ISOLATION, PATHOLOGICAL AND IMMUNOLOGICAL CHARACTERIZATION OF KENYAN INFECTIOUS BURSAL DISEASE VIRUS STRAINS FOR VACCINE DEVELOPMENT

A THESIS SUBMITTED IN FULFILLMENT OF REQUIREMENTS FOR DOCTOR OF PHILOSOPHY DEGREE OF THE UNIVERSITY OF NAIROBI [APPLIED MICROBIOLOGY (VIROLOGY OPTION)]

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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I dedicate this thesis to my loving husband, Mr. Dickson Mutinda Mwei and to my beloved children: David Usyu, Lydia Ndunge and Kevin Kioko.

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LIST OF ABBREVIATIONS

AGPT - Agar gel precipitation test

AC-ELISA - Antigen capture Enzyme linked immuno-sorbent assay

ANOVA - Analysis of Variance

BF - Bursa of Fabricius

CVL - Central Veterinary Investigation Laboratories

CAM - Chorioallantoic membrane

DsRNA - Double stranded ribonucleic acid

ELISA - Enzyme linked immuno-sorbent assay

EID_{50} - Median (50%) embryo infective dose

H & E - Hematoxylin and Eosin

IBD - Infectious bursal disease

IBDV - Infectious bursal disease virus

IBM - International Business Machines

IBV - Infectious bronchitis virus

IFN - Interferon

IgM - Immunoglobulin M

kDa - Kilodalton

KEVEVAPI - Kenya veterinary vaccine production institute

KIPPRA - Kenya Institute for Public Policy Research and Analysis

MSI - Mean symptomatic index

OIE - Organization of International Epizootics

PI - Post inoculation

RNA - Ribonucleic acid

RT-PCR - Reverse transcription polymerase chain reaction

RVIL - Regional Veterinary Investigation Laboratories

SAN - Specific antigen negative

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPF - Specific pathogen free

SPSS - Statistical programme for social studies

USA - United States of America

VP - Viral protein

vvIBDV - Very virulent infectious bursal disease virus

ABSTRACT

Infectious bursal disease (IBD), a viral disease that affects young chickens, is a disease of economic importance and a great challenge to the poultry industry world-wide. Full protection against very virulent (vvIBDV) and variant strains may not be induced by standard vaccines. In Kenya, IBD outbreaks are rarely reported in indigenous chickens. The objectives of this study were to document the severity of the disease in indigenous chicken flocks compared to layers and broilers; isolate the virus using indigenous chicken embryos; and characterize the isolated viruses with respect to pathogenicity and immunogenicity with a view to develope a local vaccine.

A t-test and Chi square analysis were used to determine difference in mortality rates between the flock types and any association between level of mortality rate and type of flock, respectively. There was no statistically significant difference ($P \geq 0.05$) in mortality rates (measure of severity) of infectious bursal disease outbreaks between broilers, layers and indigenous flock types and no association between type of flock and level of mortality rate. Indigenous chicken embryos were successfully used in virus isolation; 44 IBDV isolates were obtained. Four (E19, E9, E7 and E42) out of the 44 isolates were selected for pathogenicity testing to represent those that had caused different mortality rates in the field; low moderate and high. Each of the selected isolates was inoculated into 36 five-week old indigenous chicks at a viral dose of 10^4 EID_{50} per chicken. Symptomatic index score scale ranged from 0 (lack of signs) to 3 (prostration and death) and histology score scale ranged from 0 (no damage) to 5 (loss of all glandular architecture). The data was analyzed using analysis of variance (two-way ANOVA). Mean symptomatic index scores were highest on days 3 and 4 post inoculations for all the isolates. Highest scores were 2.4 (isolate E9, day 4), 2.2 (isolates E19 and E7 day 4 for both) and 1.6 (isolate E42, day3). Organ index score did not vary between isolates ($P \geq 0.05$). Severest damage to glandular architecture was seen in

bursa of Fabricius by days 3 and 4 post inoculation. Highest average histological score on those days was 4.5 corresponding to E7 and lowest 3.5 to E42 and E19. All four isolates were virulent pathotype with E7 and E9 showing clearly to be vvIBDV. To determine immunogenicity, formalin killed vaccines of five isolates were separately inoculated into 4-week old specific antigen negative indigenous chickens at 0.3mls of 10^4 EID_{50} per chicken via intramuscular route on days 0, 14 and 21. Respective serum was collected on days 14, 21, 28 and 35 post inoculation and Enzyme linked immune-sorbent assay used to quantify antibodies produced. All the five isolates tested for immunogenicity yielded protective titers (above 396) by day 21 post first inoculation (653 - 2143) and rose to above 6000 by day 28. Isolate E19 consistently yielded the highest average titers (2143 by day 21 rising to 9140 by day 28).

In conclusion infectious bursal disease outbreaks in indigenous flocks were as severe as in exotic chicken flocks and virulent IBDV strains were circulating in the country causing damage of organs of immune system and high mortality rate in indigenous chicken flocks. Indigenous chickens should be vaccinated routinely against infectious bursal disease and an inactivated vaccine would produce protective antibody titers. All tested isolates were possible vaccine candidates but isolate E19 which consistently yielded a very high titer was most recommended for further vaccine development.

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND INFORMATION

Agricultural sector is the backbone of Kenya's economy and the means of livelihood for most of our rural population. Sustained agricultural growth is, therefore, critical to uplift the living standards of Kenyans as well as generate rapid economic growth (KIPPRA, 2013). Farming and related enterprises are some of the most profitable businesses that Kenyans can undertake (Agriculture Sector Development Strategy, 2010). Livestock production has been identified as a useful development tool with benefits that meet the aims of the Sustainable Development Goals (FAO, 2016). However the poultry industry in many developing countries is facing serious problems due to increasing disease challenge. Unfortunately poultry production constraints, especially those due to diseases and in particular infectious bursal disease, have rendered this investment risky (Musa *et al.*, 2010). In Kenya the main viral diseases of economic importance include Marek's disease, Newcastle disease and Infectious bursal disease (Nyaga, 2007). Marek's disease and Newcastle disease are successfully controlled through vaccination. Infectious bursal disease (IBD) has been a great challenge to the poultry industry world-wide for a long time, but particularly for the past two decades following emergence of new pathotypes; variant and very virulent strains. The disease has a major setback to productivity and profitability in the poultry industries of both developing and industrialized nations (Sainsbury, 2000). Development of variant and very virulent strains resulted in disease forms that cause significant economic losses. Until 1987, strains of the virus were of low virulence, causing less than 2% specific mortality, and were satisfactorily controlled by vaccination (van den Berg, 2000). But in 1986 and 1987, vaccination failures were described in different parts of the world (van den Berg, 2000; Kegne and Chanie, 2014). In USA it was demonstrated that the new isolates resulted from antigenic drift which could

not be satisfactorily protected by classical IBD virus (IBDV) vaccines (Jackwood and Saif, 1987; Snyder *et al.,* 1992). At about the same time in Europe, the first cases of very virulent IBDV (vvIBDV) were described in chickens with protective antibody titers (Chettle *et al.,* 1989; van den Berg *et al.,* 1991). The dramatic change in the field situation in Europe was not associated with antigenic drift but strains of increased virulence (van den Berg *et al.,* 1991). The existence of African and European/ Asian vvIBDV strains have been described in Tanzania (Kasanga et al., 2007). Morbidity and mortality rates in outbreaks due to these strains can reach up to 100% depending on the pathogenicity of the virus and the susceptibility of the flock (Lukert and Saif, 2004). The emergence of vvIBDV in Europe and variant strains in USA induced heavy mortalities in poultry despite vaccination (Rautenshlein *et al.,* 2005). These changes in antigenicity and virulence have made the task of controlling IBD by vaccination more challenging (Müller *et al.,* 2012). The sudden onset of hypervirulent IBD created the need for a better characterization of the circulating strains so that vaccination strategy would protect against circulating IBDV strains. Vaccines prepared from indigenous strains have been observed to provide better protection due to more antigenic relatedness (Zorman *et al.,* 2003).

Infectious bursal disease strains that cause outbreaks in Kenya have not been characterized. In this study, Infectious bursal disease viruses were isolated from outbreaks in Kenya and characterized, with the aim of determining a vaccine strain(s).

1.2 HYPOTHESIS

Infectious bursal disease viral strains in Kenya are of diverse pathogenicity and immunogenicity.

1.3 OBJECTIVES

1.3.1 General objective

To isolate and determine pathological and immunological characteristics of Kenyan Infectious bursal disease virus strains for vaccine development

1.3.2 Specific objectives

The specific objectives of this study are to:

1. Document variations in severity of Infectious bursal disease outbreaks in indigenous chickens and compare with those of layer and broiler flocks
2. Isolate Infectious bursal disease viruses and passage them in indigenous chicken embryos
3. Determine pathogenicity of Infectious bursal disease virus isolates using indigenous chickens
4. Determine immunogenicity of local Infectious bursal disease virus isolates in indigenous chickens

CHAPTER TWO: LITERATURE REVIEW

2.1 HISTORY OF THE DISEASE

Infectious bursal disease (IBD), also known as Gumboro disease, is an immunosuppressive disease of young chickens caused by Infectious bursal disease virus (IBDV). The disease is highly contagious and characterized by destruction of lymphocytes in the bursa of Fabricius (Rautenschlein and Alkie, 2016). When Cosgrove first reported the disease in 1962 in Delaware USA, it was initially recognized as "avian nephrosis" due to lesions in the kidneys (Cosgrove, 1962). Initially there was a misconception that the disease was caused by Infectious bronchitis virus (IBV); this was because of presence of similar gross changes in the kidneys (Lasher and Davis, 1997). However in subsequent studies, the causative agent for IBD was isolated in embryonated eggs and the disease given the respective name (Winterfield and Hitchner, 1962). The clinical features of the syndrome included whitish or watery diarrhea, followed by anorexia, depression, trembling, severe prostration, and death. At necropsy, the birds exhibited dehydration, hemorrhages in the leg and thigh muscles, urate deposits in kidneys and enlargement of the bursa of Fabricius (Cosgrove, 1962).

Classical IBD continued to affect poultry and spread all over the world ever since that first outbreak reported by Cosgrove in Delaware, USA, in 1962 (Rautenschlein and Alkie, 2016). By 1970, the disease had been reported in Canada (Ide and Stevenson, 1973), Mexico (Lucio *et al.*, 1972), Europe (Gukelberger *et al.*, 1977; Landgraf *et al.*,1972), Africa (Onunkwo, 1975), the Middle east (el-Zein *et al.*, 1974) and Asia (Mohantey *et al.*, 1977).

During the period between 1984 and 1985, a significant increase in chicken mortality was experienced in the Delmarva Peninsula, broiler growing area. The clinical picture often presented

with a respiratory involvement and varied significantly from outbreaks due to classical IBDV (Craig, 1985). Lesions varied and ranged from moderate to severe, with death usually being attributed to *E. coli* infection (Craig, 1985). Using vaccinated sentinel birds, Rosenberger *et al.* (1985) obtained IBDV isolates from these chicken flocks and found that they differed from classical IBDV strains. They produced a very rapid bursal atrophy associated with minimal inflammatory response. The available killed vaccines did not confer complete protection against these new isolates. The isolates were, therefore, designated as antigenic variants and respective killed vaccines were developed, tested and proven effective against them (Schnitzler *et al.*, 1993). Currently these types of variants are widely disseminated in the world (Rautenschlein and Alkie, 2016).

In 1989, hyperacute IBD outbreaks with unusually high mortality rates in both broiler and pullet flocks were reported in Europe (Chettle *et al.,* 1989). These hyperacute outbreaks occurred even where all the hygienic and prophylactic measures had been taken. It was realized that another dramatic change had occurred in the field situation (Chettle *et al.,* 1989). Although no antigenic drift was detected, these strains of increased virulence were identified as very virulent IBDV (vvIBDV) strains (van den Berg *et al.,* 1991). They first appeared in the Netherlands and rapidly spread all over the world (Eterradosi *et al.,* 1999) including Japan (Nunoya *et al.,* 1992), Russia (Savic *et al.,* 1997), Brazil (Ikuta *et al.,* 2001) Dominican Republic (Di Fabio *et al.,* 1999) and Asia (Chen *et al.,* 1998). Outbreaks have been reported in USA but Australia and New Zealand are currently free from vvIBDV (Jackwood *et al.,* 2011).

In West Africa severe acute IBD was reported ten years prior to the emergence of European vvIBDV strains. Several reports had mentioned unusually high IBD-induced mortalities in local or imported chickens in Africa (Okoye, 1987). However, the unusually high severity of these cases

in Africa was attributed more to concurrent aggravating factors such as climatic stress or co-infection with parasites, bacteria and other viruses than to any increased virulence of the local viruses (Eterradosi *et al.,* 1999). Later researchers reported outbreaks of vvIBDV in Africa in countries like Zambia, Tanzania, Egypt and Nigeria among others (Kasanga *et al.,* 2013; Metwally *et al.,* 2009; Kasanga *et al.,* 2007). In Kenya IBDV isolates have not been characterized yet, IBDV constantly continues to evolve in the field with varying virulence (Rautenschlein and Alkie, 2016). Pathogenicity of viruses isolated using indigenous chicken embryos was determined in indigenous chickens in Kenya in this study.

2.2 ETIOLOGY

Infectious bursal disease virus, classified in *Avibirnavirus* genus under the family of viruses called *Birnaviridae*, is the causative agent of Infectious bursal disease (Murphy *et al.*, 1995; Brown, 1986). Infectious bursal disease virus particles are non-enveloped, single shelled with diameter of 60 to 70 nm (Dobos *et al.*, 1979; Hirai and Shimakura, 1974; Harkness *et al.,* 1975; Nick *et al.,* 1976; Müller *et al.* 2003). Detailed analysis of negatively stained images and of shadowed preparations shows that the architecture of the capsid of IBDV is based on the geometry of a skew T=13 icosahedral lattice **(Figure 2.1)** of the right-handed type (Ozel and Gelderblom., 1985). By cryomicroscopy, it was determined that the subunits forming the capsid are predominantly trimer clustered. Due to the conformation of the subunits, the capsid acquires a nonspherical shape (Böttcher *et al.,* 1997).

2.2.1 Viral genome structure and organization

The genome of IBDV is made up of two segments of double-stranded RNA (dsRNA) designated A and B **(Figure 2.1 and 2.2).** Segment A contains two partially overlapping open reading frames

(ORF). The first encodes a nonstructural polypeptide of 17(kDa) known as VP5, which is not necessary for replication *in vitro* but is important for virus-induced pathogenicity (Mundt *et al.,* 1997). The second ORF encodes a 109(kDa) polyprotein that is autoproteolytically cleaved into three polypeptides, VPX, VP3 and VP4. Polypeptide VPX is further processed to produce a polypeptide known as VP2 (Hudson *et al.,* 1986; Azad *et al.,* 1987). Virus capsid is formed by VP2 and VP3 **(Figure 2.1)** which are the major structural proteins (Böttcher *et al.,* 1997). VP4 is a protease responsible for proteolytic maturation of the poly-protein (Kibenge *et al.,*1997; Birghan *et al.,* 2000).

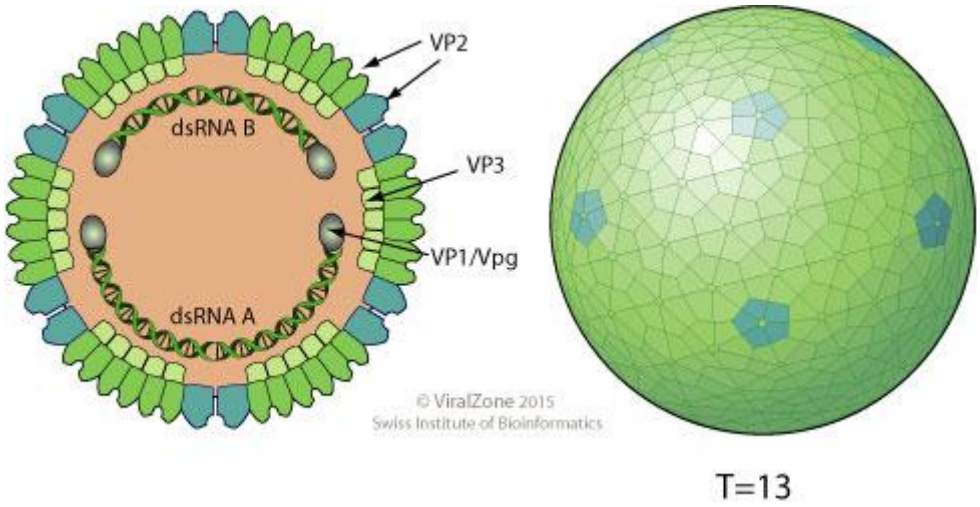


Figure 2.1: Image of a Birnaviridae virus showing non enveloped, single-shelled T=13 icosahedral symmetry virus capsid formed by viral proteins designated as VP2 and VP3 (Ref: ©ViralZone 2015, Swiss Institute of Bioinformatics)

Segment B encodes VP1, a protein which is the RNA-dependent RNA polymerase responsible for the replication of the genome and synthesis of mRNAs (Dobos, 1995; Spies *et al.,* 1987). Viral protein 1 (VP1) shares a number of primary sequence features with RNA polymerases from diverse origins (Bruenn, 1991). At the 5' and 3' ends in both genome segments of IBDV, there are direct terminal and inverted repeats that are likely to contain important signals for replication, transcription and packaging. It is however, not known whether virulence variations are due to

mutations in these regions (Nagarajan and Kibenge, 1997). A high degree of nucleotide (89%) and amino acid (93-98%) sequence similarities have been noticed between non-pathogenic serotype 2 and pathogenic serotype 1 **(Figure 2.2)** viruses. When regions of VP1 of a vvIBDV strain were exchanged with VP1 counterparts of an attenuated IBDV, the resulting recombinant virus showed reduced virulence and bursal lesions in chickens (Le Nouën *et al.,* 2012). Recent studies have identified putative virulence markers in VP1 of IBDV field isolates (Yu *et al.,* 2010).The inverted adjacent repeats at the 3' terminus on segments A and 5' terminus on segment B have the potential to form stem and loop secondary structures (Kibenge *et al.,* 1996), which are involved in the processes of RNA replication, translation and encapsidation like other RNA viruses such as poliovirus (Simoes and Sarnow, 1993). In serotype 2 viruses there is one insertion of one amino acid between hydrophilic regions 1 and 2 and deletion of one amino acid at the VP4 region **(Figure 2.2).**

