostrate and reluctant to move, with ruffled feathers and frequently watery or white diarrhea (Berg *et al.,* 2000).

2.4.2.2 Histological changes

The histological changes are observed by examining a section of tissue under a light microscope or an electron microscope where the bursa is fixed in 10% neutral buffered formalin (Van den Berg *et al.,* 1991). Sections are made and stained with hematoxylin-eosin following conventional procedures (Van den Berg *et al.,* 1991). All lesions are then scored from 0 to 5 according to the index defined by Muskett *et al.* (1979) with 0 as no damage, l as mild necrosis in isolated follicles, 2 as isolated follicles with severe depletion, 3 as over 50% of follicles with severe lymphocyte depletion, 4 as follicles remaining with few lymphocytes and 5 as loss of all follicular architecture with fibroplasias.

2.5 Epidemiology of IBDV

The IBDV was discovered in the USA in 1961 where numerous IBDV isolates were detected continually in chickens from different parts of the world (Cosgrove, 1962). The European picture has been dominated for a decade by the emergence of very virulent (vv) IBDV strains of infectious bursal disease which have now spread all over the world (Berg *et al.,* 2000). Epidemiological observations and mortality studies, clearly suggest that vvIBDV strains belong to the same genetic lineage (Eterradossi *et al.,* 1997; Yamaguchi *et al.,* 1997; Van den Berg *et al.,* 1996; Brown *et al.,* 1994). The first published sequence was strain UK661, and is now considered as the reference strain for European vvIBDVs (Brown and Skinner, 1996). The acute forms of IBD were first described in Europe at the end of the 1980s (Eterradossi *et al.,* 1992; Van den Berg *et al.,*

1991; Chettle *et al.,* 1989), then described in Japan in the early 1990s (Lin *et al.,* 1993; Nunoya *et al.,* 1992) and have rapidly spread all over Asia and to other major parts of the world (Eterradossi, 1995).

The molecular studies on IBDV detection target the VP2-HVR as it is helpful in identifying the viral strain and vaccine designing since the region appears to be appropriate for molecular diagnostics and genotyping (Kasanga *et al.,* 2008). The study conducted by Mutinda *et al.* (2013) in Kenya suggested that all types of chickens were infected by IBDV and the indigenous chickens have been shown to be severely affected as exotic chickens. It also suggested that the vaccination failures were seen in indigenous chickens as in exotic chickens. This implies that IBDV has also been found in Kenya, therefore there is need to screen imported chickens at the border due to cross-border trade and cross-border veterinary services as they are probable entry points of disease into our country.

2.6 Control of IBDV

The infectious bursal disease virus is stable and more resistant to heat, ultraviolet irradiation and photodynamic inactivation (Petek *et al.,* 1973). The virus is also resistant to ether and chloroform and is inactivated at pH 12 but unaffected at pH 2.0 (Benton *et al.,* 1967a). Because of the stability of IBDV in the environment, control through sanitation and isolation is not practical for commercial poultry production (Benton *et al*., 1967b; Parkhust, 1964; Cosgrove, 1962). The principal method for control is therefore by vaccination (Kibenge *et al*., 1988). Of the two serotypes of IBDV recognized in the U.S.A and Europe, only serotype I and its variants have been known to cause naturally occurring disease (Kibenge *et al*., 1988). Vaccines are therefore made from serotype I variants (Kibenge *et al*., 1988). Vaccines are classified as "mild" highly attenuated

vaccines and “intermediate” vaccine of moderate virulence (Kibenge *et al*., 1988). Infectious bursal disease control is performed by vaccination with attenuated or inactivated serotype 1 viruses (Ikuta *et al.*, 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Morogoro and Dar es Salaam whereby the suspected IBD cases were found. Morogoro region is located in central eastern part of Tanzania and lies between latitude 5° 58" and 10° 0" South and longitude 35° 25" and 35° 30" East. Dar es Salaam City is the economic hub of Tanzania; it is located in the eastern part of Tanzania mainland between latitudes 6° 36" and 7° 0" South and longitudes 39° 0" and 33° 33" East.

3.2 Study Design

A cross-sectional study was employed where bursal samples were collected and analysed to determine the genotype(s) of IBDV strains circulating in Tanzania using molecular techniques.

3.3 Sampling Strategies

Prospective bursal samples involved in this study were purposively collected from dead chickens which had shown clinical manifestations of IBD such as whitish watery diarrhea, ruffled feathers and enlarged bursa of Fabricius on necropsy. The entire bursas of Fabricius were aseptically collected from eight dead chickens aged 4 and 5 weeks between October 2017 and April 2018. One out of the eight samples was collected from Dar es Salaam and the remaining seven were from Morogoro. Collected bursa samples were kept in labeled sterile cryo-vial tubes containing Viral Transport Media (50% glycerol) and were immediately placed in liquid nitrogen. The samples were transported to the Biotechnology and Molecular Biology laboratory at Sokoine University of

Agriculture, College of Veterinary Medicine and Biomedical Sciences. In the laboratory, samples were stored at -80^{o}C until analysis.

3.5 Laboratory Analyses

3.5.1 Bursal suspension preparation

The procedure for sample preparation for analysis was performed in Biosafety Cabinet Class II whereby samples were chopped into small pieces using a sterile scalpel blade, then 100 mg was weighed and minced using a sterile mortar and pestle with phosphate buffered saline (1000 µl) to make 10% (100 mg /1000 µl) bursal suspension.

3.5.2 RNA extraction

RNA was extracted using RNeasy Mini kit (QIAGEN GmbH, Germany) following manufacturer′s instruction. Briefly, 460 µl of bursal suspension were mixed with 460 µl of Lysis buffer RLT containing 1% 2-mercaptoethanol (460 µl bursal suspension + 460 µl Lysis buffer RLT + 4.6 µl 2-mercaptoethanol), centrifuged for 30 min at 12 000 xg using MIKRO 220R (Andreas Hettich GmbH and Company, Germany), then the supernatant was transferred to another tube. To the tube containing mixture of bursal suspension and Lysis buffer, 460 µl 70% ethanol was added and then vortexed. From the tube, 700 µl were transferred to the RNeasy spin column and centrifuged for 15 sec at 12 000 xg and the flow-through discarded. The remaining 684.6 µl were transferred to the same RNeasy spin column centrifuged with the same conditions and flow-through discarded. To the RNeasy spin column, 700 µl of wash buffer RW1 were added and centrifuged with the same speed and time as aforesaid and then flow-through discarded. Again, 500 µl of wash buffer RPE were added to the RNeasy spin column, centrifuged with same conditions as aforementioned and flow-through were discarded. The washing procedure was repeated with 500 µl wash buffer RPE, centrifuged at maximum speed

16 000 xg for 2 min and flow-through was discarded. The RNeasy spin column was then transferred to a new collection tube and centrifuged at 12 000 xg for 1 min. Finally, RNA was eluted with 50 µl nuclease-free water into a new collection tube, centrifuged for 60 sec at 12 000 xg and stored at -80^{0}C. The eluted RNA was used for complementary DNA synthesis.