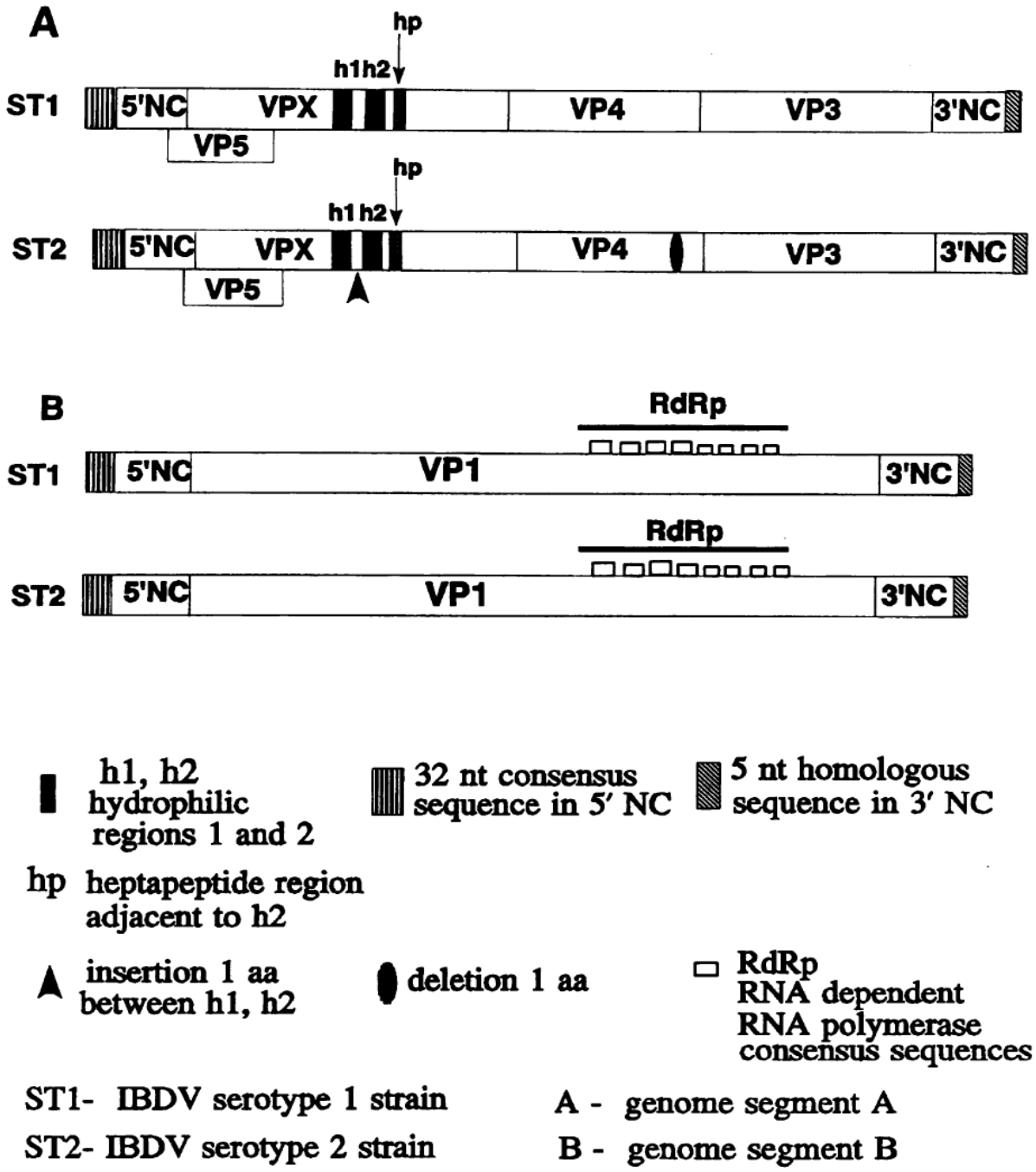


Figure 2.2: Schematic presentation of genomic organization of infectious bursal disease virus serotypes 1 and 2 (Reference: Nagarajan and Kibenge, 1997).

2.2.2 Virus replication

The virus replicates in the cytoplasm of infected cells which are usually B lymphocytes in bursa of Fabricius. Viral protein 1 is the polymerase responsible for viral RNA replication and mRNA synthesis (Wei *et al*., 2006). When a cell is infected with IBDV, virus specific ssRNA and dsRNA are synthesized. Demonstration of genome-linked proteins (VPg) in IBDV has indicated that nucleic acid replication is by a strand displacement (semi-conservative) mechanism (Müller and Nitschke, 1987; Spies *et al.*, 1987). The inner capsid space of IBDV is occupied by a ribonucleo-protein complex. This complex is formed by the dsRNA genome wrapped up by the VP3 polypeptide and covalently linked to the VPg form of the VP1 RNA-dependent RNA polymerase and by ‘‘free’’ VP1 molecules. The free VP1 molecules act both as primer and polymerase during RNA transcription (Luque *et al.*, 2009).

2.2.3 Viral Proteins

Five proteins are identified in IBDV by SDS-PAGE analysis. They are VP1 (90 kDa), VP2 (40 kDa), VP3 (35 kDa), VP4 (28 kDa) and VP5 (21 kDa) (Dobos, 1979; Nagarajan and Kibenge, 1997). Protein VP2 is the most abundant polypeptide and makes up about 51% of the virion protein (Dobos, 1979); VP3 is the second most abundant protein and makes up 40% of the virion protein; while VP4 and the VP1 are minor proteins of the virion accounting for 6% and 3% respectively.

Protein VP1 is RNA dependent RNA polymerase which has the polymerase and capping enzyme activities (Spies and Muller, 1990; Spies *et al.*, 1987). In mature virus, it is tightly bound to both ends of the genome and circularizes them (Müller and Nitschke, 1987). Thus, VP1 is present in the virion as a free polypeptide as well as a VPg (Müller and Nitschke, 1987). It is involved in the efficiency of viral replication and modulates the virulence *in vivo* (Liu and Vakharia, 2004).

Both VP2 and VP3 are responsible for the structural integrity of the virion. Protein VP3 is found inside of the capsid and carries a very basic carboxy terminal which interacts with a packaged RNA (Hudson *et al.,* 1986). Interaction between VP1 and VP3 plays a major role in efficient encapsidation (Lombardo *et al.,* 1999; Tacken *et al.,* 2000). Protein VP2 carries major neutralizing epitopes suggesting that it is at least partly exposed on the outer surface of the capsid (Bottcher *et al.,* 1997). The protein (VP2) also contains the antigenic region responsible for the production of neutralizing antibodies and is highly conformation dependent (Azad *et al.,* 1987; Betcht *et al.,* 1988). Neutralizing monoclonal antibodies against VP2 can be used to differentiate the serotypes and strains (Becht *et al.,* 1988). The protein (VP2) is also responsible for antigenic variation (Brown *et al.,* 1994), virulence (Yamaguchi *et al.,* 1996) and tissue culture adaptation (Lim *et al.,* 1999). Viral antigenicity is controlled by a few amino acids located in the exposed P domain of VP2 (Letzel *et al.,* 2007). The VP3 protein on the inner side of the capsid contains group-specific epitopes and serotype-specific epitopes that elicit non-neutralizing and non-protective antibodies (Kochan *et al.,* 2003).

Proteins VP2 and VP5 are involved in induction of apoptosis in chicken B-lymphocytes (Yao and Vakharia, 2001). Protein VP4 is the viral protease (Jagadish *et al.,* 1988) and contributes to processing of precursor polypeptide (Azad *et al.,* 1987; Jagadish *et al.,* 1988). The IBDV VP4 utilizes a serine lysine catalytic dyad (Ser –652 and Lys 692) (Lejal *et al.,* 2000; Mundt, 1999). Final VPX –VP2 processing is associated with the final maturation or release steps of the virus (Kibenge *et al.,* 1999) and correct scaffolding of the VP3(Müller *et al.,* 2003).

Protein VP5 is a membrane-associated protein involved in viral release (Lombardo *et al.,* 1999; Méndez *et al.,* 2015) it is, however, not essential for viral replication (Mundt *et al.,* 1997). It is

highly basic, cysteine rich and conserved among all serotypes of IBDV strains (Yao and Vakharia, 2000).

2.3 TRANSMISSION

Infectious bursal disease is a highly contagious disease that may be transmitted through direct contact between infected and susceptible young chickens or indirectly through fomites. Infected chickens begin to shed IBDV in faeces one day after infection and can transmit the disease for at least 14 days post infection (Vindevogel *et al.,* 1976). Under natural conditions, the most common mode of infection is via the oral route (Sharma *et al.*, 2000). Direct transmission of the virus occurs via contact with infected birds, contaminated litter or faeces (Office International des Epizooties, 2004). Transmission can also occur through airborne dissemination of virus-laden feathers or poultry house dust (Benton *et al.*, 1967).

Indirect transmission of virus usually occurs via fomites such as feed, equipment, people's clothing and shoes (Benton *et al.*, 1967). The virus is not transmitted through the egg or transovarian route and there is no carrier state in recovered birds (Lukert and Saif, 2004). Infectious bursal disease virus is very persistent in the environment of a poultry house and can be difficult to eradicate from premises which housed infected chickens (Benton *et al.*, 1967).

2.4 HOST SUSCEPTIBILITY

Chicken is the only bird among the avian species known to be susceptible to IBDV where the virus induces clinical disease and causes IBD characteristic lesions (Lukert and Saif, 2004). Turkeys, ducks and ostriches are susceptible to infection with IBDV but do not show signs of clinical disease (McNulty *et al.*, 1979; Lukert and Saif, 1997). Several species of free-living and captive birds of prey yield positive results when examined for antibodies to IBDV (Eterradossi and Saif, 2008).

Antibodies to IBDV have been detected in wild birds and several rare avian species including Antartic penguins, ducks, gulls, crows and falcons (Eterradossi and Saif, 2008). The genome of IBDV has been detected in free living pigeons and guinea fowls (Kasanga *et al.,* 2008). Wild birds therefore may play a role in epidemiology of IBD.

Domestic fowl are the natural host of IBDV (Hemboldt and Garner, 1964). All breeds of chicken are affected but there is variation in severity of the disease between breeds (Mutinda *et al.,* 2013). White Leghorns exhibit the most severe disease and have the highest mortality rate (Lukert and Saif, 1997). Variation in severity of the disease between exotic and indigenous chickens in Kenya was determined in this study.

2.5 PATHOGENESIS

Chickens acquire IBDV infection orally or by inhalation. The virus is transferred from the gut to the other tissues by phagocytic cells like macrophages. In macrophages of the gut associated tissues it could be detected as early as 4 hours after oral inoculation using immunofluorescence (Müller *et al.,* 1979). The virus then reaches the bursa of Fabricius via the blood where the most extensive virus replication occurs. By 13 hours post inoculation (PI) most follicles are positive for virus and by 16 hours PI a second and pronounced viremia occurs accompanied by secondary replication in other organs resulting in disease and death (Van den Berg *et al.,* 2000).

Cells that produce IgM (IgM+ cells) are the target lymphocytes for the virus. During the acute phase of the disease, there is a reduction in the circulating IgM + cells (Hirai *et al.,* 1981; Rodenberg *et al.,* 1994) but circulating IgG level remains the same (Giambrone *et al.,* 1977; Kim *et al.,* 1999). The bursa undergoes atrophy as the bursal follicles get depleted of B cells. Virus replication causes extensive damage to lymphoid cells in medullary and cortical regions of the

follicle. Apoptosis of the neighboring non infected B cells augments the destruction of the bursa morphology. By this time an ample amount of viral antigen can be detected in other lymphoid organs like caecal tonsils and spleen (Sharma *et al.,* 2000; Tanimura and Sharma, 1998). The destruction of lymphocyte populations associated to the infection causes immune suppression and hampers the immunological maturation of infected birds (Sharma *et al.,* 2000).

Although T cells are resistant to infection by IBDV (Hirai *et al.,* 1979), they play a significant role in the pathogenesis of IBD (Muller *at al.,* 2003). Transient lesions appear in the thymus during the acute phase of the disease (Sharma *et al.,* 2000). A dramatic influx of T cells is reported in and around the site of virus replication (Tanimura and Sharma, 1997). The infiltrated T cells could be detected from 1st to 12th week post inoculation although the viral antigen disappears by the 3rd week. Intra bursal T cells and T-cell-mediated responses play significant role in viral clearance and promoting recovery from infection. The IBDV induced cytotoxic T cells limit the spread of the virus by destroying the cells expressing the viral antigen and thus initiate the recovery process (Muller *et al.,* 2003). At the same time IBDV induced T cells enhance the viral lesions by producing inflammatory cytokines; T helper cells produce inflammatory cytokines like IFN-γ which activate the macrophages to produce nitric oxide (NO) (Sharma *et al.,* 2000). The NO production after IBD virus infection exerts antiviral effect since it has been shown that immune-suppressed chickens which failed to induce NO have more severe disease and higher degree of virus replication. Production of NO does not however seem to correlate with the hemorrhagic lesions which result from the reaction of host-factors (anticoagulant) and the determinants responsible for virus virulence and virus clearance (Poonia and Charan, 2000).

Humoral immunity is the primary mechanism of the protective immune response. Antibody production is stimulated at the primary site of viral replication in gut associated tissue and they

can be detected as early as 3 days PI. These antibodies prevent the spread of the virus to other tissues. Due to the rapid onset of antibodies, the necrotic foci that form in the bursa of Fabricius stop expanding and are completely eliminated (Becht, 1980).

Both humoral and cellular arms of the immune system are compromised during IBDV infection due to lysis of the B cells and altered antigen-presenting cells. The IBDV induced damage to humoral immunity is reversible. Antibody production correlates with the morphologic restoration of the bursal follicles and return to normal levels of mitogenic response of T cells. Chickens that survive the disease, clear the virus and recover from its pathologic effects (Sharma *et al.*, 2000).

2.6 CLINICAL PRESENTATION

There are three principal clinical presentations of Infectious bursal disease: - hyperacute form, classical and immuno-suppressive forms. Clinical presentation depends on the virulence of the infecting strain, presence of IBDV specific antibody as well as the age and breed of chickens in the affected flock. The incubation period of IBD ranges from 2 to 4 days after exposure. In general the chickens may show variable morbidity and mortality with severe depression lasting about 5-7 days (Office International des Epizooties, 2004). Usually, mortality starts at the second and third day after infection, reaches a peak at day 4 and then drops quickly (Office International Des Epizooties, 2004). The surviving chickens recover rapidly with a state of apparent health after 5 days (van den Berg *et al.*, 2000). The main clinical signs are white watery diarrhoea, vent feathers are stained with urates, ruffled feathers, reluctance to move, anorexia, trembling and prostration (Office International des Epizooties, 2004).

The classical form, as described since early 1960s, is caused by the classic moderate virulent strains of IBDV. The disease is characterized by acute depression followed by typical signs and

lesions. Classical IBDVs induce approximately 10–50% mortality (Office International des Epizooties, 2004). The feed intake is depressed but water consumption may be elevated. Severely affected birds become dehydrated and die (Cosgrove, 1962; Faragher, 1972).

The hyperacute form described initially in Europe, and then spread to Asia, Africa and some countries in Latin America, is caused by hypervirulent strains of IBDV also referred to as very virulent strains. This form is characterized by a hyperacute progressive clinical disease leading to high mortality rates on affected farms. Very virulent IBDVs induce approximately 50–100% mortality with typical signs and lesions (Office International des Epizooties, 2004). The initial outbreaks in Europe were characterized by high morbidity (80%) and significant mortality attaining 25% in broilers and 60% in pullets over a 7-day period (Chettle *et al.,* 1989; van den Berg *et al.,* 1991; Nunoya *et al.,* 1992). Later studies reported that mortality induced by vvIBDV could range between 40–100% in fully susceptible specific pathogen free (SPF) chickens, 60% in layers, and 30% in broilers (van den Berg *et al.,* 1991; van den Berg and Meulemans, 1991; Jackwood *et al.,* 2009).

The immunosuppressive form, principally described in the United States, is caused by low-pathogenicity strains of IBDV, as well as by variant strains, such as the Delaware variant strains, which partially resist neutralization by antibodies against the so-called "classic" or standard strains (Snyder, 1990). Variant IBDVs induce little if any clinical signs and no mortality but marked bursal lesions (Office International des Epizooties, 2004).Variation in severity of IBD outbreaks in layers, broilers and indigenous chickens in Kenya was determined in this study.

2.7 PATHOLOGICAL FINDINGS

2.7.1 Gross lesions

The tissue distribution and severity of lesions is dependent on the subtype and pathogenicity of the virus (Rosenberger and Cloud, 1986; Tanimura *et al.,* 1995). Chickens which die acutely of primary IBD infection show dehydration of the subcutaneous fascia and musculature of the thigh, inguinal and pectoral areas (Cosgrove, 1962; Hirai *et al.,* 1981). Hemorrhages occur in thigh and pectoral muscles and sometimes on the mucosa at the proventriculus junction and on the serosal surface and plicae of the bursa (Hanson, 1962; Huff *et al.,* 2001). Kidneys show enlargement and pallor with accumulation of crystalline urate in tubules (Cosgrove, 1963). Splenic enlargement has been documented, with small gray foci uniformly dispersed through the parenchyma (Hirai *et al.,* 1981).

The cloacal bursa is the target organ for the replication of IBDV and hence the most severely affected. It is the main organ in which lesions develop following exposure to IBDV (Cheville, 1967). Characteristic pattern of bursal changes observed during the course of infection differ for the classic and variant viruses (Rosenberger and Cloud, 1986). During the infection with classic viruses, the bursa increases transiently in size accompanied with inflammation. When the inflammation subsides, rapid bursal atrophy occurs. Chickens that die at early stages after the infection show a doubling in size of the bursa due to oedema. The bursa is pale yellow in colour and shows striations. By the 5th day the bursa returns to normal weight, but it continues to atrophy, and from the 8th day forward it is approximately one-third its original weight (Hirai *et al.*, 1981).

The vvIBDV strains are able to cause greater decrease in thymic weight index and more severe lesion in cecal tonsils, thymus, spleen, and bone marrow, but the bursal lesions are similar (Lukert and Saif, 2004).

Variant strains have been reported that do not induce an inflammatory response (Rosenberger and Cloud, 1986; Sharma *et al.*, 1989). However, it was reported that one variant strain was able to induce inflammatory lesions (Hassan and Saif, 1996).Variation in pathogenicity of IBDV has also been observed between different breeds of chickens where generally light layer breeds have been reported to be more susceptible than heavy broilers (van den Berg and Meulemans, 1991). Pathogenicity of IBDV strains isolated from outbreaks in this study was determined in indigenous chickens.

2.7.2. Histological lesions

The virus causes extensive destruction of lymphocytes producing lesions in the bursa of Fabricius and other lymphoid tissues (Mahgoub, 2012). Infection with standard and variant strains results in death of bursal B lymphocytes. Necrosis of lymphocytes in the medulla of the organ can be detected within one day of infection (Mahgoub, 2012). By the third day an inflammatory response with oedema, heterophil infiltration, congestion and hemorrhage is present in infections by standard strains. At this time the follicles may be reduced to a necrotic center surrounded by heterophils. From the fourth day after infection the acute inflammatory reaction declines, and as necrotic debris is cleared by phagocytosis, cystic cavities are formed. Fibroplasia occurs in the surrounding connective tissue and the covering epithelium becomes infolded and irregular (Cheville, 1967; Pope, 1996). Sharma *et al.* (1989) observed that the infection with the variant

strain did not result in an acute inflammatory response, and follicular lymphoid necrosis was evident at three days after infection.

The development of lesions by IBDV in thymus depends on the pathotype of the virus (Inoue *et al.,* 1994; Tanimura *et al.,* 1995). Thymocyte depletion induced by IBDV in the cortex of the thymus is caused by apoptosis (Inoue *et al.,* 1994). The highly pathogenic strains of IBDV are associated with severe thymocyte loss when compared to less pathogenic strains (Tanimura *et al.,* 1995).

The spleen shows hyperplasia of the reticuloendothelial cells around the adenoid sheath arteries during the early stages of infection. Lymphoid necrosis occurs in the peri-arteriolar lymphoid sheath by 3 days post infection. Recovery of the spleen occurs without any sustainable damage to the germinal follicles (Cheville 1967; Lukert and Saif, 2003).

The Harderian gland may be severely affected by IBDV infection. Normally this gland is infiltrated and populated with plasma cells as the chicken ages. Infection with IBDV prevents this infiltration (Survashe *et al.,* 1979). Harderian gland of the chickens infected at 1 day of age has 5-10 folds fewer plasma cells than those of uninfected chickens for up to 7 weeks of age (Dohms *et al.,* 1981). However, lymphoid follicles and heterophil populations in the harderian gland may not be affected by IBDV infection and even necrotic or degenerative changes may not be found in the acini or excretory ducts. In contrast, broilers infected at 3 weeks of age have a 51 % reduction in plasma cell content at 5-14 days post infection (Dohms *et al.,* 1988). This reduction is temporary and levels become normal after 14 days.

In caecal tonsils, there may be acute heterophilic inflammation, destruction of lymphocytes, with regeneration on the fifth day after infection (Hemboldt and Garner, 1964).Characteristic

congestion of liver tissue and fatty degeneration of hepatocytes have been reported in IBDV infection (Ma *et al.,* 2013).

The effect of isolated Kenyan IBDV strains to histology of bursa of Fabricius, thymus gland, caecal tonsils, Harderian gland and spleen of indigenous chickens in Kenya was determined in this study.

2.8 IMMUNOSUPPRESSIVE PROPERTIES OF IBDV

Both clinical and subclinical IBDV infections cause immunosuppression, compromising humoral and cellular immune responses (Sharma *et al.,* 2000). Cho (1970) demonstrated that white leghorn chickens exposed to IBDV at one day of age were consistently more susceptible to develop visceral tumors and nerve enlargement by Marek's disease virus. Further Allan *et al.*(1972) reported that IBDV infections at early age were immunosuppressive; they severely depressed the antibody response to Newcastle disease.

Virus replication in bursa of Fabricius leads to extensive lymphoid cell destruction in the medullary and cortical region of the follicles (Tanimura and Sharma, 1997). The acute lytic phase of the virus is associated with a reduction in circulating IgM+ cells (Hirai *et al.,* 1981; Rodenberg *et al.,* 1994). Exposed chickens produce suboptimal levels of antibodies against a number of infectious and noninfectious antigens (Kim *et al.,* 1999; Cho, 1970; Faragher *et al.,* 1974; Wyeth, 1975).

Only the primary antibody response is impaired, the secondary responses remain intact (Rodenberg *et al.,* 1994; Sharma *et al.,* 1989) and this humoral deficiency may be reversible. Although destruction of antibody producing B cells may be one of the principal causes of humoral deficiency, other possible mechanisms may include the adverse effect of IBDV on antigen-presenting and helper T cell functions (Sharma *et al.,* 2000).

The virus has effect on T-cells although the cells are resistant to infection with IBDV and there is no evidence that the virus actually replicates in thymic cells (Tanimura and Sharma 1998; Sharma *et al.,* 1989). However, there is evidence that *in vitro* mitogenic proliferation of T cells of IBDV exposed birds is severely compromised(this mitogenic inhibition is mediated most probably by macrophages);it is not clear how IBDV induces macrophages to exhibit this suppressor effect (Sharma *et al.,* 2000). Sharma *et al.,* (2000) detected a dramatic infiltration of T-cells in the bursa of Fabricius during the acute phase, accompanied by the precipitous drop in the number of IgM+ cells. The infiltrating cells were predominantly CD8+; seen from the seventh day of infection. It was suggested that T-cells modulate the infection, limiting the viral replication in the bursa in the early phase of the disease, and they also promote bursal tissue damage and delay recovery, possibly through the release of cytokines and cytotoxic effects (Rautenschlein *et al.,* 2002). Cytotoxic T cells may exasperate virus-induced cellular destruction by lysing cells expressing viral antigens. T cells may also promote the production of inflammatory factors increasing the tissue destruction such as nitric oxide (Sharma *et al.,* 2000). Immunogenicity of IBDV isolates was determined in indigenous chickens in Kenya in this study.

2.9 DIAGNOSIS OF IBDV

Initial diagnosis of an outbreak of IBD in a flock is based on the clinical signs and grossly visible lesions on the bursa of Fabricius. Differential diagnoses, with respect to clinical signs, include Newcastle disease, inclusion body hepatitis, mycotoxicosis and infectious bronchitis. In subclinical and immunosuppressive forms of IBD, Marek's disease and chicken anemia are also considered (Lasher and Shane, 1994; Lukert and Saif, 1997); however, normally, these can easily be differentiated at post-mortem examination. Microscopic lesions caused by IBDV are well characterized (Cheville, 1967; Pope, 1996; Sharma *et al.,* 1989) and histopathological diagnosis

has the advantage of giving valuable information about the virulence of IBDV strain involved and the possible time when the infection occurred. Current serological tests include agar gel precipitation test (AGPT), virus neutralization test and enzyme linked immunoassay (Marquadt *et al.,* 1980; Rosenberger *et al.,* 1998; OIE, 2008). Enzyme linked Imunoassay (ELISA) is widely used because it is a sensitive and rapid method and it enables handling of high amounts of samples. Serological techniques make it possible to detect the immunologic response in an outbreak or evaluate vaccination programs (Odor, 1995). The use of monoclonal antibodies in the antigen-capture ELISA (AC-ELISA) allows for more precise antigenic characterization (Snyder *et al.,* 1992). The virus can be isolated in embryonated eggs, cell cultures or by inoculation of susceptible birds. Inoculation in birds is the best method, because the other methods may modify the original characteristics of the IBDV field strains (Rosenberger *et al.,* 1998).

Other tests that have been used are fluorescent antibody techniques, immunohistochemistry and molecular techniques. The reverse transcription-polymerase chain reaction (RT-PCR) allows for the detection of viral RNA from infected clinical samples (Jackwood *et al.,* 1996; Lee *et al.,* 1992; Wu *et al.,* 1992). Differentiation of the strains is possible if the RT-PCR products are further analyzed using restriction enzymes (Jackwood and Jackwood, 1994; Jackwood and Jackwood 1997; Jackwood and Sommer, 1998). Other molecular techniques include the use of DNA probes (Jackwood, 1990; Jackwood *et al.,* 1992).

2.10 VACCINATION

Control of infectious bursal disease in chickens requires the application of sound biosecurity measures alongside effective vaccinations of chicks and parent flocks (Müller *et al.,* 2012). There are both live attenuated and inactivated vaccines for control of IBDV infections. Precise timing is

crucial in administration of live vaccines to chicks due to interference of maternally derived antibodies on the performance of live vaccines (Müller *et al.,* 2012). On the other hand, high parental immunity is beneficial in protecting young chicks from field virus challenge during the critical first 2 weeks of life when the bursa is highly vulnerable to damage caused by IBDV (Hitchner, 1976).

Inactivated vaccines do not replicate in the bird and are costly to produce and administer but have been found useful in administration to parent flocks prior to lay to provide passive immunity to offspring via maternally derived antibodies. The inactivated vaccines must have an antigenic content that is high enough to induce high immunity in parent flocks that can be passed to progeny at protective levels (Rosenberger *et al.,* 1987). Usually inactivated vaccines work best when administered in a prime-boost regimen, where attenuated live IBDV vaccines are first used for priming.

Live vaccines commonly used in chicks are suitable for mass vaccinations, do not require an adjuvant and can replicate in the bird to induce both humoral and cell mediated immunity (Müller *et al.,* 2012). One of the main side effects of the live vaccines is reversion to virulence resulting in disease and loss of production. Most conventional live vaccines are subjectively classified as mild, intermediate and intermediate plus or "hot" vaccines depending on the level of attenuation (Rautenschlein *et al.,* 2005). The mild vaccines do not neutralize high levels of maternally derived antibodies and in contrast some of the intermediate and most of the hot vaccines cause severe bursal lesions and could easily revert back to virulence (Hair-Bejo *et al.,* 2004).

Vaccinations have not been very successful in different parts of the world due to progressive changes in antigenicity and virulence of the virus and poor handling of the vaccines (Mutinda, *et*

al., 2014; Müller et al., 2012). In view of this, however, vaccination still remains the single most important method of controlling IBDV in the field besides biosecurity. Other vaccines either being developed or already developed but not extensively used due to varied reasons include genetically engineered vaccines, subunit vaccines, viral vector vaccines and immune complex vaccines (Müller *et al.*, 2012).

In Kenya, use of imported live attenuated vaccines in exotic commercial chicken flocks and inactivated vaccines in breeding flocks has not successfully controlled the disease since outbreaks continue to be reported in vaccinated flocks (Mutinda *et al.,* 2014). In addition, there is very scanty information on outbreaks or control of the disease in free range indigenous chickens which constitute about 76% of the poultry population in Kenya (King'ori *et al.,* 2010). This information gap was addressed in this study and potential vaccine virus candidates isolated and characterised.

2.11 ECONOMIC SIGNIFICANCE

Infectious bursal disease virus is worldwide in distribution and is an important virus in the poultry industry as it causes immune suppression and mortality in infected chickens (Jackwood *et al.,* 2011). The disease is a major set-back to productivity and profitability in the poultry industries of both developing and industrialized nationals. Direct losses linked to specific mortality depend on the dose and virulence of infecting IBDV strain, age and breed of the chicken and presence or absence of immunity (van den Berg *et al.,* 2000). Indirect economic impact of the disease, when quantified, is considerably significant (Musa *et al.,* 2012). It occurs due to virus induced immune-suppression and the interactions of IBDV and other viruses, bacteria or parasites (Farooq *et al.,* 2003). Losses occur due to secondary infections, growth retardation and condemnation of carcasses at the slaughter houses (Farooq *et al.,* 2003). Until the emergence of variant and very

virulent strains of IBDV in 1986, the significance of IBD was confined to the immunosuppression (van den Berg *et al.,* 2000). In broilers, immunosuppression is denoted by the high prevalence of viral respiratory infections and elevated mortality due to airsacculitis and colisepticaemia. In addition, the condemnation rate in processing plant may be increased tenfold from 0.5% to over 5% in affected flocks.

A decrease of 10% in profit margin was attributed to relative depression in body mass and feed conversion efficiency due to IBD in a study on economic effect of subclinical IBD (Mcllroy *et al.,* 1989). In studies on vvIBDV, it was shown that infection with strain 849VB in 38-day-old hybrid Leghorn pullets, resulted in 60% losses in form of mortality rate, whereas, broiler chicks infected at the same age showed 17% mortality (van den Berg and Meulemans, 1991). The virus does not affect man and has no direct public health significance (Lukert and Saif, 2004).

CHAPTER THREE: INFECTIOUS BURSAL DISEASE OUTBREAKS IN INDIGENOUS CHICKEN AND OTHER FLOCK TYPES IN KENYA

3.1 INTRODUCTION

The first case of IBD in Kenya was reported in 1991 in commercial birds at the Kenyan coast (Mbuthia and Karaba, 2000). The disease, commonly known as Gumboro disease, is caused by Infectious bursal disease virus (IBDV) and is an important threat to commercial poultry industry worldwide (Müller *et al.,* 2003). The subclinical form of the disease is common in chicks less than three weeks of age and it is characterized by severe immune-suppression and lack of clinical signs (Lukert and Saif, 2003). Clinical form of the disease mainly affects 3-6 week old chicks and usually has a sudden onset and a short incubation period. Affected birds manifest prostration, vent pecking, profuse watery diarrhea, followed by death or rapid recovery; in severe cases, there is extensive destruction of lymphocytes especially in the bursa of Fabricius. This form is characterized by variable morbidity and mortality rates, both of which can reach up to 100% depending on the pathogenicity of the virus and the susceptibility of the flock (Lukert and Saif, 2004). Heavy mortality, reduction in growth rate and immuno-suppression in affected flocks contribute to economic importance of the disease (Shome *et al.,* 1997). The disease has been studied more in exotic commercial chickens than in indigenous chickens (Mutinda *et al.*, 2014). The purpose of this study was to document the severity of natural outbreaks in indigenous chicken, and compare them with those of broiler and layer flocks in Kenya.

3.2 MATERIALS AND METHODS

3.2.1 Study area and study material

The study covered all the former provinces (previous administrative boundaries) of Kenya where there were reports of outbreaks of infectious bursal disease. Data and samples were collected from outbreaks reported in Coast (Kwale, Mombasa, Taita taveta, Kilifi), Central (Nairobi, Kiambu), Eastern (Machakos, Mbeere, Meru), Riftvalley (Kericho, Kajiado), and Nyanza (Rachuonyo) regions. North Eastern and Western parts of Kenya did not report any outbreaks during the period of the study (December 2011 to June 2013).

3.2.2 Permission to carry out the study

Permission to carry out the experiments was accorded by Faculty Biosafety, Animal Use and Ethics Committee via approval dated 29th October 2012, Ref wanz5101012. The Director of Veterinary Services granted permission to utilize Veterinary Services Departmental field staff and institutions to identify outbreaks of infectious bursal disease in flocks of chicken in Kenya.

3.2.3 Study design

Letters were written and follow up calls made to personnel in Regional Veterinary Laboratories and private clinics in the country to identify and report any suspected outbreaks of the disease. The National Central Veterinary Laboratories (CVL), Kabete; Regional Veterinary Investigation Laboratories (RVIL) at Mariakani, Ukunda and Nakuru, University of Nairobi Poultry clinic and Nakuru Veterinary Resource Center were engaged to identify outbreaks and assist in collecting samples. These institutions were selected based on previous reports of the disease in their areas of jurisdiction. All the institutions were supplied with sample collection bottles, cool boxes and other

sampling materials. Carcasses of chicken submitted to these institutions for diagnosis were opened and full postmortem examination done (Charlton *et al.,* 2006). History of the outbreak was collected and outbreak farms visited for collection of more fresh carcasses. On postmortem diagnosis of infectious bursal disease, bursa of Fabricius samples were aseptically collected, placed in sterile universal bottles and transported in a cool box to the University of Nairobi virology laboratory. Disease confirmation was done using AGPT

3.2.4 Farm visits and collection of fresh carcasses

Visits to the outbreak farms were made as outbreaks were reported. Clinical signs presented by the sick birds were observed and recorded. For each outbreak, information on when the outbreak started, type of birds affected, number affected, number dead, duration of outbreak, vaccination history and the clinical signs observed was collected. Fresh carcasses were collected for postmortem examination **(Figures 3.1 and 3.2).** A follow up phone call was made at the end of the outbreak to establish the total number dead.

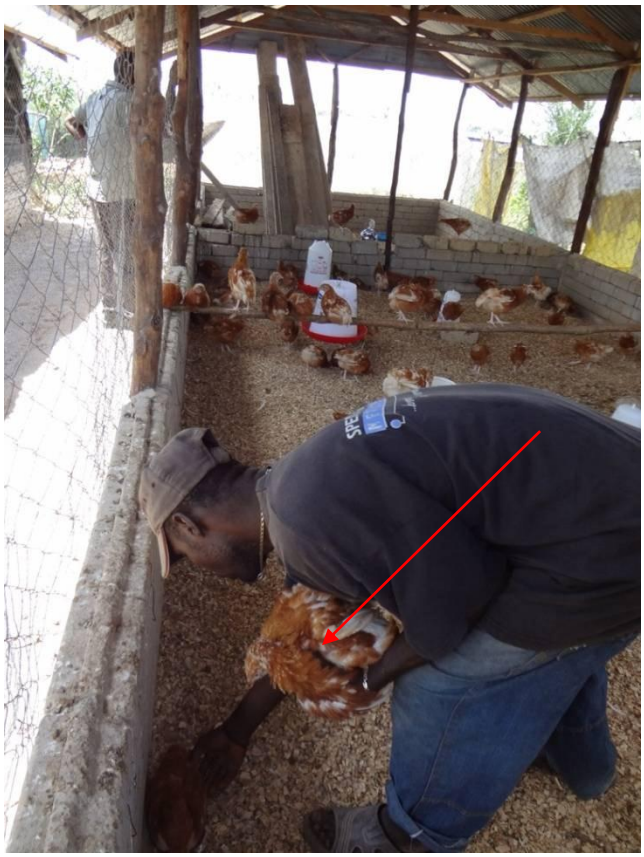


Figure 3.1: A farmer collecting dead and sick birds (red arrow) in a flock of pullects affected by Infectious bursal disease

Figure 3.2: Selection of fresh carcasses (yellow arrow) for postmortem examination from among dead birds in an Infectious bursal disease outbreak farm

3.2.5 Postmortem examination, sample collection and processing

Carcasses were opened in the laboratories where cases were submitted and postmortem examinations performed as described by Charlton *et al*. (2006). From each fresh carcass opened the bursa of Fabricius was aseptically collected, placed in a sterile universal bottle and chilled. The samples were transported in a cool box to the virology laboratory at the University of Nairobi. Bursal tissues were homogenized into 50% (w/v) suspension in phosphate buffered saline. The homogenate was centrifuged at 2000 rpm for 30 minutes, the supernatant harvested tested for viral antigen by Agar gel precipitation test (OIE, 2008; Chuahan and Roy, 1998) and kept for virus isolation experiments in a later study (Chapter 4).

3.2.6 Agar gel precipitation test

This test was carried out as described by Okoye and Uzoukwu (1981) with minor modifications as described by Mutinda (2011). Agar gels were prepared as described in the OIE (2008) manual of standards. In a layer of agar on a petri-dish, a hexagonal pattern of one central and six peripheral rounded wells 6 mm in diameter 3mm apart were cut using a template and tubular cutter (OIE, 2008), to make a hexagonal pattern. The well at the center was filled with 50µl of standard antiserum; while the five peripheral wells were filled with test sample bursal homogenates (prepared as described in **section 3.2.5**) alternated with known standard antigen. Results were read by checking for an opaque white line of precipitation between the central well and peripheral wells; this happened in places where homologous antigens and antibodies met in optimal concentration (OIE, 2008). Standardized antigens J.nr. 75.60353 (freeze-dried bursa) and standardized antisera, CUX 52/70 75.60352, which had been supplied for a previous study to RVIL Mariakani by A.M. Bojesen of The Royal Veterinary Agricultural University, Denmark, were used.