3.5.3 Complementary DNA (cDNA) synthesis

The cDNA was synthesized by using Revert Aid first strand cDNA synthesis kit (Thermo Scientific, Lithuania (EU). The requirements for cDNA synthesis included RNase free water, double stranded RNA (dsRNA), Dimethyl sulfoxide (DMSO), 5X Reaction buffer, deoxynucleotides (dNTPs), Random Hexamer primer, RNase inhibitor, RevertAid RT (Reverse Transcriptase enzyme), ice and incubator.

Table 1: Preparation of master mix for cDNA synthesis

cDNA synthesis reagents	1X (µl)	11X (µl)
5X Reaction Buffer	4	44
Random Hexamer Primer	1	11
RNase Inhibitor	1	11
dNTPs mix	2	22
RevertAid RT(Enzyme)	1	11
RNase free water	9	99
Total volume		198/11 = 18

Briefly, 3 µl of each nine extracted dsRNA were mixed with 1.5 µl of dimethyl sulfoxide (DMSO) and incubated at 97^{0}C for 5 min then immediately chilled on ice. To the tube containing RNA-DMSO mixture the following reagents were added, 4 µl of 5X Reaction buffer, 1 µl of Random Hexamer Primer, 1 µl of RNase Inhibitor, 2 µl of dNTPs Mix, 1 µl of RevertAid RT (enzyme) and 9 µl of RNase-free water to a final volume of 22.5 µl

(Table 1). Reverse transcription reactions were performed in TAKARA PCR Thermo cycler whereby the reaction tubes were incubated for 60 min at 42^0C then reverse transcriptase enzyme inactivated for 5 min at 70^0C and cooled at 4^0C. The synthesized cDNA was stored at 4^0C and used as template for Polymerase chain reaction (PCR).

3.5.4 Polymerase Chain Reaction (PCR) for VP2-HVR

3.5.4.1 Preparation of primer stock solution

The IBDV Stock primer solution of 100 pM/µl concentration was prepared by adding 250 µl of Nuclease free water to the tube containing lyophilized primer. The prepared stock primer was then labeled and stored at -20^0C.

3.5.4.2 Preparation of primers working solution

From stock primer concentration (100 pM/µl), the working primer solution of 25 pM/µl concentration was prepared by using dilution Law ($C_1V_1 = C_2V_2$). The 25 pM/µl was prepared by adding 25 µl of 100 pM/µl to 75 µl of Nuclease free water to final volume (V_2) 100 µl. The prepared working primer solution was then labeled and stored at -20^0C. The information about working primer concentration for IBDV detection was adapted from Kasanga *et al*. (2008).

Table 2: Primer names and their sequence

Primer name	Sequence	Expected outcome
IBDV forward primer	5′-CCA GAG TCT ACA CCA TAA-3′	472bp
IBDV reverse primer	5′- CCT GTT GCC ACT CTT TCG TA-3′	

The cDNA templates were carried out using Bioneer PCR PreMix® readymade Kit (Bioneer Corporation, South Korea), the IBDV VP2-HVR was amplified by TAKARA PCR Thermo Cycler GP (Takara, Japan) whereby IBDV forward primer (5′-CCA GAG

TCT ACA CCA TAA-3′) and IBDV reverse primer (5′- CCT GTT GCC ACT CTT TCG TA-3′) were used and DNA copies produced (Table 2). These primers were highly sensitive and specific hence targeted the VP2-HVR on segment A.

Table 3: Preparation of Master Mix for PCR

PCR reagents	1X (µl)	11X (µl)
Forward primer	1	11
Reverse primer	1	11
Nuclease free water	16	176
Total volume		198/11 = 18

In summary, to each 11 Bioneer Premix tubes, the following were added, 1 µl IBDV forward primer, 1 µl IBDV reverse primer, 16 µl nuclease free water (Table 3) and 2 µl cDNA to a final volume 20 µl. The amplification reactions were carried out in PCR machine (TAKARA PCR Thermo cycler) where PCR cycle parameters were as follows; one cycle for pre-denaturation holds for 3 min at 95^{0}C, 35 cycles each undergoes three sequential phases i.e. (denaturation for 30 sec at 94^{0}C, primer annealing for 30 sec at 58^{0}C and primer extension for 30 sec at 72^{0}C) and one cycle for final extension at 72^{0}C for 5 min (Table 4). The PCR products were then separated on 1.5% agarose gel by electrophoresis and stained with E-Z vision.

Table 4: PCR cycling condition

Steps	Temperature (^{0}C)	Time	No. of cycles
Pre-denaturation	94	3 min	1
Denaturation	94	30 sec	
Annealing	58	30 sec	35 cycles
Extension	72	30 sec	
Final extension	72	5 min	1
Withheld	4	Infinity	None

3.5.5 Purification of post PCR product

The amplified PCR products were purified using Illustra GFX PCR purification kit and pure DNA obtained. The DNA was purified from DNA polymerase, salts, unused primers and dNTPs used during PCR reaction.

In brief, the GFX spin column was placed in a collection tube whereby 100 µl of PCR product was transferred to, followed by addition of Capture buffer then centrifuged at 13 000 xg for 60 sec to allow DNA binding. The flow-through was then discarded and the GFX column was placed into the same collection tube. Thereafter, 500 µl Wash buffer Type 1 was added to the GFX spin column to wash unused PCR reagents, then centrifuged with same conditions as aforementioned and flow-through discarded. The GFX spin column was then placed into a clean tube where 50 µl Elution Buffer Type 6 was added to the center of the GFX, centrifuged for 1 min and pure DNA eluted. The purified DNA was then run in 1.5% agarose gel and visualized using E-Z vision stain.

3.5.6 Cycle sequencing

The purified DNA was then cycle sequenced by using Big Dye terminator Kit where the cycle sequencing Master Mix and 1.6 pmol primer were prepared.

Table 5: Master Mix for cycle sequencing

Cycle sequencing reagents	1X (µl)
RNase free water	3.5
5X Sequencing Buffer	2
Big dye Terminator	0.5
Primer (at 1.6pmol)	3
Purified DNA template	1
Total volume	10

Briefly, 3 µl of 1.6 pM/µl forward and reverse primer was placed in separate PCR tubes followed by addition of 1 µl purified DNA, 3.5 µl nuclease free water, 2 µl sequencing buffer and 0.5 µl big dye to final volume of 10 µl (Table 5). The reaction tubes were subjected to PCR, for 25 cycles carrying out denaturation at 96^0C for 10 sec, primer annealing at 50^0C for 0.05 sec and extension at 60^0C for 4 min to obtain labeled DNA fragments of different size (Table 6).

Table 6: PCR conditions for cycle sequencing

Steps	Temperature	Time	Number of cycles
Pre-denaturation	96^0C	1min	1
Denaturation	96^0C	10sec	
Annealing	50^0C	5sec	25 cycles
Extension	60^0C	4min	
Withheld	10^0C	Hold	None

3.5.7 Sequencing

The cycle sequenced DNA was purified by means of ethanol precipitation. The procedure was done by adding 5 µl mM EDTA and 60 µl 100% ethanol to the cycle sequenced products, vortexed, left in dark for 15 min and centrifuged for 30 min at 13 000 xg and then the supernatant was removed. Afterwards, 60 µl 70% ethanol was added to the tube, vortexed and centrifuged for 30 min at 13 000 xg then supernatant was removed again. The tubes were then vacuum-dried in the dark for 15 min to get rid of ethanol, followed by addition of 20 µl Hi-Di Formamide and kept again in the dark place for 15 min. Lastly the samples were transferred to the sequencing plate and loaded into the Sanger sequencing capillary electrophoresis technology using AB 3500 Genetic analyzer whereby the order of nucleotides were obtained. Each DNA fragment was sequenced in both directions (forward and reverse directions) to generate a reliable consensus sequence. The sequenced nucleotides of VP2-HVR of isolates from chicken

(Kuchi ecotype) and vaccine were assembled and edited using Geneious software program. The assembled and edited obtained sequences were then deposited into the NCBI GenBank database to determine alignment with VP2-HVR of IBDV that are well characterized.

3.6 Data Analyses

For sequence and phylogenetic analyses; the published nucleotide sequences were selected for amino acids comparison and phylogenetic analysis of TZ/DSM/2018 and vaccine isolate. Some known IBDV strains (vvIBDVs, cvIBDVs, Variant strain and classical vaccines) were also included for amino acid sequence comparison. The obtained nucleotide sequence of chicken isolate was analyzed with the aid of Geneious software and MEGA X to produce deduced amino acid and phylogenetic tree. Nucleotide sequence was subjected to BLAST search to determine identity with other published IBDV strains available in Pubmed. The nucleotide sequence was aligned using Clustal Wallis in MEGA X software. The phylogenetic analysis was done using NJ method (Saitou and Nei, 1987) utilizing Kimura-2- parameter (Kimura, 1980) included in MEGA X (Kumar *et al.*, 2018). The phylogenetic tree was generated based on the nucleotide sequences of HV-VP2 which involved 34 nucleotide sequences whereby one from field isolate, one from vaccine isolate (Virgo 7 strain) and 32 from references strains obtained from the Genbank. The topology of the tree was estimated by the bootstrap method of analysis of 1000 replicates.

CHAPTER FOUR

4.0 RESULTS

4.1 IBDV HVR-VP2 by RT-PCR in Chickens

From eight chickens, the bursal of Fabricius were collected based on clinical manifestation and postmortem examinations. One out of eight chickens was RT-PCR positive for IBDV (Table 7) and showed a specific band at 472 bp in an agarose gel (Fig. 3). The chicken (Kuchi ecotype) detected with IBDV by RT-PCR was from Dar es Salaam city and designated as TZ/DSM/2018 whereas the vaccine isolate (Virgo 7 strain) used as positive control was designated as PC/VAC.

Table 7: Description of chickens used in the study showing respective PCR results in selected areas in Tanzania

Field sample number	Category	Vaccination (yes/no)	Chicken status	Management	Location	PCR
S1	Local	No	Dead	Scavenging	Morogoro	-
S2	Local	No	Dead	Scavenging	Morogoro	-
S3	Local	No	Dead	Scavenging	Morogoro	-
S4	Local	No	Dead	Scavenging	Morogoro	-
S5	Local	No	Dead	Scavenging	Morogoro	-
S6	Broiler	No	Dead	Caged	Morogoro	-
S7	Broiler	No	Dead	Caged	Morogoro	-
S8	Kuchi ecotype	Yes	Dead	Caged	Dar es Salaam	+

The chicken (Kuchi ecotype) that was PCR positive for VP2-HVR shown in figure 3

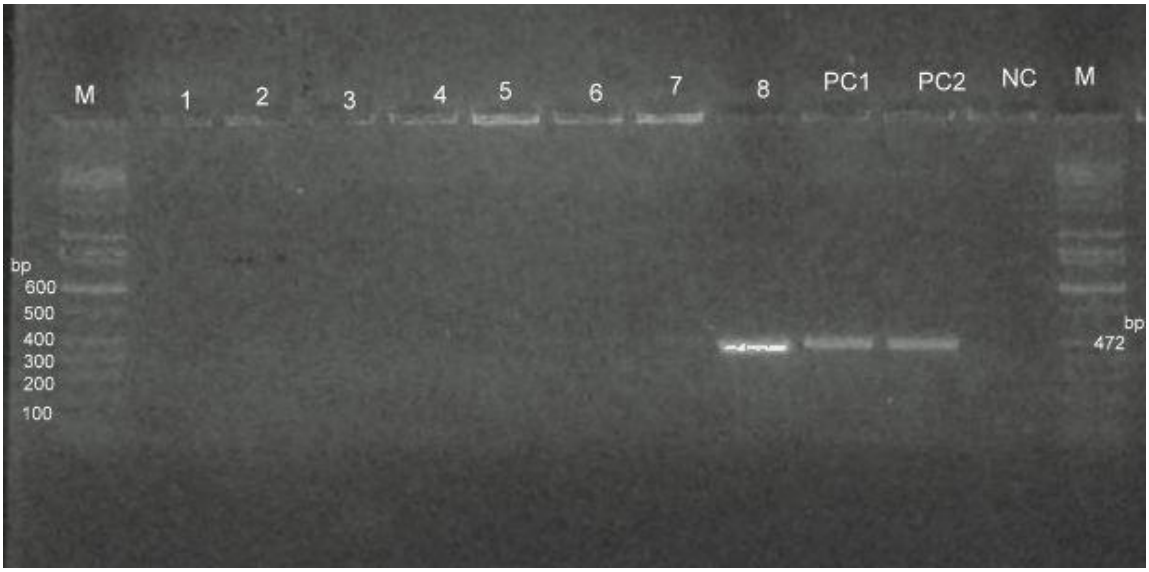

Figure 3: Amplification of the IBDV VP2-HVR of field isolate (TZ/DSM/2018), PC1 (KMRG 48) and PC2 (Vaccine- PC/VAC).