3.2.7 Data analysis with respect to mortality rates

Descriptive statistics generated included the mortality rates for each farm and the average mortality rates for the vaccinated and unvaccinated flocks. A t-test was used to determine difference in mortality rates between the flock types. Chi square analysis was used to check for any association between level of mortality rate (20% and above was considered high) and two independent variables; type of flock and vaccination history. A p-value of less than 0.05 ($P<0.05$) was considered statistically significant (Preacher, 2001).

3.3 RESULTS

3.3.1 Description of flocks with confirmed outbreaks

Post-mortem examination was done on sample birds from 82 flocks with suspected outbreak; out of these, 39 had lesions, consistent with the disease and were confirmed to be IBD outbreaks by AGPT; they were, therefore, included in the study. Out of the 39 flocks with confirmed outbreaks, 19 were layer, 5 were broiler and 15 were indigenous chicken flocks. The layer, broiler and 8 of the indigenous flocks were being produced commercially under intensive production system while 7 of the indigenous flocks were under free range extensive system where they were scavenging for food. The flock sizes ranged from 100 to 1100 birds among commercial flocks and 8 to 40 birds among the indigenous free-range birds. The birds were of mixed ages ranging from 3 weeks to 14 weeks. Twenty two of the flocks were vaccinated against IBDV by the farmers while seventeen were not.

3.3.2 Clinical signs

In all outbreak flocks, birds showed drowsiness, depression **(Figures 3.3 and 3.4),** drooling of saliva, ruffled feathers, white watery diarrhea, severe prostration and death. The affected birds were from layers, broilers and indigenous flocks, vaccinated and unvaccinated.

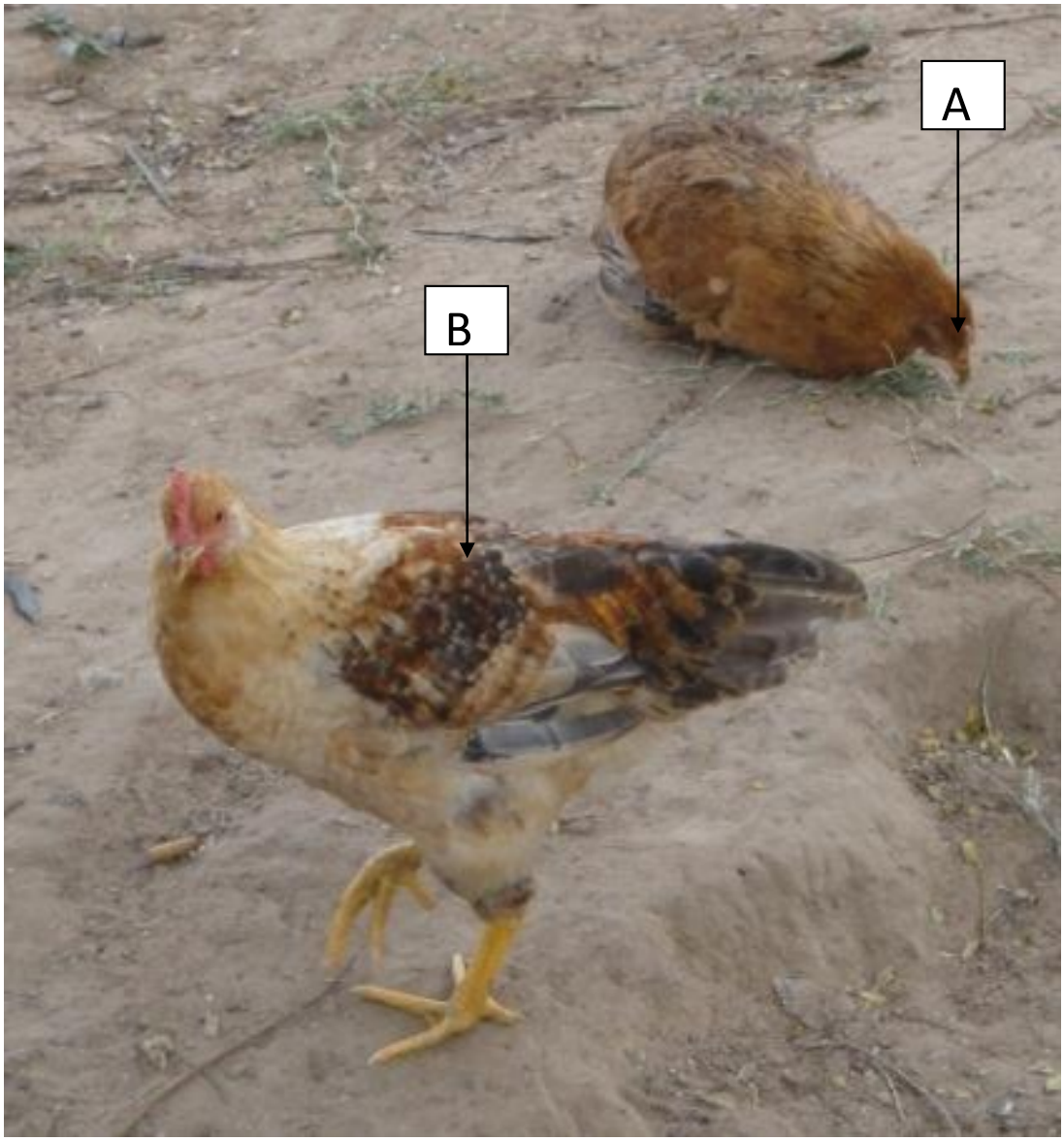


Figure 3.3: Depressed indigenous chicken pullet (Arrow A) suffering from Infectious bursal disease compared to the normal appearing bird (Arrow B).

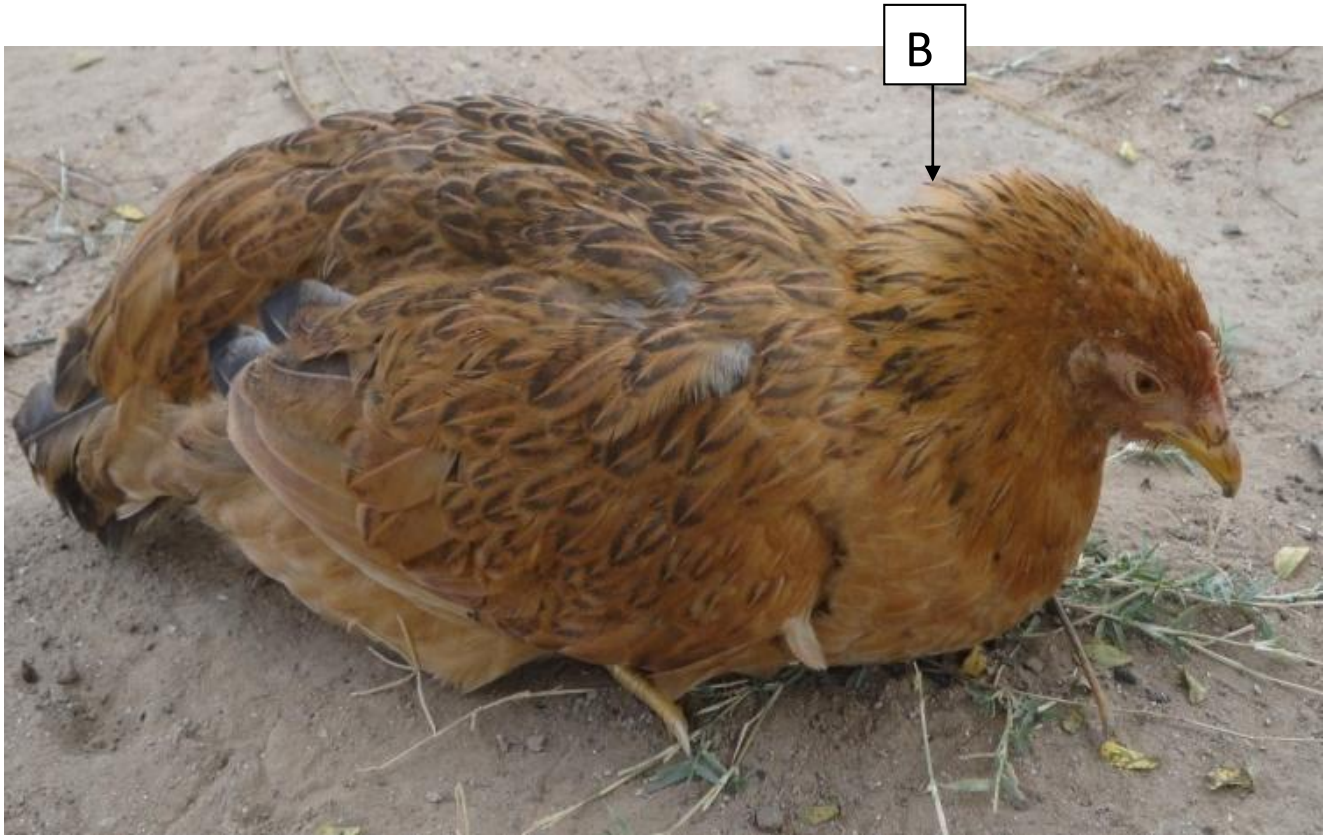


Figure 3.4: Ruffled feathers (Arrow B) in a depressed indigenous chicken pullet suffering from infectious bursal disease

3.3.3 Gross findings at postmortem examination

Lesions observed in the carcasses that were opened, from all three flock types, were typical of IBD infection; haemorrhages were observed on the bursa of Fabricius, thigh and breast muscles **(Figure 3.5, 3.6 and 3.7).** Bursae of Fabricius were either enlarged, due to oedema, and hyperaemic; or shrunken, with necrotic debri. Liver and spleen were enlarged. Kidneys were pale and swollen with dilated tubules. Haemorrhages were also seen in proventriculus.

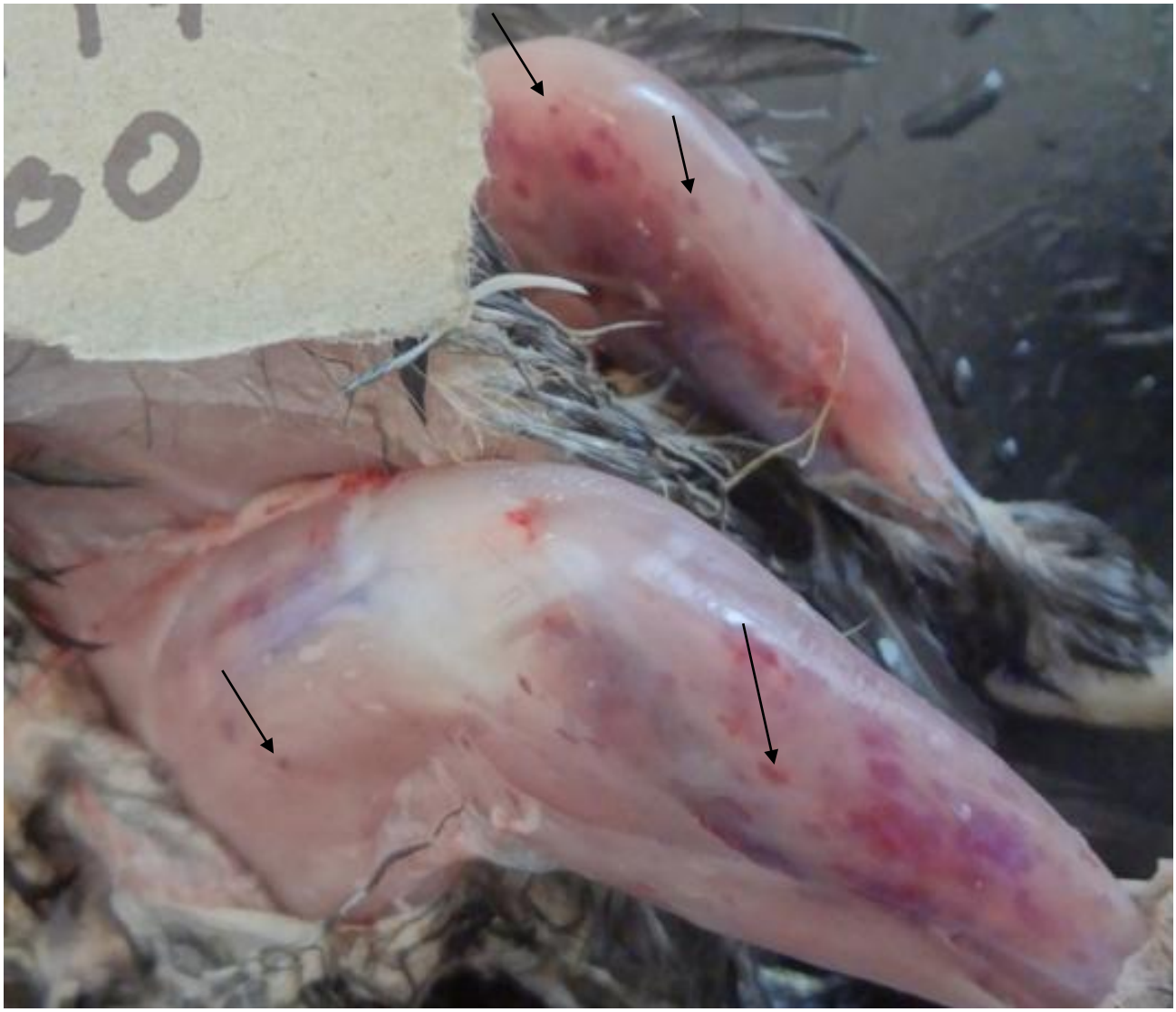


Figure 3.5: Haemorrhages on thigh and leg muscles of an indigenous chicken (black arrows) from an outbreak of Infectious bursal disease

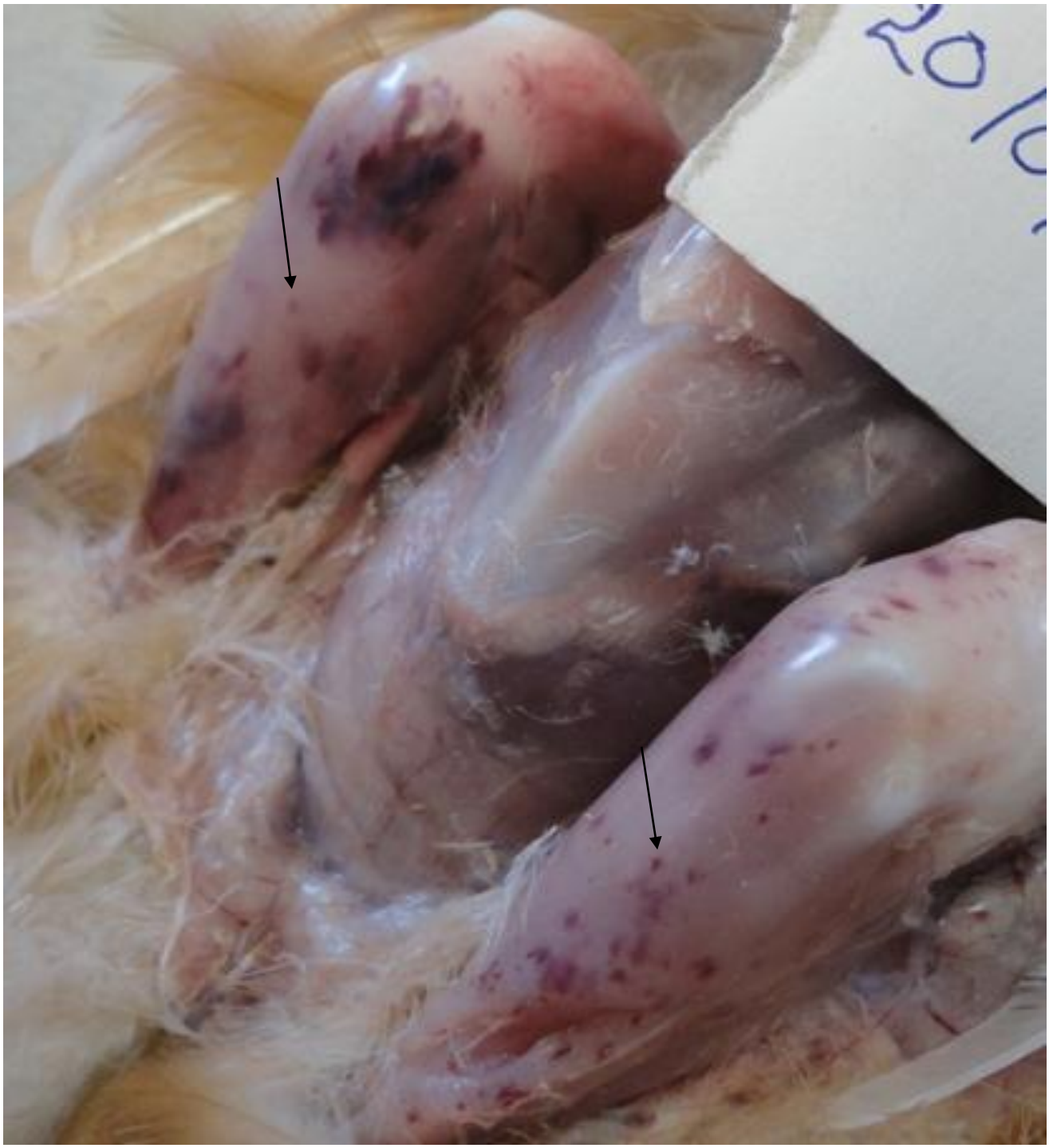


Figure 3.6: Haemorrhages on thigh and leg muscles of a chicken layer (black arrows) from an outbreak of infectious bursal disease