The positive sample is 472 base pair (bp) in size; Lane M, 100-bp DNA ladder; Lanes 1–8, tested samples identification numbers; Lane PC1, Known positive control available in the laboratory (KMRG 48), Lane PC2, Positive control vaccine available in the market (PC/VAC) and Lane NC, negative control (RNase free water)

4.2 Nucleotide Sequences of TZ/DSM/2018 Isolate and PC/VAC

The VP2-HVR sequence of TZ/DSM/2018 was 434 bp long. A BLAST search for nucleotide sequences revealed the maximum identity of 96% with LUSC 47-2016 strain from Lusaka, Zambia. The VP2-HVR nucleotide sequences of TZ/DSM/2018 from chicken (Kuchi ecotype) and PC/VAC from IBDV vaccine (Virgo 7 strains) were aligned with nucleotide sequences of selected reference strains from Genbank. The nucleotide alignment revealed that there are insertions of new nucleotides (Adenine at position 389 and Cytosine at position 390) in field isolate. Moreover, the nucleotide substitution was observed in field strain at different positions; Thymine substituted by Cytosine at 119 and 263, Adenine substituted by Thymine at 180, Guanine substituted by

Adenine at 185, Adenine substituted by Guanine at 214, 323 and 427, Cytosine substituted by Thymine at 290 and Adenine substituted by Cytosine at 388.

4.3 Deduced Amino Acid Sequences

Deduced amino acid sequences were obtained from nucleotide sequences by using Geneious software program. The analysis of deduced amino acid sequences of VP2-HVR allowed identification of critical residues that constitutes particular patterns characteristics of the different groups/clusters. A BLAST search for deduced amino acid sequences revealed the maximum identity of 99% between TZ/DSM/2018 and a very virulent strain LUSC47-2016 isolated in Lusaka in Zambia.

The deduced amino acid sequences of the HVR of VP2 gene from TZ/DSM/2018 (field strain) and PC/VAC (positive control vaccine) isolates used in this study was determined and compared to that of selected serotype 1 IBDV strains available in Pubmed. The antigenic and virulence sites of the amino acids that spans from 210 to 350 within VP2-HVR of recently circulating IBDV strains in Tanzania were analyzed in comparison with some of the published IBDV strains; Classical IBDV (IBA vaccine, classical virulent, Australian strain, and D78), variant IBDV (variant E) strain and VV-IBDV (Ivory coast strain, UK661, NIE/96/090/C). The compared strains revealed that IBDV strains that are recently circulating in Tanzanian had the conserved putative virulence marker at positions 222(A), 242(I), 256(I), 294(I) and 299(S) which are also found in very virulent IBDV (Table 8b). The potential antigenic site of TZ/DSM/2018 isolate strain was very similar to KMZA-78 very virulent IBDV strain which clustered in VV2 and found at position 219(Q), 222(A), 242(I), 254(S), 256(I), 279(D), 284(A), 294(I), 299(S) and 300(A) (Shown in table 8a). The unique amino acid residue was as well observed at positions 269S and 338P in TZ/DSM/2018 isolate which was not found in any of the compared amino acid sequences.

Table 8a: Antigenic relatedness of IBDV strains to the vaccine candidate virus/strain

IBDV strains	Deduced aa* positions determining antigenicity within VP2-HVR									
	219	222	242	254	256	279	284	294	299	300
IBA vaccine	Q	P	V	G	V	N	T	L	N	E
52/70	.	.	I	.	.	D	A	.	.	.
Variant E strain	.	T	.	S	.	.	A	.	.	.
002-73	.	.	.	.	.	G	A	.	S	.
88180	.	Q	I	.	I	D	A	.	.	Q
UK661	.	A	I	.	I	D	A	I	S	.
NIE/96/090/C:	.	A	I	S	I	D	A	I	S	Q
KMZA-78:	.	A	I	S	I	D	A	I	S	A
Vaccine D78	.	.	.	.	.	.	.	.	.	.
PC/VAC	**.**	**A**	**I**	**.**	**1**	**.**	**A**	**I**	**S**	**.**
TZ/DSM/2018	**.**	**A**	**I**	**S**	**1**	**D**	**A**	**I**	**S**	**A**

Legend: Letters indicate deduced amino acids
Dots indicate similar amino acid(s) to that of the IBA vaccine

Table 8b: Virulence relatedness of IBDV strains to the vaccine candidate virus/strain

	Deduced aa* positions determining virulence				
IBDV strains	222	242	256	294	299
IBA vaccine	P	V	V	L	N
52/70	.	.	.	.	.
Variant E	T	.	.	.	.
002-73	.	.	.	.	S
88180	Q	I	I	.	.
UK 661	A	I	I	I	S
NIE/96/090/C:	A	I	I	I	S
(KMZA-78:	A	I	I	I	S
Vaccine D78	.	.	.	.	.
PC/VAC	**A**	**I**	**I**	**I**	**S**
TZ/DSM/2018	**A**	**I**	**I**	**I**	**S**

Legend: Letters indicate deduced amino acids
Dots indicate similar amino acid(s) to that of the IBA vaccine

From the table 8a and 8b, *aa = amino acid, IBA vaccine with its amino acids was used as a reference strain. The compared amino acids sequences and corresponding accession numbers used in this study includes; attenuated classical vaccine strain (IBA AJ586965); Standard classical virulent strain (52/70,Y14958); Variant E, (D10065); Australian classic strain (002-73, AF148073); Ivory coast atypical VV strain (88180,AJ001941); European/Asian VV 3 (UK661, Z25480); African VV-1 (NIE/96/090/C, AJ586926); African VV-2,(KMZA-78, AB200985); and Vaccine D78 (AJ586963). The isolates used in this study include; Vaccine Virgo 7 strains (PC/VAC) and field strain (TZ/DSM/2018).

4.4 Phylogeny of IBDVs Detected in Chickens

The sequences used in the analysis clustered into very virulent IBDV (VVIBDV) and classical strain IBDV of serotype 1 strains and the clustering indicated genetic relatedness of the IBDV strains. The strains that clustered in the same group are genetically closely related and might have the same ancestral origin. In the phylogeny, the TZ/DSM/2018 isolate from Tanzania showed the highest degree of nucleotide identity of 96% with very virulent IBDV (LUSC 47-2016 strain) isolated from Lusaka, Zambia. The TZ/DSM/2018 isolate strain was clustered in the same group with LUSC47-206 strain from Lusaka, Zambia (Fig. 4). As well, the TZ/DSM/2018 isolate strain was genetically related with other African type of VV-IBDVs in VV2 cluster including KMRG-46, KDSM-32, KMRG-48, KAR-53, KMRG-38, KMRG-40, KMZA-78 and KMRG-79. The field isolate (TZ/DSM/2018) was genetically unrelated to the vaccine strain (PC/VAC) available in the market for IBD control.

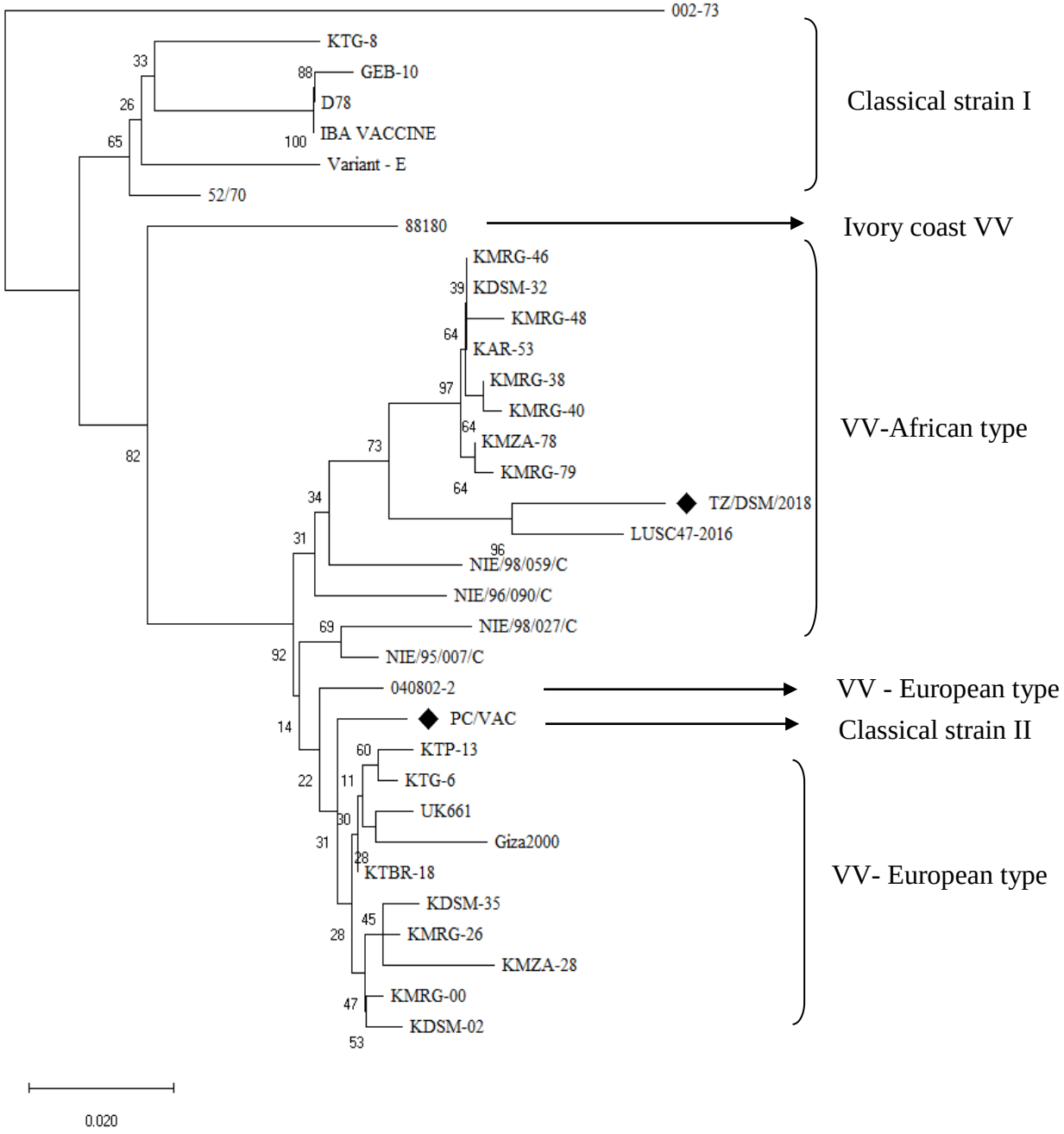

Figure 4: The phylogenetic tree of Tanzanian IBDV isolate, vaccine isolate and selected reference strains

The tree (Fig. 4) was constructed by the neighbor-joining method based on nucleotides on VP2-HVR of serotype 1 strains using aligned nucleotide sequences in Clustal W utilizing Kimura 2-parameter (Kimura, 1980) in MEGA X (Kumar *et al.,* 2018). The accession numbers used in tree construction are Classical strains (IBA vaccine,

AJ586965); Standard classical virulent strain (52/70, Y14958); Variant E, (D10065); Vaccine D78 (AJ586963); KTG-8 AB306715; GEB-10 AB200987; Australian classic strain (002-73, AF148073); African type VV-1 (NIE/96/090/C, AJ586926; NIE/98/059/C: AJ586950; NIE/98/027/C, AJ586948; NIE/95/007/C, AJ586917); European/Asian very virulent type (KMRG-00 AB200975; KMRG-26 AB200978; KMZA-28, AB200979; KTP-13, AB306716; KTG-6, AB306714; KTBR-18, AB200977; GIZA2000, AY318758; UK661, Z25480, 040802-2, AB200988; KDSM-02, AB200976; KDSM-35 AB200980); African type VV-2(KMZA-78, AB200985; KMRG-79, AB200986; KMRG-40, AB200982; KAR-53, AB200984; KMRG-38, AB200981; KMRG-48, AB200983; KMRG-46, AB201125; KDSM-32 AB201124); Ivory coast atypical VV strain (88180, AJ001941). The isolates used in this study include; Vaccine Virgo 7 strain (PC/VAC) and field strain (TZ/DSM/2018).