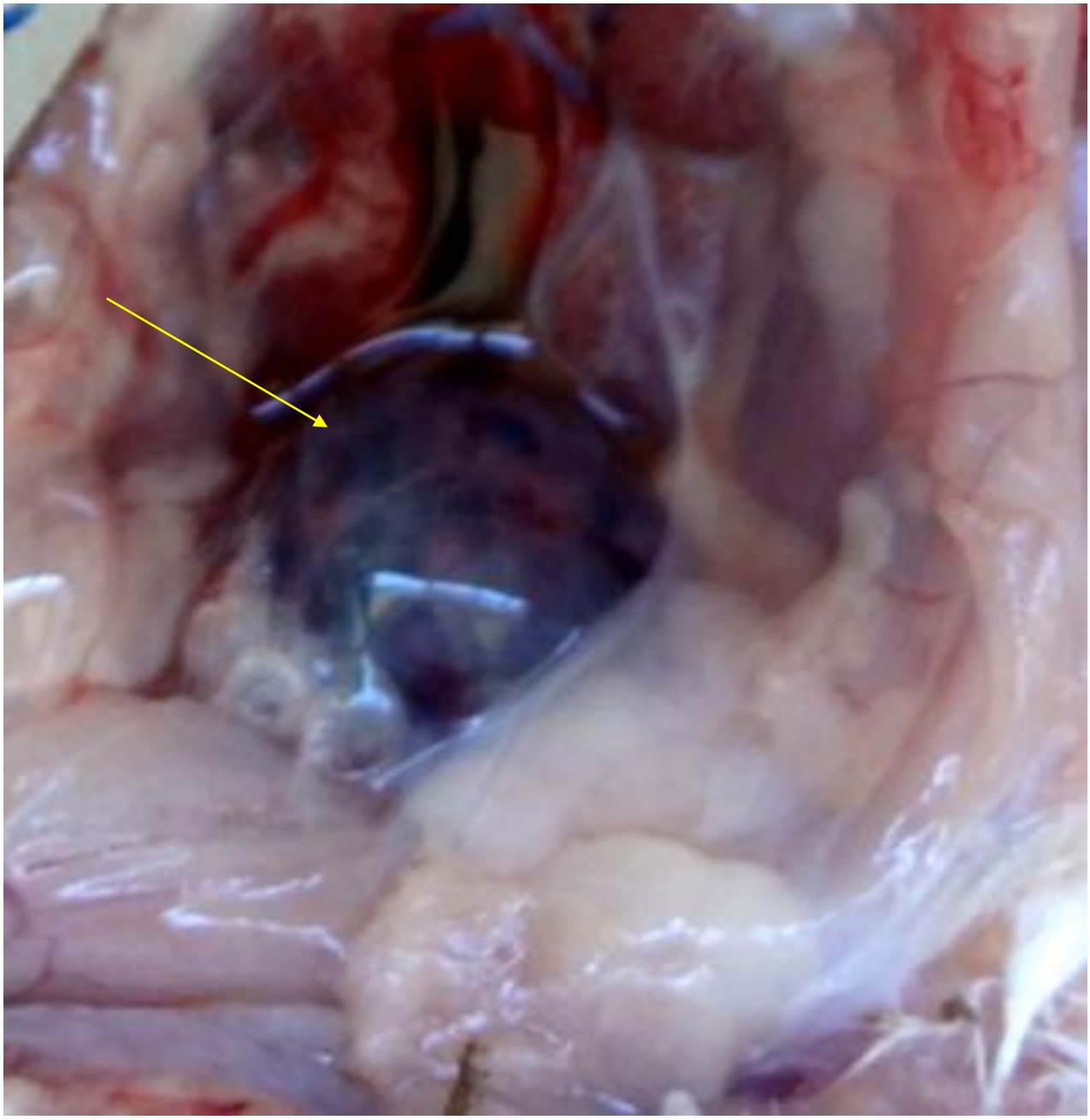


Figure 3.7: Haemorrhagic bursa of Fabricius in broiler chicken (yellow arrow) from an outbreak of Infectious bursal disease

3.3.4 Confirmation of outbreaks

Bursal suspensions prepared from the outbreak cases yielded clear white lines of precipitation in AGPT **(Figure 3.8).** This was a confirmation of the presence of IBD viral antigen in the bursae of the birds. Thirty nine flocks of chickens (layers broilers and indigenous birds) were positive on AGPT.

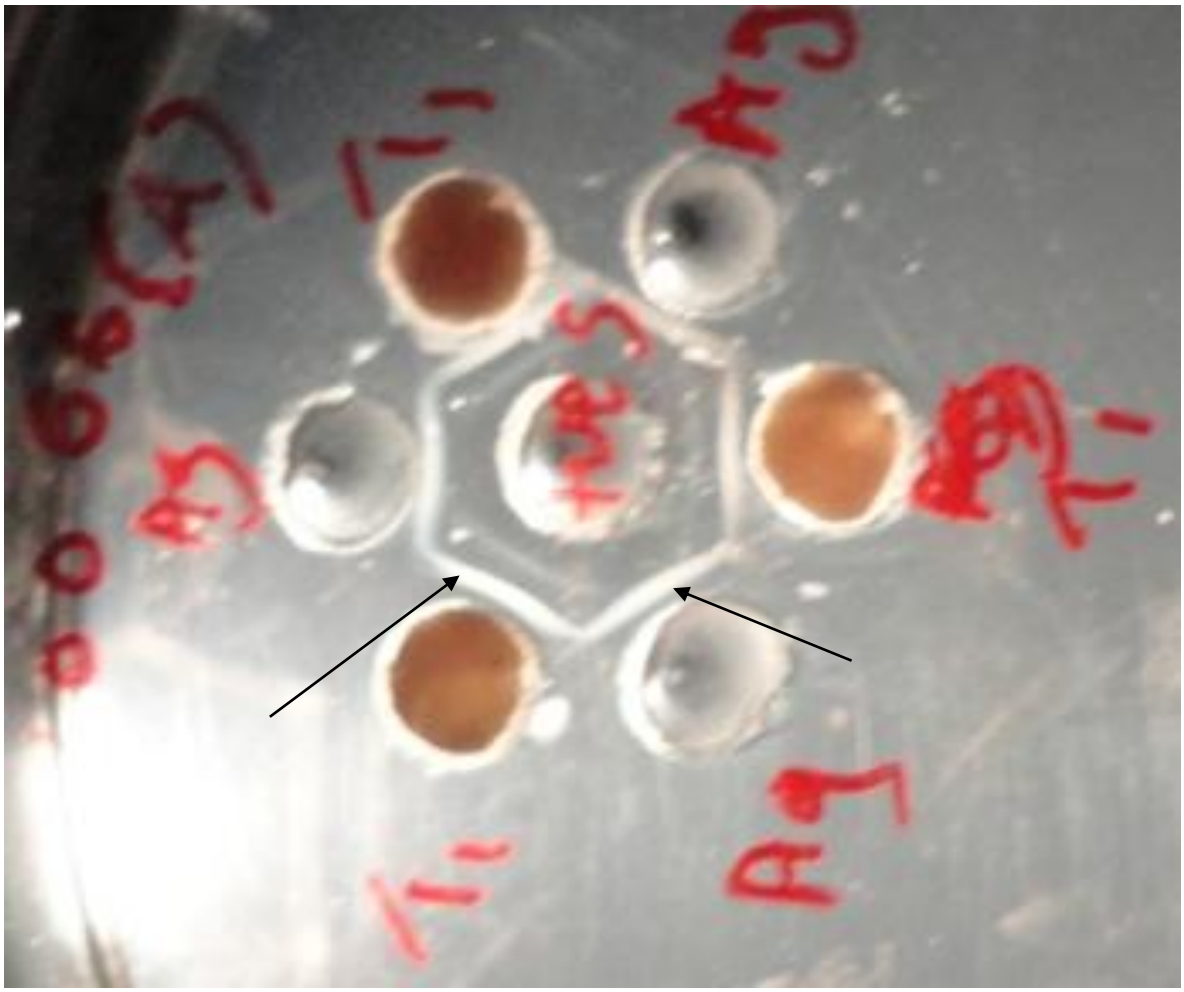


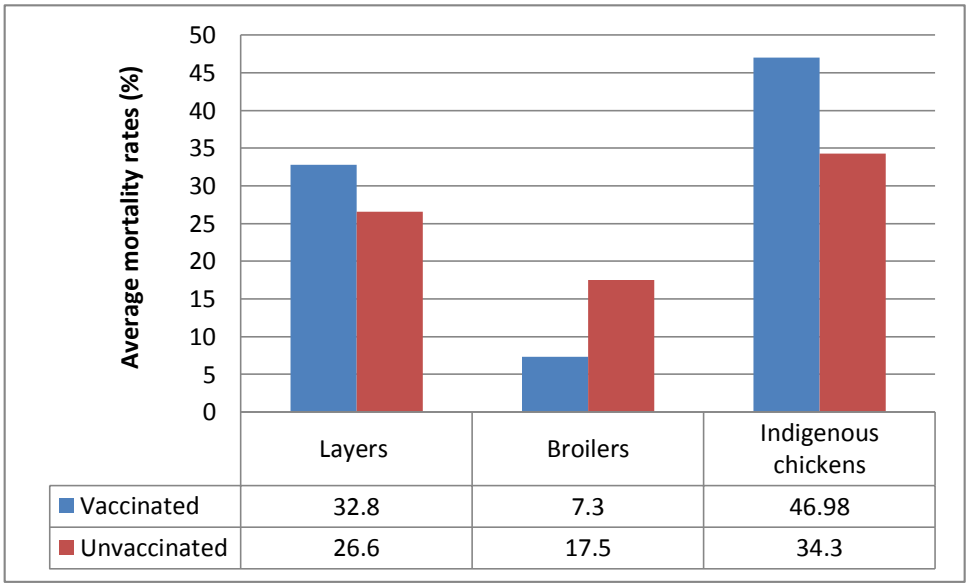
Figure 3.8: Agar gel precipitation test plate showing precipitation lines (arrows) in positive Infectious bursal disease cases

3.3.5 Mortality rates and history of vaccination

Flocks of indigenous chickens had the highest mortality rates which ranged from 1.3% to 100%, with an average of 39.2%; followed by layers with an average of 31.1%. Mortality rates in layers ranged from 0.67% to 86%. **Figure 3.9** shows carcasses collected from an outbreak in vaccinated layers. Flocks of broilers had the least mortality rates ranging from 0.4% to 33.3%, with an average of 13.4%. The difference in mortality rates between indigenous flocks and layers was not statistically significant (P=0.5076); between indigenous flocks and broilers was also not statistically significant (P=0.1240). The same was the case for difference between

Figure 3.9: Chicken (8 weeks old pullets) carcasses collected from an outbreak of infectious bursal disease in vaccinated chicken layers

Indigenous flocks and exotic flocks (broilers and layers combined) (P=0.309). In this study 73.7% of the outbreaks in layers were in vaccinated flocks. In comparison 40% of the outbreaks in broilers and indigenous flocks were in vaccinated flocks. This study established that most of the farmers who vaccinated their indigenous chickens were raising them commercially, through intensive production. Those keeping the birds through the traditional extensive free range system, where chickens were kept for subsistence purposes, did not vaccinate their birds. Outbreaks in vaccinated indigenous flocks recorded the highest average mortality rate (46.98%), followed by unvaccinated indigenous flocks (34.3%), vaccinated layers (32.8%) and unvaccinated layers (26.6%), as shown in **Figure 3.10.** Mortality rates recorded in outbreaks in broilers were the reverse: 7.3% for vaccinated and 17.5% for unvaccinated ones **(Figure 3.10).** Statistically, vaccination was not associated with low level of mortality rate (less than 20%) in outbreak flocks (P=0.709).

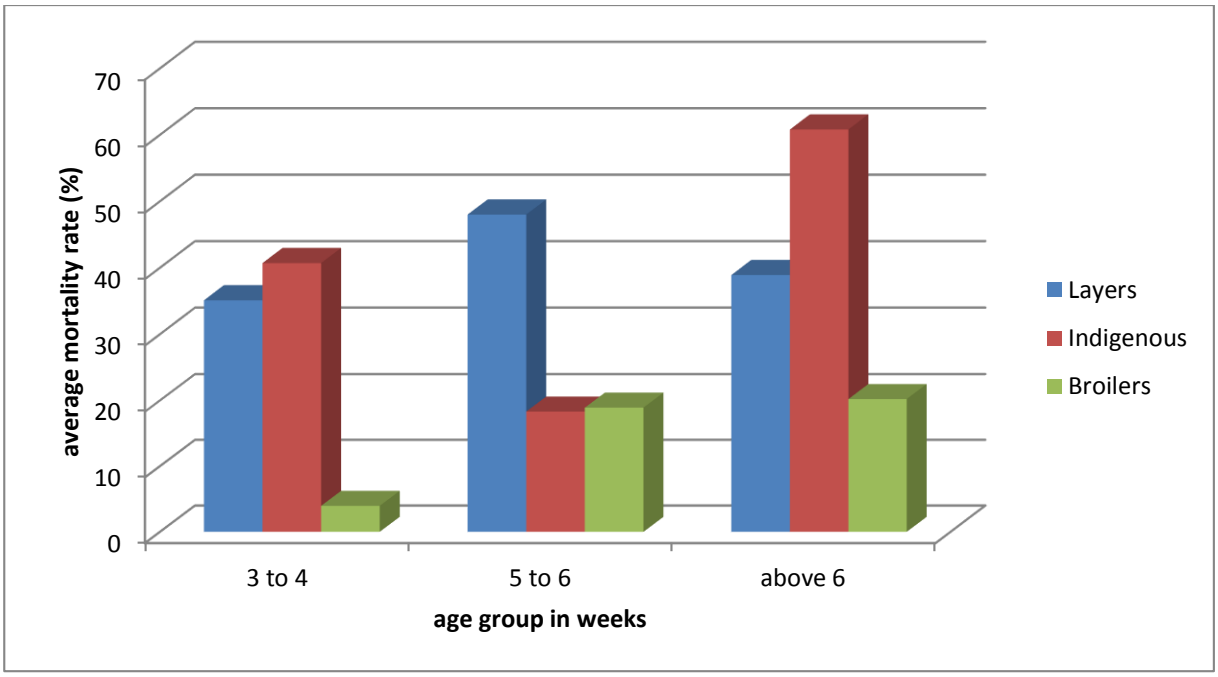


Legend: (%) - Percentage

Figure 3.10: Average mortality rates in infectious bursal disease outbreaks in vaccinated and unvaccinated flocks of commercial layers, broilers and indigenous chickens

When compared between age groups, indigenous chickens had highest mortality rate in the 3-4 weeks and above 6 weeks age groups. Highest mortality rate in layers was recorded in the 5-6

weeks old age group (**Figure 3.11**). Broiler flocks had the least mortality rate in all the age groups compared to flocks of layers and indigenous chickens. In broilers, the mortality rate increased with age. In this study, level of mortality rate (high or low) was not associated with any flock type (P>0.05). Vaccination was also not associated with low level of mortality (P=0.709).



Legend: (%) – Percentage

Figure 3.11: Average mortality rate per chicken age group

3.4 DISCUSSION

Severity of IBD outbreak depends on the virulence of the virus and the susceptibility of the chicken among other factors. Highly pathogenic strains of IBDV were reported to cause high mortalities exceeding 90% in highly susceptible chicken flocks in Japan (Nunoya *et al.*, 1992). This compares with findings of this study, where some outbreaks recorded mortality rates of 100% in indigenous flocks and 86% in exotic layers. In another study, higher mortality was observed in Fayoumi than

in white leghorn chicks infected with IBD (Chakraborty *et al.*, 2010). After finding variable susceptibility to very virulent IBDV (vvIBDV) among various Egyptian chickens, it was suggested that innate non immunogenic factors may play a critical role in resistance to the disease (Hassan *et al.*, 2002). Findings in this study suggest that some Kenyan indigenous flocks are highly susceptible to the disease. In contrast indigenous chickens and cross breeds in Nigeria were found susceptible to IBD but the mortality rates between the two, though not different, was significantly lower than in the exotic chickens (Okoye *et al.*, 1999; Oluwayelu *et al.*, 2002). In Bangladesh IBD was reported in local Sonali and Fayoumi chicks reared under semi scavenging system and in indigenous chicks under confinement (Biswas *et al.*, 2005; Chakraborty *et al.*, 2010).

Genetic differences in susceptibility of chicken lines to infection with IBDV have been documented to occur (Bumstead *et al.*, 1993). Earlier researchers found that the disease caused higher mortality rates in lighter breeds than in heavy breeds (Bumstead *et al.*, 1993; Lukert and Saif 1991; Abdul, 2004), while certain lines of Brown and White Leghorns have been shown to be highly susceptible to IBDV infection (Bumstead *et al.*, 1993).

The findings in this study show that all three chicken types in Kenya are susceptible to IBD. Though mortality rates ranged from low to high in outbreaks in indigenous as well as in the exotic chickens, the highest average mortality rate was in outbreaks affecting indigenous flocks. Indigenous village chickens are normally not immunized against IBDV. The chicks are highly susceptible since they do not have maternal antibodies. This explains why there was high mortality rates recorded in the 3-4 weeks age group in indigenous flocks. Survivors gained immunity; hence the low mortality rates in 5-6 weeks age group. The observation made in this study, that as the chicks grew older they seemed to lose respective immunity, could be because there was no vaccination done in the indigenous flocks. This increased susceptibility is demonstrated by the

high mortality rate in birds above 6weeks age group. Outbreaks in young indigenous chicks are not usually reported to the Veterinary department. Failure to suspect IBD, coupled with the assumption that indigenous chicken are hardy and resistant to many diseases as compared to exotic ones (Kitalyi, 1998) could be the reason why few farmers vaccinate their indigenous chickens against IBD.

The present finding that outbreaks occur even in vaccinated flocks agrees with earlier research (Yahia *et al.,* 2008; Islam *et al.,* 2005; Mutinda, 2011). Vaccination failures have been reported in many different parts of the world (Müller *et al.* 2012; Adamu *et al.,* 2013) and attributed to different reasons. Some of the possible reasons are improper handling and administration of vaccine (Mutinda *et al.,* 2014), virus antigenic variations, live vaccine potency, and residual maternal antibody interference (Islam *et al.,* 2008). Although earlier studies showed that farmers failed to store, reconstitute, and administer the vaccines properly, it is important to note that most vaccines currently used in this country, are imported (Mutinda *et al.,* 2014); there is, therefore, a chance that they differ antigenically from the local strains. It is therefore important to isolate the strains circulating in Kenya and characterize them with the aim to determine vaccine candidate strains that can be used to develop a local vaccine. Isolates of IBDV strains from these outbreaks have been adapted to grow in chicken embryos as described in chapter four.

CHAPTER FOUR: ISOLATION OF INFECTIOUS BURSAL DISEASE VIRUSES FROM DISEASE OUTBREAKS AND THEIR ADAPTATION TO INDIGENOUS CHICKEN EMBRYOS

4.1 INTRODUCTION

Isolation of IBDV can be done in cell cultures, embryonating eggs from specific antibody-negative sources or in specific antibody-negative chickens. However, some difficulty may be experienced when using the earlier two systems as the virus does not readily adapt to them (OIE, 2008). Most of the strains isolated in the field, and in particular, the hypervirulent strains, cannot be multiplied in cell cultures, since they require either previous passages on embryonated eggs or several blind passages in cell cultures before a cytopathogenic effect is obtained. This adaptation, however, tends to be accompanied by an attenuation of the strain. Different propagation methods have different effects in modifying the pathogenicity of IBDVs; when passaged in birds IBDV strains maintain their pathogenicity whereas viruses propagated in chicken embryos may lose or maintain their pathogenicity (Rodriguez-Chaves *et al.,* 2002). Isolation in embryonated eggs does not require adaptation of the virus by many serial blind passages, and is suitable for vvIBDVs. In the absence of lesions, the embryos from the first passage should be homogenised in sterile conditions and clarified, and two additional serial passages should be performed (Hitchner, 1970; van den Berg 2000).