CHAPTER FIVE

5.0 DISCUSSION

This study aimed at determining the IBDV genotype(s) of recently circulating strains in Tanzania and examining the genetic relatedness of circulating IBDV strains to that of vaccine candidate. The investigation revealed chicken designated TZ/DSM/2018 to be positive by RT-PCR targeting VP2-HVR of IBDV (Fig. 3). This observation indicates that serotype 1 IBDV prevail in Dar es Salaam and contribute to the occurrence of IBD, which ultimately lead to mortality of chicken. The low IBDV detection rate of 12.5% in Tanzania could be ascribed to low sample size and unknown epidemiology of IBDV in Tanzania. The BLAST search of TZ/DSM/2018 isolate showed the highest nucleotide similarity of 96% with LUSC47-2016 strain isolated in Lusaka in Zambia. The closeness in genetic relatedness of Tanzanian field strain (TZ/DSM/2018) to that of Zambian strain (LUSC47-2016) could be associated with similar possible drivers of mutations in the two countries like adaptation to new host and environment, genetic factors and host migration (Sanjuán and Domingo-Calap, 2016) which are exacerbated by transporting chicken across the border. The TZ/DSM/2018 isolate was found to have a specific mutation involving an insertion of new nucleotides (Adenine at position 389 and Cytosine at position 390) and a substitution mutation at positions 263 (T to C), 214 (A to G) and 388 (A to C) that has not been observed in other selected published strains. These observations suggest that TZ/DSM/2018 virus might be re-emerging strain due to geographically restricted mutation(s). Further studies are required to elucidate this hypothesis.

The deduced amino acid sequences when compared with some selected published strains revealed that the field strains had conserved putative virulence marker at 222(A), 242(I),

256(I), 294(I) and 299(S) positions corresponding to Very virulent IBDVs characteristics (Table 8b). The TZ/DSM/2018 isolate strain was antigenically100% similar with KMZA-78 very virulent IBDV strain clustered in VV2, suggesting that the field strain was very virulent strain. The functions and structures of protein built from unique amino acids observed at positions 269S and 338P in TZ/DSM/2018 were not examined in this study. Therefore, further investigation is needed to unravel whether these amino acids bring changes on protein structure and function and whether it affects virus antigenicity and/or pathogenicity. The unique amino acid at position 269S found between minor hydrophilic region I and II whereas amino acid at position 338P found beyond major hydrophilic region B of TZ/DSM/2018. These unique amino acids from TZ/DSM/2018 isolate may or not lead to virus modification and hence alter the virulence and antigenicity of the virus. The amino acid substitution observed within HV-VP2 in TZ/DSM/2018 might have modified the virus ability to escape the neutralizing antibodies (Liu *et al.*, 2013; Jackwood and Sommer-Wagner, 2011). The further investigations are needed to examine whether the observed unique amino acids could change the pathogenicity of IBDV variants.

The phylogenetic analysis of TZ/DSM/2018 revealed that the field strain was in the same cluster with very virulent LUSC47-2016 strain isolated in Lusaka in Zambia and had highest degree of 96% nucleotide identity (Fig. 4). The TZ/DSM/2018 isolate strain was also genetically related with other African type VV2-IBDVs, implying that the recently circulating IBDV strain in Dar es salaam, Tanzania belong to VV-IBDV lineage. The isolate recently detected in Dar es Salaam, Tanzania was antigenically different from vaccine isolate (PC/VAC) available in market indicating that the vaccines available in the market do not confer immunity against circulating IBDV strains in the region.

The few previous studies conducted on molecular characterization of IBDV genome confirmed that IBDV strains circulating in Tanzania were classical, African very virulent and European/Asian very virulent strains (Kasanga *et al.,* 2007; Kapaga *et al.,* 1988). The other study conducted in Tanzania (Kasanga *et al.,* 2008) was on detection of IBDV in free-living pigeon and guinea fowl. The study proposed that the VV-IBDV (VV3) and classical strains were circulating in Tanzania and they could even be detected in free-living pigeon and guinea fowls. The findings of the current study which detected VV-IBDV in Dar-es-Salam are in agreement with the previous studies.

This study has therefore provided important information which can be useful in developing appropriate vaccine against the currently circulating IBDV strains because the viral genome has undergone mutations since it was last characterized in 2007. The study has also demonstrated the importance of regular molecular monitoring of the IBDV evolution in order to understand the dynamics of IBDV strains in Tanzania, which is critical for control of IBD in the country.

CHAPTER SIX

6.0 CONCLUSION, RECOMMENDATIONS AND WAY FORWARD

6.1 Conclusion

The present study has shown that the African very virulent IBDV genotype exist in Tanzania and contribute to the occurrence of IBD in the country. Also, the detected IBDV genotype was genetically and antigenically different from the virus strain incorporated in vaccines used against IBD in Tanzania.

6.2 Recommendations

(i) Molecular characterization of IBDV field strains should be carried out continuously so as to monitor the emergence and re-emergence of IBDV variants prior to importation of vaccine against circulating IBD viruses.

(ii) There should be an intensive screening of imported chickens and their products at borders for possible harbouring of IBDV infections before distributing to our internal markets.

6.3 Way Forward

(i) Further investigation(s) are required to uncover genetic mutations and antigenic variation(s) that could influence the virulence and antigenicity of IBDV field strains, respectively.

(ii) The detection of very virulent IBDV strain(s) in vaccinated chickens suggests that further work on examining the efficacy of vaccine and designing of vaccination programs need to be performed so as to establish the appropriate control method of IBD in Tanzania and the region at large.

REFERENCES

Adamu, J., Owoade, A. A., Abdu, P. A., Kazeem, H. M. and Fatihu, M. Y. (2013). Characterization of field and vaccine infectious bursal disease viruses from Nigeria revealing possible virulence and regional markers in the VP2 minor hydrophilic peaks. *Avian Pathology* 42(5): 420-433.

Allan, G. M., McNulty, M. S., Connor, T. J., McCracken, R. M. and McFerran, J. B. (1984). Rapid diagnosis of infectious bursal disease infection by immunofluorescence on clinical material. *Avian Pathology* 13(3): 419-427.

Azad, A. A., Jagadish, M. N., Brown, M. A. and Hudson, P. J. (1987). Deletion mapping and expression in *Escherichia coli* of the large genomic segment of a birnavirus. *Virology* 161(1): 145-152.

Becht, H., Muller, H. and Muller, H. K. (1988). Comparative studies on structural and antigenic properties of two serotypes of infectious bursal disease virus. *Journal of General Virology* 69(3): 631-640.

Benton, W. J., Cover, M. S. and Rosenberger, J. K. (1967a). Studies on the transmission of the infectious bursal agent (IBA) of chickens. *Avian Diseases* 11(3): 430-438.

Benton, W. J., Cover, M. S., Rosenberger, J. K. and Lake, R. S. (1967b). Physicochemical properties of the infectious bursal agent (IBA). *Avian Diseases* 11(3): 438-445.

Berg, T. P. V. D. (2000). Acute infectious bursal disease in poultry: a review. *Avian Pathology* 29(3): 175-194.

Bottcher, B., Kiselev, N. A., Stel Mashchuk, V. Y., Perevozchikova, N. A., Borisov, A. V. and Crowther, R. A. (1997). Three-dimensional structure of infectious bursal disease virus determined by electron cryo-microscopy. *Journal of Virology* 71(1): 325-330.

Brown, M. D. and Skinner, M. A. (1996). Coding sequences of both genome segments of a European 'very virulent' infectious bursal disease virus. *Virus Research* 40(1): 1-15.

Brown, M. D., Green, P. and Skinner, M. A. (1994). VP2 sequences of recent European 'very virulent' isolates of infectious bursal disease virus are closely related to each other but are distinct from those of 'classical' strains. *Journal of General Virology* 75(3): 675-680.

Burkhardt, E. and Muller, H. (1987). Susceptibility of chicken blood lymphoblasts and monocytes to infectious bursal disease virus (IBDV). *Archives of Virology* 94(3): 297-303.

Cao, Y. C., Yeung, W. S., Law, M., Bi, Y. Z., Leung, F. C. and Lim, B. L. (1998). Molecular characterization of seven Chinese isolates of infectious bursal disease virus: classical, very virulent, and variant strains. *Avian Diseases* 42(2): 340-351.

Chettle, N., Stuart, J. C. and Wyeth, P. J. (1989). Outbreak of virulent infectious bursal disease in East Anglia. *Veterinary Record* 125(10): 271-272.

Ching Wu, C., Rubinelli, P. and Long Lin, T. (2007). Molecular detection and differentiation of infectious bursal disease virus. *Avian Diseases* 51(2): 515-526.

Cosgrove, A. S. (1962). An apparently new disease of chickens: avian nephrosis. *Avian Diseases* 6(3): 385-389.

Da Costa, B., Chevalier, C., Henry, C., Huet, J. C., Petit, S., Lepault, J. and Delmas, B. (2002). The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of VP2. *Journal of Virology* 76(5): 2393-2402.

Dobos, P., Hill, B. J., Hallett, R., Kells, D. T., Becht, H. and Teninges, D. (1979). Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. *Journal of Virology* 32(2): 593-605.

Eterradossi, N. (1995). Progress in the diagnosis and prophylaxis of infectious bursal disease in poultry. In: *Comprehensive Reports on Technical Items presented to the International Committee or to Regional Commissions*. Office International des Epizooties, Paris. pp75-82.

Eterradossi, N., Picault, J. P., Drouin, P., Guittet, M., L'Hospitalier, R. and Bennejean, G. (1992). Pathogenicity and preliminary antigenic characterization of six infectious bursal disease virus strains isolated in France from acute outbreaks. *Zoonoses and Public Health* 39(10): 683-691.

Eterradossi, N., Rivallan, G., Toquin, D. and Guittet, M. (1997). Limited antigenic variation among recent infectious bursal disease virus isolates from France. *Archives of Virology* 142(10): 2079-2087.

Fahey, K. J., O'donnell, I. J. and Bagust, T. J. (1985). Antibody to the 32K structural protein of infectious bursal disease virus neutralizes viral infectivity in vitro and confers protection on young chickens. *Journal of General Virology* 66(12): 2693-2702.

Hirai, K. and Shimakura, S. (1974). Structure of infectious bursal disease virus. *Journal of Virology* 14(4): 957-964.

Hirai, K., Shimakura, S., Kawamoto, E., Taguchi, F., Kim, S. T., Chang, C. N. and Iritani, Y. (1974). The immunodepressive effect of infectious bursal disease virus in chickens. *Avian Diseases* 1974: 50-57.

Hudson, P. J., McKern, N. M., Power, B. E. and Azad, A. A. (1986). Genomic structure of the large RNA segment of infectious bursal disease virus. *Nucleic Acids Research* 14(12): 5001-5012.

Ikuta, N., El-Attrache, J., Villegas, P., Garcia, M., Lunge, V. R., Fonseca, A. S. K., Oliveira, C. and Marques, E. K. (2001). Molecular characterization of Brazilian infectious bursal disease viruses. *Avian Diseases* 45(2): 297-306.

Jackwood, D. H. and Saif, Y. M. (1987). Antigenic diversity of infectious bursal disease viruses. *Avian Diseases* 31(4): 766-770.

Jackwood, D. J. and Sommer, S. E. (1999). Restriction fragment length polymorphisms in the VP2 gene of infectious bursal disease viruses from outside the United States. *Avian Diseases* 43(2): 310-314.

Jackwood, D. J. and Sommer-Wagner, S. E. (2011). Amino acids contributing to antigenic drift in the infectious bursal disease Birnavirus (IBDV). *Virology* 409(1): 33-37.

Kapaga, A. M., Msami, H. M. and Mella, P. N. P. (1989). Infectious bursal disease (Gumboro disease) in Tanzania. In: Tanzania Veterinary Association Scientific Conference (TVA), AICC, Arusha, Tanzania. *TVA* 7: 37–42.

Kasanga, C. J., Yamaguchi, T., Wambura, P. N., Maeda-Machang'u, A. D., Ohya, K. and Fukushi, H. (2007). Molecular characterization of infectious bursal disease virus (IBDV): diversity of very virulent IBDV in Tanzania. *Archives of Virology* 152(4): 783-790.

Kasanga, C. J., Yamaguchi, T., Wambura, P. N., Munang'andu, H. M., Ohya, K. and Fukushi, H. (2008). Detection of infectious bursal disease virus (IBDV) genome in free-living pigeon and guinea fowl in Africa suggests involvement of wild birds in the epidemiology of IBDV. *Virus Genes* 36(3): 521-529.