Embryos at nine to eleven days of age are most suitable. Inoculation by the chorio-allantoic membrane (CAM) route gives a greater yield of virus and is preferable to the classical allantoic route (Hitchner, 1970; Takase *et al.,* 1996). Embryo death occurs three to seven days following inoculation. The affected embryos are oedematous, congested, with a gelatinous appearance of the

skin, and haemorrhages are often present in the toes or the encephalon. The variants from the USA cause less embryonic mortality, splenomegaly and no marked lesions of hepatic necrosis. Among the different compartments of the inoculated egg, the embryo is the place where the highest titers of virus occur. The liver shows scattered petechiae and foci of necrosis, and is the organ that is the richest in viral particles (McFerran, 1993). The purpose of this study was to isolate and propagate IBDV strains from outbreaks in Kenya. Initially white leghorn chicks were used, since they are the ones documented to be susceptible to the virus, but later adaptation of the virus to indigenous chicken embryos was explored as a locally available resource.

4.2 MATERIALS AND METHODS

4.2.1 Study design

Bursae of Fabricius (BFs) collected from birds and fresh carcasses from Infectious bursal disease (IBD) outbreaks were used as sources of respective virus samples and those from the same farm were pooled and homogenized together. Presence of the viral antigen in the BFs was confirmed using agar gel precipitation test (AGPT). Positive bursa samples were used to inoculate4-week old SPF white leghorn chicks. Chickens were preferred for initial sample inoculation, meant to amplify the virus from field samples, since other methods could modify the original characteristics of the IBDV field strains (Rosenberger *et al.*, 1998). White leghorn chicks have been shown to have the highest IBDV antigen load in the bursal tissues compared to other IBDV infected chicks (Aricibasi, 2010). After 72 hours, respective BFs were aseptically harvested into universal bottles and stored at -20^{o}C. For convenient referencing, the set of bursae from outbreak cases were referred to as "first generation bursae'' while those harvested from SPF white leghorns were referred to as "second-generation bursae and viruses". The second-generation bursae were used as sources of

virus for propagation through indigenous-chicken embryos. The viruses were serially passaged three times in indigenous-chicken embryos, after which the embryo homogenate harvest from 3rd passage was inoculated into 4-week old indigenous chicks, which served as indicators for viral presence and virulence. Presence of virus in the bursae of these birds was confirmed by AGPT using known antiserum. The bursae and viruses obtained from inoculated indigenous-chickens were referred to as "third-generation bursae/viruses"

4.2.2 Experimental birds

Both white leghorn and indigenous chicks were hatched and raised to the age of 4 weeks at the University of Nairobi, Kabete campus. The white leghorn chicks were hatched from embryonated eggs obtained from a specific pathogen free (SPF) flock maintained at Kenya Veterinary Vaccine Production Unit (KEVEVAPI) of the Government of Kenya. Indigenous chicks were hatched from fertile eggs obtained from indigenous chickens that were kept in an isolated farm with no history of IBD outbreak and were maintained unvaccinated against IBDV. They were mainly normal feathered birds with a few naked neck types. Before start of experiment, sera from these indigenous chickens were confirmed to be free from IBDV antibodies, through AGPT (OIE, 2008).

4.2.3 Experimental embryos

Indigenous-chicken embryos were utilized at 11 day old for the virus propagation and serial passage experiment. They were obtained from the same farm of indigenous chickens described above that supplied the eggs hatched to indigenous chicks. Strict bio-security measures were observed all the time.

4.2.4 Source of samples

One hundred and fifty three (153) bursa of Fabricius samples were collected from Infectious bursal disease reported outbreaks in layers, broilers and indigenous chickens in Kenya described in **section 3.3.1** of this document. The samples were aseptically collected and submitted to the University of Nairobi Virology Laboratory under cold chain as described in **sections 3.2.4 and 3.2.5.**

4.2.5 Preparation of samples

Aseptically collected bursa of Fabricius prepared as described in **section 3.2.5** were tested for IBDV antigen by AGPT as described in **section 3.2.6** and positive samples were selected for virus isolation. Selected samples were treated with penicillin (1000units) and streptomycin at 1000 µg/ml each per sample (OIE, 2008).

4.2.6 Amplification of virus in specific antibody negative white leghorn chicks

Four week-old white leghorn susceptible chicks were inoculated with 100 µl of the antibiotic-treated AGPT-positive, first-generation, bursal samples via intranasal and eye-drop routes. Fifty microlitre volume was given intranasally and another fifty microlitre volume given by intraocular route (Hoque *et al.*,2001). Three birds were used for each sample. The chicks were killed 72 hours after inoculation, and the carcasses were examined for lesions, and the BFs harvested aseptically. Non-haemorrhagic BFs harvested from birds inoculated with same sample were pooled and homogenized together. Presence of second-generation virus was confirmed by AGPT test. The AGPT positive samples were treated with antibiotics, as previously described in **section 4.2.5** and stored at -20^{o}C for further use.

4.2.7 Passage in indigenous chicken embryos

Each AGPT-positive second-generation bursal suspension obtained as described in **section 4.2.6** was inoculated into chorio-allantoic membrane of three 11-day-old SAN indigenous-chicken embryos at a rate of 200 µl per embryo (OIE, 2008). The inoculated embryonated eggs were incubated at 37^{o}C for six days and candled twice daily. The embryos that died within 48 hours of incubation were discarded while those that died after 48 hours were recorded and chilled at 4^{o}C. The experiment was terminated on day six post inoculation and the remaining live embryonated eggs were chilled at 4^{o}C overnight. Three serial embryo passages were performed. At each passage, embryos were harvested aseptically into a petri dish, examined and lesions recorded. Their head and limbs were discarded and the main body homogenized in PBS to make an embryo suspension (OIE, 2008). Harvested supernatant was treated with penicillin and streptomycin at 1000 µg/ml per sample (OIE 2008) and passaged by repeating the process three times. At the end of every passage AGPT was done to check for presence of IBDV in the embryo homogenate.

4.2.8 Virus propagation in indigenous chickens

Harvested virus homogenate from the 3rd embryo passage was inoculated into 4 week old SAN indigenous chicks. Each chick was inoculated with 1ml of harvested homogenate via the oral and oculo-nasal routes (Hoque *et al.,* 2001). Three birds were inoculated per sample. Birds were observed for clinical signs of IBD and BFs harvested aseptically 72hrs post inoculation from each inoculated bird. Presence of IBDV in the bursa was confirmed by AGPT and labeled (third generation bursa/virus).

4.2.9 Agar gel precipitation test (AGPT)

This was done as described in **section 3.2.6** of this document. Standardized antigen, Cat No. RAA0123 (IBDV Antigen) Lot No. BR28/08 and standardized antisera, Cat No. RAB0124 (IBDV Type 1+ve serum) Lot no. BR25/08 from Animal Health and Veterinary Laboratories Agency, United Kingdom were used.

4.3 RESULTS

4.3.1 Antigen detection in samples submitted from field clinical cases

Out of one hundred and fifty three (153) samples collected from reported field outbreaks, 67 (43.8%) were positive for IBDV on AGPT test (**Table 4.1).** When the 67 IBDV positive samples were inoculated into white-leghorn chicks 44 (65.7%; 44/67) infected the chicks and caused disease; viral antigen in the BFs harvested was detected in all the 44 samples by AGPT.

Table 4.1: Virus antigen detection by Agar gel precipitation test in bursa of Fabricius collected from field outbreaks and experimental chicks

Source of bursa of Fabricius	**Agar gel precipitation test results**				
	No. Positive	**No. Negative**	**Total samples**	**Percentage positive (%)**	**Percentage negative (%)**
Chicks from suspected outbreaks (1st generation)	67	86	153	43.8	56.2
SPF chicks inoculated with field material (2nd generation)	44	23	67	65.7	34.3

Key: (%) – Percentage, No. - Number

4.3.2 Clinical signs and lesions on White Leghorn chicks

Clinical signs observed in white leghorn chicks within 72hours post inoculation were: inappettance, ruffled feathers, white watery diarrhea, depression and death. On opening the carcasses, typical IBD lesions were observed; they included: enlarged BFs which were also oedematous, haemorrhagic and sometimes necrotic and atrophied, with caseous cheesy exudates in the lumen, haemorrhages in the thigh, leg and breast muscles, proventriculus, caecal tonsils, thymus and spleen. In addition the spleen, caecal tonsils and thymus were swollen.

4.3.3 Lesions and antigen detection in indigenous chicken embryos

A summary of the lesions observed when the isolates were passaged in 11 day old indigenous chicken embryos are shown in **Table 4.2.** The effects of isolates on embryos were similar. The most common observation was death of the embryos. The embryo mortality rate was 88% on primary inoculation; it then rose to 94% in 1st passage and 91% in 2nd passage, then came down to 67% in 3rd passage **(Figure 4.1).** Embryo mortality was high between day 3 and 4 post inoculation. Dwarfed embryos **(Figure 4.2)** with oedema and congestion followed as the next common lesion. The livers were swollen and mottled (with patchy congestion **(Figures 4.3 and 4.4)** and pale yellow tending to green). Kidneys and spleen were also enlarged and mottled characterized by patchy congestion and paleness. Oedematous chorio-allantoic membranes (CAMs) were observed with or without congestion or haemorrhages, as shown in **Table 4.2** and **Figure 4.2.** In general there was an overall reduction of lesions observed in the embryos with increased passage as shown in **Table 4.2.** Agar gel precipitation test done to confirm the presence of the virus in the embryos yielded faint precipitation lines which, though hardly visible, were present in all the passages.

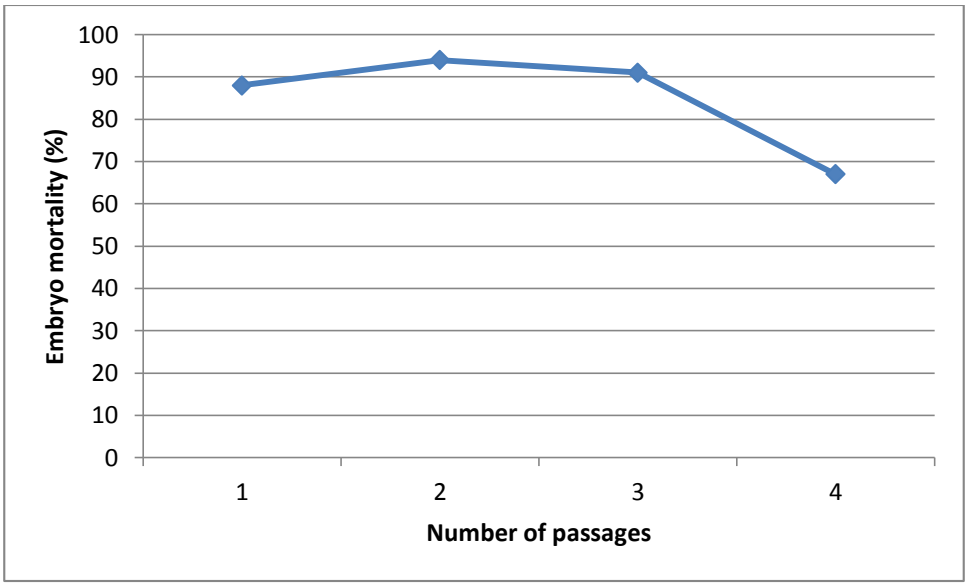


Figure 4.1: Percent embryo mortality in different passages of embryos inoculated with Infectious bursal disease virus.

Table 4.2: A summary of the lesions observed on Infectious bursal disease virus inoculated embryos at different passages

Lesion observed	**Primary**	**Passage 1**	**Passage 2**	**Passage 3**
Dwarfed embryo	112/132 (85%)	103/132 (78%)	106/132 (80%)	100/132 (76%)
Dead embryo	116/132(88%)	124/132 (94%)	120/132 (91%)	88/132 (67%)
Congested embryo	45/132 (34%)	29/132 (22%)	20/132 (15%)	25/132(19.0%)
Oedematous embryo	57/132 (43%)	77/132 (58%)	73/132 (55.0%)	75/132(57%)
Haemorrhagic embryo	40/132 (30%)	53/132 (40%)	61/132 (46%)	63/132(48%)
Enlarged Mottled Liver	26/132 (20%)	25/132 (19%)	18/132 (14%)	13/132(10%)
Enlarged Kidneys	4/132 (3%)	0%	0%	0%
Congested CAM	40/132 (30%)	36/132 (27%)	37/132 (28%)	32/132 (24%)
Haemorrhagic CAM	9/132 (7%)	15/132 (11%)	1/132(1%)	13/132 (10%)
Oedematous CAM	71/132 (54%)	83/132 (63%)	87/132 (66%)	100/132 (76%)

Key: CAM – chorioallantoic membrane, (%) - Percentage

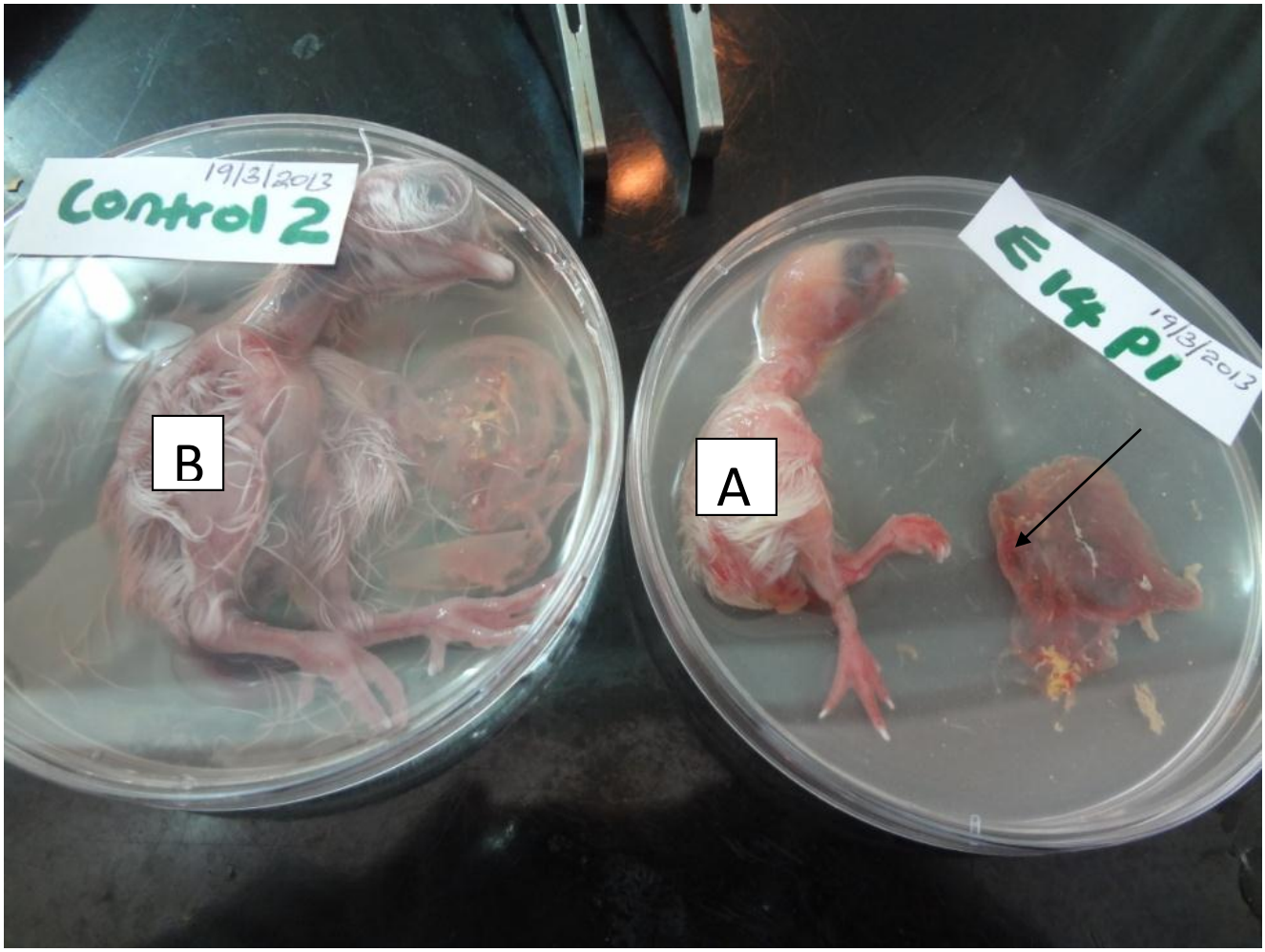


Figure 4.2: Eighteen day old dwarf congested infectious bursal disease virus infected chicken embryo (A) with haemorrhagic chorio-allantoic membrane (black arrow in A) as compared to the uninfected 18 day old control chicken embryo (B)

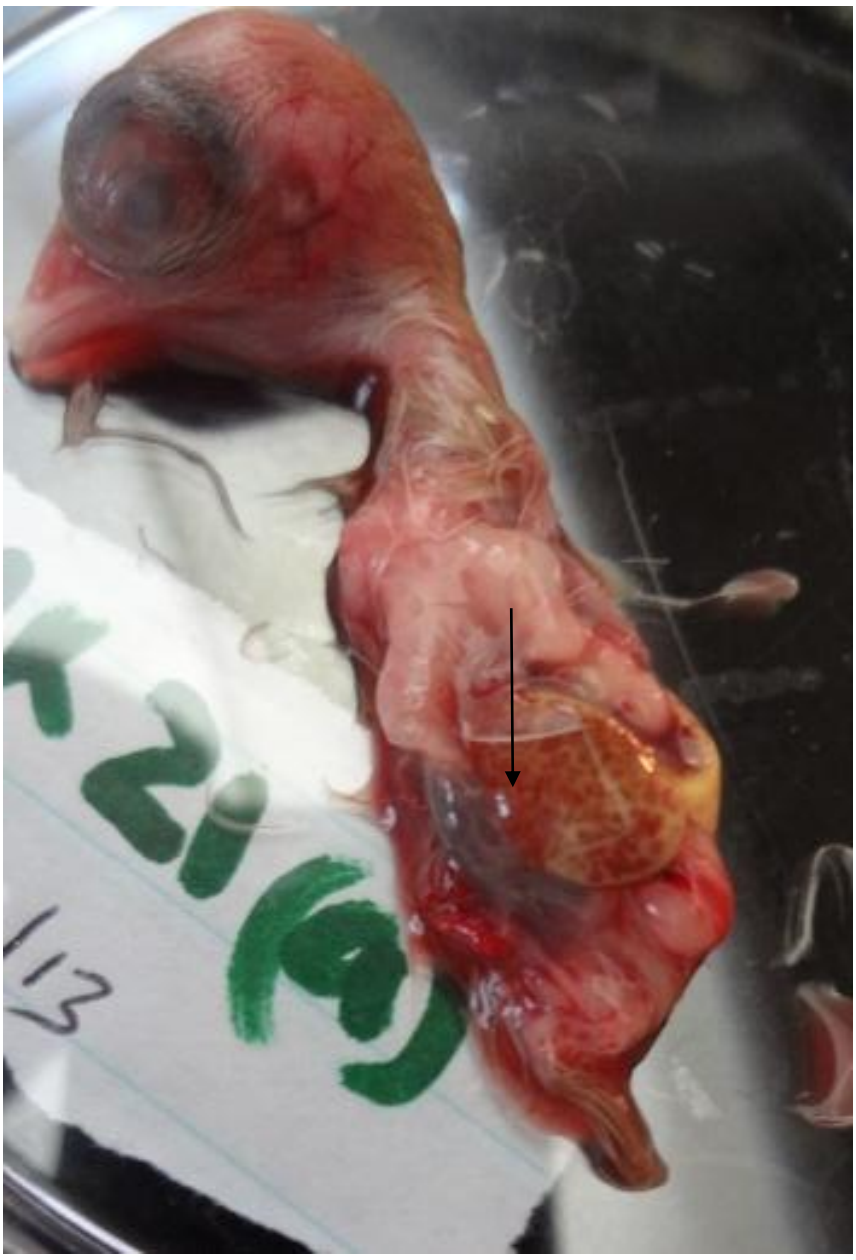


Figure 4.3: Swollen liver with patchy congestion and pale yellow-green colouration (black arrow) in an indigenous chicken embryo inoculated with Infectious bursal disease virus