Kibenge, F. S., Dhillon, A. S. and Russell, R. G. (1988). Biochemistry and immunology of infectious bursal disease virus. *Journal of General Virology* 69(8): 1757-1775.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111-120.

Kumar, S., Stecher, G., Li M., Knyaz, C. and Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35(6): 1547-1549.

Lejal, N., Da Costa, B., Huet, J. C. and Delmas, B. (2000). Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. *Journal of General Virology* 81(4): 983-992.

Lin, Z., Kato, A., Otaki, Y., Nakamura, T., Sasmaz, E. and Ueda, S. (1993). Sequence comparisons of a highly virulent infectious bursal disease virus prevalent in Japan. *Avian Diseases* 37(2): 315-323.

Liu, D., Zhang, X. B., Yan, Z. Q., Chen, F., Ji, J., Qin, J. P., Li, H.Y., Lu, J. P., Xue, Y., Liu, J. J. and Xie, Q. M. (2013). Molecular characterization and phylogenetic analysis of infectious bursal disease viruses isolated from chicken in South China in 2011. *Tropical Animal Health and Production* 45(5): 1107-1112.

Lombardo, E., Maraver, A., Espinosa, I., Fernandez-Arias, A. and Rodriguez, J. F. (2000). VP5, the nonstructural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. *Virology* 277(2): 345-357.

Mazariegos, L. A., Lukert, P. D. and Brown, J. (1990). Pathogenicity and immunosuppressive properties of infectious bursal disease "intermediate" strains. *Avian Diseases* 34(1): 203-208.

Mekuriaw, A., Bitew, M., Gelaye, E., Mamo, B. and Ayelet, G. (2017). Infectious bursal disease: outbreak investigation, molecular characterization, and vaccine immunogenicity trial in Ethiopia. *Tropical Animal Health and Production* 49(6): 1295-1302.

Muller, H. (1986). Replication of infectious bursal disease virus in lymphoid cells. *Archives of Virology* 87(3): 191-203.

Muller, H., Islam, M. R. and Raue, R. (2003). Research on infectious bursal disease the past, the present and the future. *Veterinary Microbiology* 97(1): 153-165.

Muller, H., Schnitzler, D., Bernstein, F., Becht, H., Cornelissen, D. and Lutticken, D. H. (1992). Infectious bursal disease of poultry: antigenic structure of the virus and control. *Veterinary Microbiology* 33(4): 175-183.

Mundt, E., Beyer, J. and Muller, H. (1995). Identification of a novel viral protein in infectious bursal disease virus-infected cells. *Journal of General Virology* 76(2): 437-443.

Muskett, J. C., Hopkins, I. G., Edwards, K. R. and Thornton, D. H. (1979). Comparison of two infectious bursal disease vaccine strains: efficacy and potential hazards in susceptible and maternally immune birds. *The Veterinary Record* 104(15): 332-334.

Mutinda, W. U., Nyaga, P. N., Njagi, L. W., Bebora, L. C. and Mbuthia, P. G. (2013). Gumboro disease outbreaks cause high mortality rates in Indigenous chickens in Kenya. *Bulletin of Animal Health Production Africa* 61: 571-578.

Ndashe, K., Simulundu, E., Hang'ombe, B. M., Moonga, L., Ogawa, H., Takada, A. and Mweene, A. S. (2016). Molecular characterization of infectious bursal disease viruses detected in vaccinated commercial broiler flocks in Lusaka, Zambia. *Archives of Virology* 161(3): 513-519.

Nunoya, T., Otaki, Y., Tajima, M., Hiraga, M. and Saito, T. (1992). Occurrence of acute infectious bursal disease with high mortality in Japan and pathogenicity of field isolates in specific-pathogen-free chickens. *Avian Diseases* 36(3): 597-609.

Okoye, J. O. A. and Uzoukwu, M. (1981). An outbreak of infectious bursal disease among chickens between 16 and 20 weeks old. *Avian Diseases* 25(4): 1034-1038.

Ozel, M. and Gelderblom, H. (1985). Capsid symmetry of viruses of the proposed birnavirus group. *Archives of Virology* 84(4): 149-161.

Parkhurst, R. T. (1964). On-the-farm studies of Gumboro disease in broilers. *Avian Diseases* 8(4): 584-596.

Petek, M., D'apirile, P. N. and Cancellotti, F. (1973). Biological and physico-chemical properties of the infectious bursal disease virus (IBDV). *Avian Pathology* 2(2): 135-152.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Sanjuán, R. and Domingo-Calap, P. (2016). Mechanisms of viral mutation. *Cellular and Molecular Life Sciences* 73(23): 4433-4448.

Sharma, J. M., Kim, I. J., Rautenschlein, S. and Yeh, H. Y. (2000). Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. *Developmental and Comparative Immunology* 24(2): 223-235.

Shwed, P. S., Dobos, P., Cameron, L. A., Vakharia, V. N. and Duncan, R. (2002). Birnavirus VP1 proteins form a distinct subgroup of RNA-dependent RNA polymerases lacking a GDD motif. *Virology* 296(2): 241-250.

Swiss Institute of Bioinformatics (2015). Infectious bursal disease virus structure and genome [http://viralzone.expasy.org/viralzone/allbyspecies/572.html] site visited on 15/06/ 2017.

Vakharia, V. N., He, J., Ahamed, B. and Snyder, D. B. (1994). Molecular basis of antigenic variation in infectious bursal disease virus. *Virus Research* 31(2): 265-273.

Van den Berg, T. P., Gonze, M. and Meulemans, G. (1991). Acute infectious bursal disease in poultry: isolation and characterization of a highly virulent strain. *Avian Pathology* 20(1): 133-143.

Van den Berg, T. P., Gonze, M., Morales, D. and Meulemans, G. (1996). Acute infectious bursal disease in poultry: immunological and molecular basis of antigenicity of a highly virulent strain. *Avian Pathology* 25(4): 751-768.

Yamaguchi, T., Ogawa, M., Miyoshi, M., Inoshima, Y., Fukushi, H. and Hirai, K. (1997). Sequence and phylogenetic analyses of highly virulent infectious bursal disease virus. *Archives of Virology* 142(7): 1441-1458.

Yao, K., Goodwin, M. A. and Vakharia, V. N. (1998). Generation of a mutant infectious bursal disease virus that does not cause bursal lesions. *Journal of Virology* 72(4): 2647-2654.