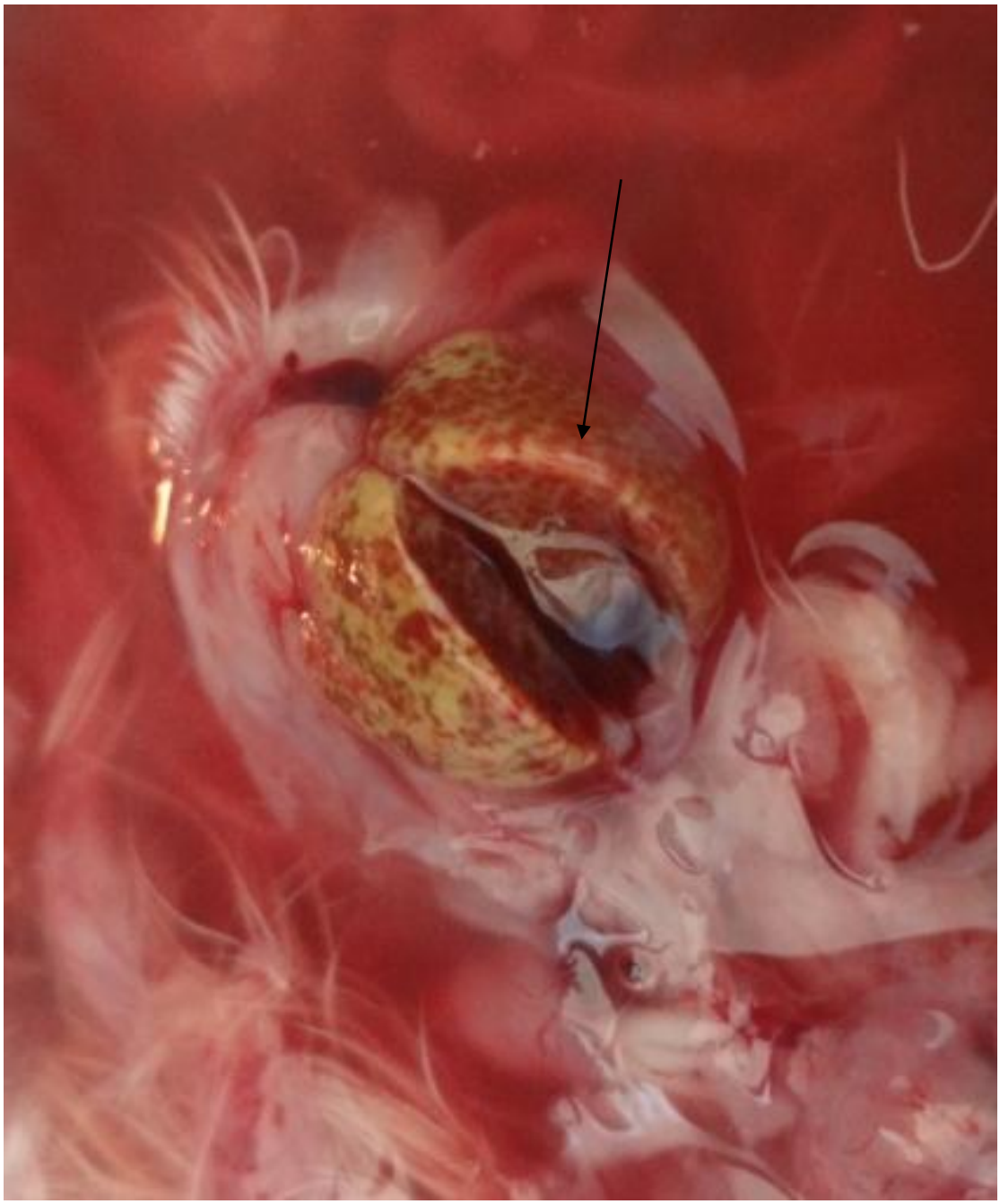


Figure 4.4: A closer view of swollen liver with patchy congestion and pale yellow-green colouration producing a mottled effect in an Indigenous chicken embryo inoculated with infectious bursal disease virus

4.3.4 Lesions seen and virus detection in chicken bursae.

When inoculated into indigenous chicks (as indicators), all the 44 embryo-passaged viral isolates (after the third passage) produced disease. The main clinical signs observed were: watery diarrhoea, ruffled feathers, reluctance to move, anorexia, trembling and prostration. Post-mortem lesions included dehydration of the skeletal muscles with multifocal ecchymotic haemorrhages **(Figure 4.5),** enlargement of the kidneys with urate-distended tubules. The bursa of Fabricius showed lesions characteristic of IBD, was enlarged and turgid. Caseous necrotic debris was observed in the lumen of the bursa. Intrafollicular haemorrhages **(Figure 4.6)** were present and, in some cases, the bursa was completely haemorrhagic. Peri-bursal straw-coloured oedema was present in many bursae. Also, bursae from all the inoculated indigenous chicks had viral antigen confirmed by the AGPT; they yielded clear distinctly visible precipitation lines against the reference antiserum.

Figure 4.5: Haemorrhages on thigh and pectoral skeletal muscles (black arrows) in an indigenous chicken inoculated with Infectious bursal disease virus (isolate E17) that was passaged three times in indigenous chicken embryos.

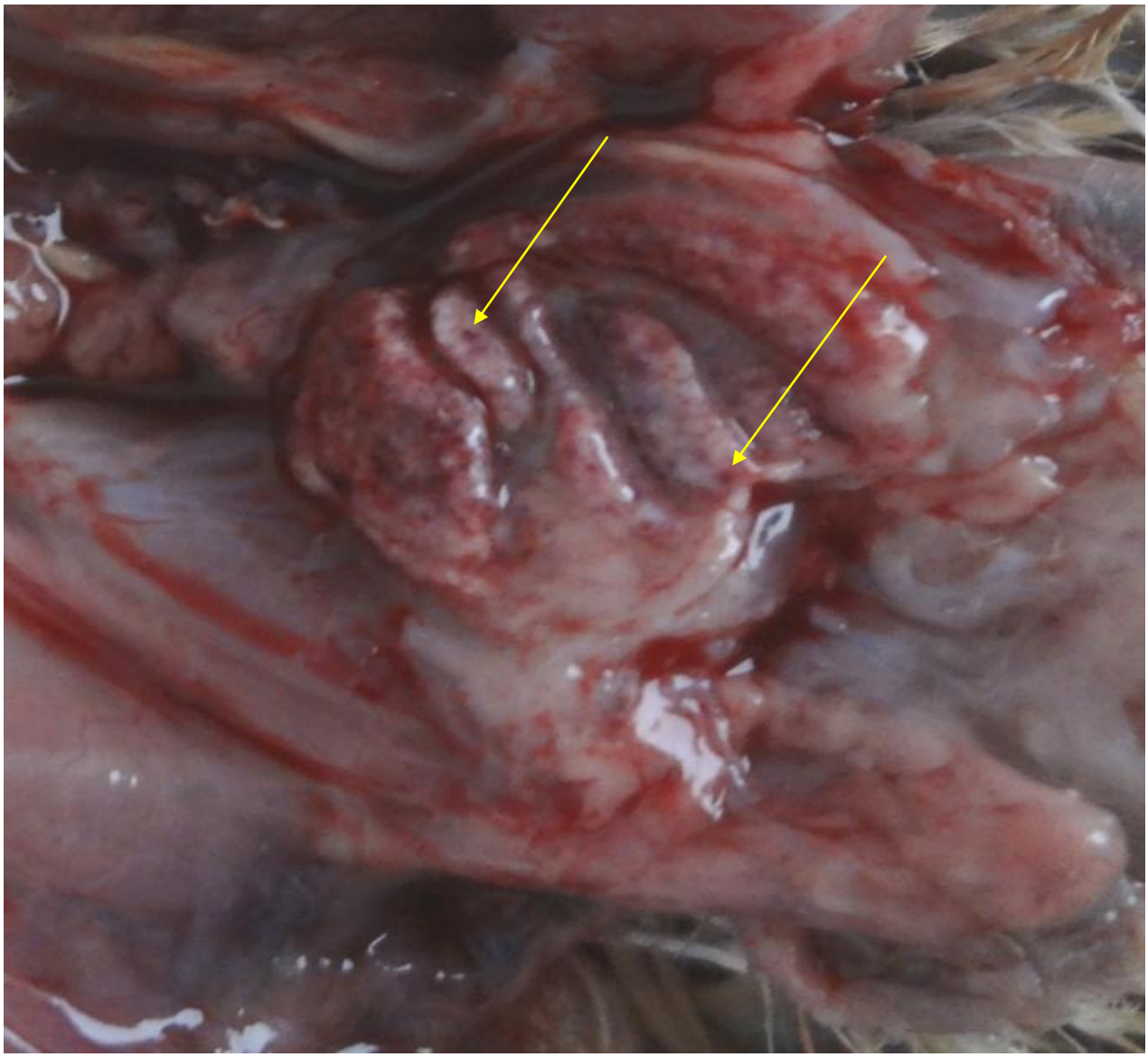


Figure 4.6: An open swollen haemorrhagic oedematous bursa of Fabricius (yellow arrows) in an indigenous chicken inoculated with Infectious bursal disease virus (isolate E17) that was passaged three times in indigenous chicken embryos.

4.4 DISCUSSION

The virus was successfully isolated from 44 out of the 67 AGPT positive samples in this study.

White leghorn layers normally show very high susceptibility to IBDV and have been used by most

investigators in IBDV experiments (van den Berg 1991; Lukert and Saif 2003). Failure to isolate the virus from 23 of the 67 AGPT positive field samples could be as a result of the virus having been inactivated, noting that AGPT detects viral antigen even in samples where the virus has been inactivated (OIE 2008). This, therefore, serves as a caution towards proper handling of the samples, even though the virus tends to be hardy (Benton *et al.,* 1967).

Clinical manifestations and post-mortem findings of dead and sick birds may aid in the diagnosis of a disease. However, laboratory diagnosis is necessary for confirmation of the disease (Banda 2002; OIE 2008). In this study, typical gross lesions and clinical signs of IBD were reported to have been observed in all outbreaks where 153 samples were collected from, but viral antigen was only detected in 67 of the 153 samples submitted. Erroneous clinical diagnosis can occur in cases where postmortem examinations are not performed. In such cases outbreaks could be due to diseases other than IBD; diseases like Newcastle and acute coccidiosis can clinically be confused for IBD due to the high mortality (Banda, 2002). Inclusion body hepatitis is another differential diagnosis for IBD due to the liver changes observed at postmortem examination. However, it is also possible that the outbreaks, picked in this study, were due to IBDV but at the time of sampling the virus was not detectable by AGPT. The agar gel precipitation test (AGPT) detects viral antigen in the bursa of Fabricius in the early stages of the infection before the development of an antibody response (OIE 2008).

The purpose of cultivating IBDV isolates in chicken embryos was to determine whether the virus could be adapted through passaging in the indigenous chicken embryos. The results compare well with other studies in which indigenous chicken embryos were reported to be good for virus isolation (Abdul, 2004). In this study all the 44 isolates recovered from the white leghorn chicks grew in indigenous chicken embryos. Snedeker *et al.,* 1967 and Izawa *et al.,* 1978 made serial

passages of the virus in chicken embryos resulting in attenuation of very virulent and classic strains. Furthermore, Ahmad *et al.* (2005) reported reduction in mortality as virus was passaged in embryonated eggs until very low mortalities were seen in the sixth passage. Lesions observed in indigenous chicken embryos inoculated with the Kenyan IBDV isolates were as reported for IBDV strains inoculated in embryos of other types of chicken (Hoque *et al.,* 2001; OIE 2008). High mortality of inoculated embryos, oedema, congestion and haemorrhages observed in embryos in this study have in the past been associated with hypervirulent and classic strains (Hitchner 1970; Omer *et al.,* 2008). Dwarfing of embryos, enlarged liver and spleen, observed in this study, were comparable to observations reported by Lukert and Saif (2003). Very weak precipitation lines which were hardly visible when AGPT was used to test for the presence of viral antigen in the harvested embryos were an indication that virus quantity was low in the embryos. This finding agrees with findings by other researchers that IBDV strains do not grow easily in embryonated chicken eggs and take time to adapt (Anjum *et al.,* 2010). However, when the Kenyan embryo homogenates from the last passage were inoculated into indigenous chicks, the disease was reproduced by all the isolates and AGPT done to detect viral antigen in the bursa of inoculated chicks yielded highly visible and very strong precipitation lines. The isolates were found to be still virulent after 3 serial passages in indigenous chicken embryos. Variations in the embryo passage numbers leading to IBDV attenuation have been observed by researchers and attributed to experimental conditions, strain of the virus and internal environment in the eggs (Anjum *et al.,* 2010). Attenuation of IBDV in embryos was obtained after 43 passages by Lazarus *et al.* (2008), after 8 serial passages by Yamaguchi *et al.* (1996) and after 13 serial passages by Izawa *et al.* (1978). Thus, it is possible that further passages of between 8 and 43 passages of the Kenyan IBDV

in the indigenous embryonated eggs could yield an attenuated virus that could be a candidate for a local vaccine.

This is the first time in Kenya that IBDV has been isolated from outbreaks in chickens, and after being repeatedly grown in embryos shown to induce the disease in susceptible chickens, fulfilling Koch's postulate (Fredricks and Relman, 1996). In previous studies in Kenya, clinical samples from outbreaks were inoculated in embryos, in an attempt to demonstrate pathogenicity of the virus in SPF embryos (Mutinda, 2011), but did not attempt to induce the disease in susceptible chickens to demonstrate successful isolation of the virus.

The results of our study have shown, for the first time that indigenous chicken embryos in Kenya, support the growth of IBDV and can be used to propagate the virus producing the typical lesions. With more passages these could yield an attenuated vaccine. Pathogenicity testing of IBDV isolates in indigenous chickens was recommended as one way of characterizing the isolates and this has been reported in chapter five.

CHAPTER FIVE: PATHOGENICITY OF KENYAN INFECTIOUS BURSAL DISEASE VIRUS ISOLATES IN INDIGENOUS CHICKENS

5.1 INTRODUCTION

Infectious bursal disease virus (IBDV), a non-enveloped, bi-segmented RNA virus that belongs to the *Avibirnavirus* genus within the *Birnaviridae* family, is an immunosuppressive virus that primarily targets B-lymphocytes in the bursa of Fabricius of affected chickens (van den Berg *et al.,* 2000). There are 2 distinct serotypes of IBDV that are distinguished by virus neutralization and cross-protection tests. The 2 serotypes are designated as serotype I and II; only serotype 1 strains are pathogenic (Jackwood *et al.,* 1985). Variation in range of pathogenicity is observed with various serotype I IBDV strains with the most virulent strains being designated as "very virulent" (van den Berg *et al.,* 1991). The IBDV strains are generally subdivided as avirulent (serotype II viruses), subclinical, classical virulent (also referred to as standard), and very virulent (also referred to as hypervirulent) pathotypes (Jackwood *et al.,* 2011). Criteria for designation of IBDV strains as the vvIBDV pathotype are based on a combination of molecular, antigenic, and phenotypic characteristics (van den Berg *et al.,* 2004). The reported mortality induced by vvIBDV can range between 40–100% in fully susceptible specific pathogen free (SPF) chickens, 60% in layers, and 30% in broilers (van den Berg *et al.,* 1991; van den Berg and Meulemans, 1991; Jackwood *et al.,* 2009). The vvIBDV pathotype was first reported in Belgium in the late 1980s and has since rapidly spread throughout Europe, Middle East, Asia, and South America. Previous to the emergence of vvIBDV, less virulent strains of IBDV were well controlled by vaccination, and mortality of less than 2% was typically observed in the field (van den Berg, 2000). Variation in pathogenicity of IBDV has also been observed between different breeds of chickens where generally light layer breeds have been reported to be more susceptible than heavy broilers (van

den Berg and Meulemans, 1991). Earlier studies on outbreaks of IBD in Kenya showed very high mortality rates that varied from 5% to 75% in vaccinated broiler flocks and from 13% to 56% in the unvaccinated ones while in vaccinated layers, mortality ranged from 35% to 80% (Mutinda, 2011). Difference between mortality rates in outbreaks of IBD in indigenous chicken, broiler and layer flocks encountered in the field in an earlier study was not statistically different (Mutinda *et al.,* 2013). Most of the IBDV pathogenicity studies have been conducted in SPF Leghorn-type birds (Kim *et al.,* 1998; Rautenschlein *et al.,* 2002; Rautenschlein *et al.,* 2003), which are highly susceptible to IBDV, inducing lesions and mortality. The purpose of the current study was to determine pathogenicity of Kenyan IBDV isolates in specific antibody negative (SAN) indigenous chickens.

5.2 MATERIALS AND METHODS

5.2.1 Viruses

Four of the 44 isolates obtained in **section 4.2.8** were selected and designated as isolates E7, E9, E19 and E42. The isolates were selected to represent those that had caused different mortality rates in the field outbreaks: low, moderate and high **(Table 5.1).** Isolate E7 was originally isolated from a vaccinated flock of 9week old layer pullets in Nairobi area that had come down with Gumboro in 2012. Out of 1154 birds in the flock, all were affected and more than 930 died (>80%) in the outbreak. Isolate E19 was from a sample submitted to CVL Kabete from a layer farm in Kiambu county where 50 pullets had died of Gumboro in a flock of 200 (25%). Isolate E9 came from an outbreak of Gumboro that was in Kwale in a flock of 4 weeks old vaccinated layer pullets. Out of 400 pullets in the flock all were affected and 300 died (75%) in the outbreak. Isolate E42 was from

a flock of vaccinated indigenous chickens that came down with IBD in Kilifi County. All the 100 chickens in the flock were affected and all (100%) died in the outbreak.

Table 5.1: Description of the flocks from which the viruses selected for pathogenicity testing were obtained

Virus isolate	**Source**	**Type of chicken affected**	**Age affected**	**Number affected**	**Mortality rate (%)**
E7	Nairobi	Vaccinated layers	9	1154	80 (more than)
E19	Kiambu	Layers	4	200	25
E9	Kwale	Vaccinated layers	4	300	75
E42	Kilifi	Vaccinate Indigenous	14	100	100

5.2.2 Titration of the viruses

A 20% viral suspension was made for each of the selected virus as described in **section 4.2.3** above. The suspension was then diluted serially to make seven 10-fold dilutions. Out of each dilution five 11 day old indigenous chicken embryos were inoculated, via the CAM route, with 0.2ml of the suspension per embryo. The embryos were candled twice daily for seven days and the embryo mortality and infectivity (indicated by congestion and / or hemorrhages) recorded. The 50% end point dilution was calculated using the method of Reed and Muench (1938) and 0.2ml suspension of this dilution considered to contain one 50% embryo infective dose (EID_{50}).The

reciprocal of this dilution was the number of EID_{50} units in 0.2 ml volume of the original undiluted inoculum inoculated into each egg.

5.2.3 Experimental chickens

One day old SAN indigenous chickens were obtained and reared, as described in **section 4.2.2** above, to 5 weeks when they were used in this experiment. They were then transferred to inoculation room allotted to experimental groups. Feed and water were provided *ad-libitum.*

5.2.4Sample size determination

The formula used to determine the number of birds required was as described elsewhere (Martin *et al.*1987; Nahamya *et al.,* 2006).

$$N = \frac{[z\acute{a}(2pq)^{1/2} - z\beta(peqe+pcqc)^{1/2}]^2}{(pe-pc)^2}$$

Where zα=1.96, p=0.5, q=0.5, pe=0.5, qe=0.5, pc=0.2 and qc=0.8 and zβ=0.84

$$N = \frac{[1.96(2x0.5x0.5)^{1/2} - 0.84(0.5x0.5+0.2x0.8)^{1/2}]^2}{(0.5-0.2)^2}$$

About 20 birds per experiment

The calculated number of birds (N) approximated 20 when zα=1.96, p=0.5, q=0.5, pe=0.5, qe=0.5, pc=0.2 and qc=0.8 and zβ=0.84 but was adjusted to 18 birds when time and expense were considered (Martin *et al.,* 1987).

5.2.5 Experimental design

Two hundred and sixteen (216) 5week-old SAN chickens were allotted to nine groups designated numbers 1 to 8, having 18 birds each. Groups 1 to 4 were used for the pathogenicity study, while groups 5 to 8 were used for the mortality study. Each chicken from the first to the eighth groups was inoculated via the oral oculo-nasal route with 10^4 EID_{50} of the respective isolate. One isolate was handled at a time to avoid cross contamination and for each isolate, a total of 18 birds were left as controls and termed as the ninth group. In the pathogenicity study, three birds from each group were weighed and necropsied at 1, 3, 4, 8, 11, and 14 days post-inoculation. Bursa of Fabricius, spleen, thymus, caecal tonsil and Harderian gland were collected, weighed and the average organ/body weight ratios calculated. For the mortality study, the birds were kept for 2 weeks post inoculation and monitored daily. Clinical signs were scored daily as described by Le Nouën *et al.* (2012) with minor modifications. Dead birds were necropsied, samples collected and processed. The bursa of Fabricius was also collected for histopathological examination.

5.2.6 Quantification of clinical signs

Clinical signs were quantified by symptomatic index as previously described with minor modifications (Le Nouën *et al.*, 2012). Scores on this index ranged from 0 to 3 with increasing severity, as shown in **Table 5.2**. The mean symptomatic index (MSI) score of the surviving chickens was calculated daily (once a day; every morning). To ensure consistency in the evaluation of the symptomatic index, each score was determined by 2 trained independent observers. The results of the two observers were compared and a consensus score determined

.

Table 5.2 Scores based on clinical signs

Scores	Description of clinical signs
0	lack of signs
1	typical IBD signs (ruffled feathers) conspicuous in quiet bird only, the bird stimulated by a sudden change in environment (light, noise, or vicinity of experiment observer) appears normal, motility is not reduced
2	typical IBD signs conspicuous even when bird is stimulated, dehydration is apparent, diarrhea, dullness evident and motility may be slightly reduced
3	typical severe IBD signs with prostration or death

Legend: According to Le Nouën *et al.,* (2012).

5.2.7 Mortality rate

Mortality rate for each group among groups 5 to 8 was calculated at the end of the experiment as a percentage of the number of birds inoculated in that group. Number of chicks recorded dead for each group was divided by number of chicks challenged then multiplied by 100, as described by Babiker *et al.,* (2008).

$$\text{Mortality rate} = \frac{\text{Number of chicks dead} \times 100}{\text{Number of chicks challenged}}$$

5.2.8 Organ to body weight ratio

The lymphoid organs sampled (bursa of Fabricius, spleen, thymus, caecal tonsils and Harderian gland) were weighed separately and average of each organ/body weight ratios or indices determined by the following formula: (organ weight in grams / body weight of individual bird in grams) × 1000 (Tanimura *et al.,* 1995).

5.2.9Histology

Sampled organs were fixed in 10% neutral buffered formalin. Sections were made and stained with haematoxylin-eosin following conventional procedure as described by Luna (1968) and in later studies by Mutinda (2011). Histological lesions were scored from 0 to 5 according to the index defined by Muskett *et al.* (1979) and modified in later studies by van den Berg *et al.*,(1991) and Mutinda (2011): 0 = no damage; l=mild necrosis in isolated follicles; 2 = moderate generalized lymphocyte depletion or isolated follicles with severe depletion; 3=over 50% of follicles with severe lymphocyte depletion; 4=outlines of follicles remaining with few lymphocytes and increase in connective tissue, cysts and thickened corrugated epithelium; 5=loss of all follicular architecture with fibroplasia.

5.2.10 Data analysis

Descriptive statistics were generated and a Two-Way ANOVA (IBM SPSS software) used to compare the effect of different isolates on body weight, bursal index, thymic index, splenic index, Harderian gland index and caecal tonsils index scores on different days post inoculation. The interaction effect was tested for statistical significance to determine whether the effect of number of days post inoculation on body weight or any of the organ index scores depended on type of

isolate inoculated. A p-value of less than 0.05 ($P<0.05$) was considered statistically significant (Laerd, 2013).

5.3 RESULTS

The results showed that the four isolates tested were very virulent and produced severe disease with high symptomatic index scores **(Table 5.3)** in indigenous chicks with extensive damage to the bursa of Fabricius along with the other organs of the immune system.

Table 5.3: A summary of the major effects of the selected isolates on indigenous chickens

Isolates	**E19**	**E42**	**E7**	**E9**
Symptomatic index scores on day 4*	2.2	1.4	2.2	2.4
B/F Histology lesions on day 4*	3.5	3.5	4.5	4.3
B/F index score on day 4*	2.3	3.1	3.1	2.9
Mortality rates				
Field	25%	100%	80%	75%
Experimental	27.8%	16.7%	61.1%	66.7%
Titers of isolates	$10^{4.23}$	$10^{3.32}$	$10^{4.33}$	$10^{4.5}$

Legend: B/F – Bursa of Fabricius, % - Percentage, day 4* - this was the day when the birds were very sick; symptomatic index scores were highest.

5.3.1 Titers of the isolates

The titers of the viruses varied **(Table 5.4)** with isolate E9 having the highest titer ($10^{4.5}$ EID_{50}per 0.2mls) and E42 the lowest ($10^{3.32}$ EID_{50} per 0.2mls). Isolate E19 and E7 had titers of $10^{4.23}EID_{50}$ and $10^{4.33}EID_{50}$ per 0.2mls respectively.

Table 5.4 Titers of the various isolates

Isolate	Titer (per 0.2mls)
E19	$10^{4.23}$
E42	$10^{3.32}$
E7	$10^{4.33}$
E9	$10^{4.5}$

Note: Isolate E9 had highest titer followed by E7, E19 and finally E42 with lowest titer.

5.3.2 Clinical signs and mortality rates

Clinical signs of IBD were observed in all inoculated chickens with slight variation on severity. Highest mortality rate was in chickens inoculated with isolate E9 at 66.7% followed by E7 at 61.1%, E19 at 27.8% and lowest mortality was in chickens inoculated with isolate E42 at 16.7%. Highest symptomatic index score was recorded on day 4 for all the isolates and it was highest in isolate E9 at 2.4, followed by E7 and E19 at 2.2 each and lastly isolate E42 at 1.4 **(Figure 5.1).**

Chickens inoculated with isolate E9 showed clinical signs from day 3 to day 9 post inoculation; all the birds were sick on days 3, 4 and 5 and recovery was observed from day 6 post inoculation **(Table 5.5).** Chickens inoculated with isolate E7 showed clinical signs of disease from day 2 to day 6 post inoculation; all the birds were sick from day 2 to day 6 and recovery was recorded in all the birds on day 7. Chickens inoculated with isolate E42 showed clinical signs of disease from day 2 to day 6; all the birds were sick on days 2, 3 and 4 and recovery was observed starting from day 5 - all the birds were fully recovered starting from day 7. Chickens inoculated with isolate E19

showed clinical signs of disease from days 2 to 7 with apparent recovery being observed from day 8; all the birds were sick on days 3, 4, 5 and 6 **(Figure 5.1).**

Table 5.5: Mortality rates and duration of clinical signs caused by different isolates

Isolate	**Day of on-set of clinical signs inoculation**	**Duration of clinical signs (days)**	**Mortality rate (%)**
E9	3	7	66.7
E7	2	5	61.1
E42	2	5	16.7
E19	2	6	27.8

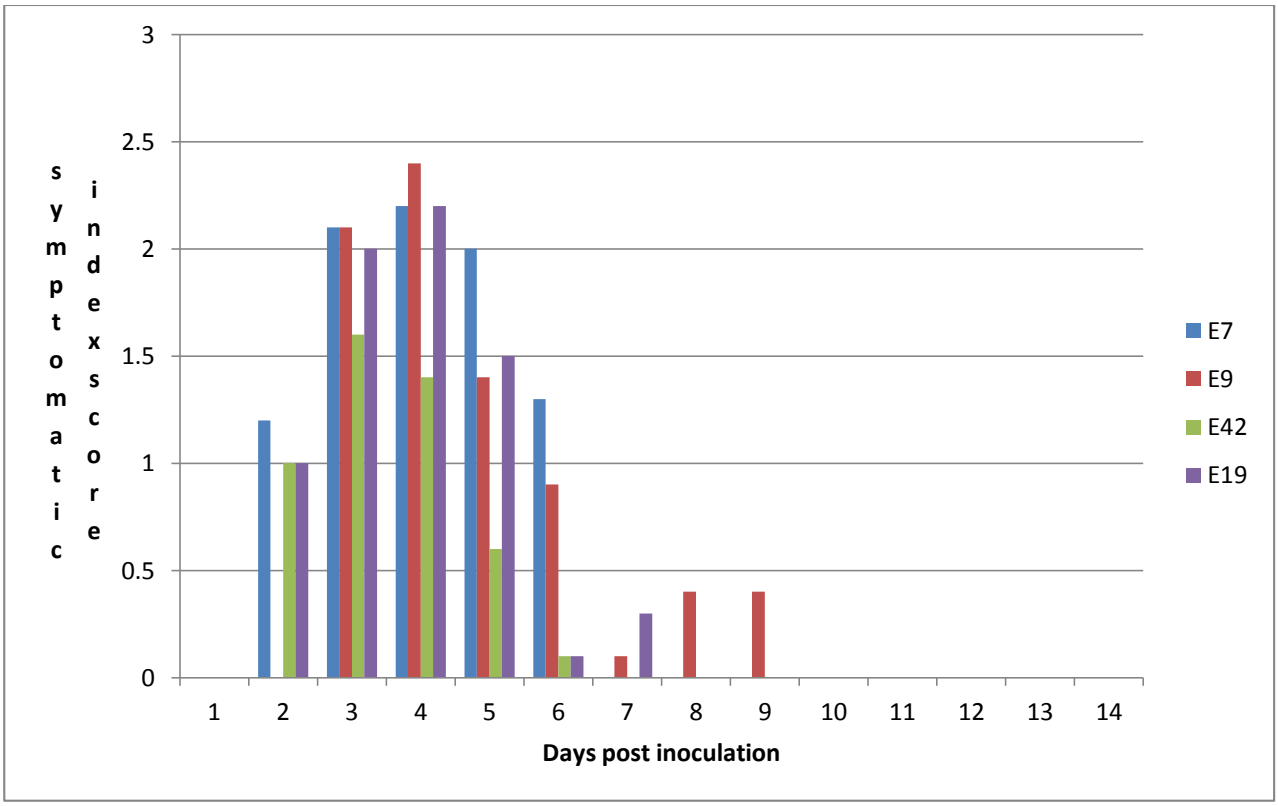
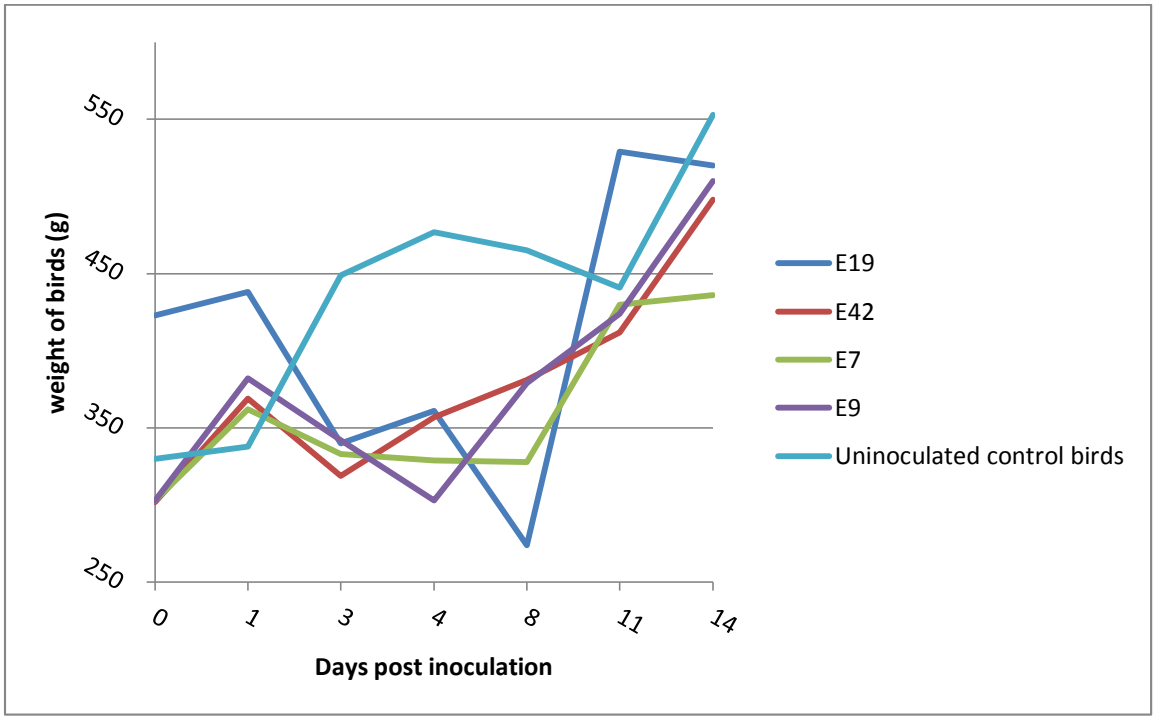


Figure 5.1: Symptomatic index score per day per isolate

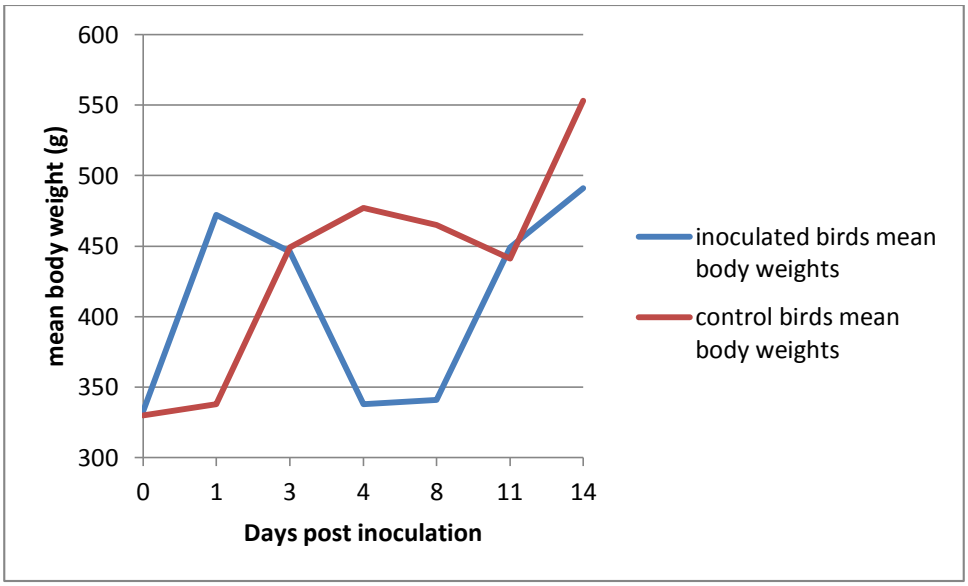
5.3.3 Effect of isolates on weights of bursa, spleen, thymus, caecal tonsils and body weight

There was general trend for reduction of body weight in all the inoculated birds compared to control birds **(Figures 5.2 and 5.3).**Reduction in body weight was clearly evident from day 3 post inoculation and would continue decreasing till day 8 then rise shortly after that and was comparable to the weight of the control birds by day 14. Results from ANOVA test showed that there was no statistically significant difference in mean body weight between the isolates (P=0.54) but there were statistically significant differences between days post inoculation (P<0.0005). The effect of number of days post inoculation on weight of bird did not depend on type of isolate inoculated and statistically there was no significant interaction between the factors F (15, 52) = 0.757, p=0.716.



Legend: (g) - grams

Figure 5.2: Variation of body weight (g) against days post inoculation with different isolates.



Legend: (g) - grams

Figure 5.3: Mean body weight of birds for all inoculated birds against days post inoculation compared to control

The bursa index decreased starting from day 3 post inoculation and continued to day 14 compared to control chickens **(Figure 5.4 and 5.5).**Analysis of variance(ANOVA) test results showed that there was no statistically significant difference in mean bursal index between the isolates (P=0.414) but there were statistically significant differences between days post inoculation (P<0.0005).There was no statistically significant interaction between isolate and number of days post inoculation on bursal index score F (15, 54) =1.063, p=0.41. The effect of number of days post inoculation on bursal index did not depend on type of isolate inoculated.

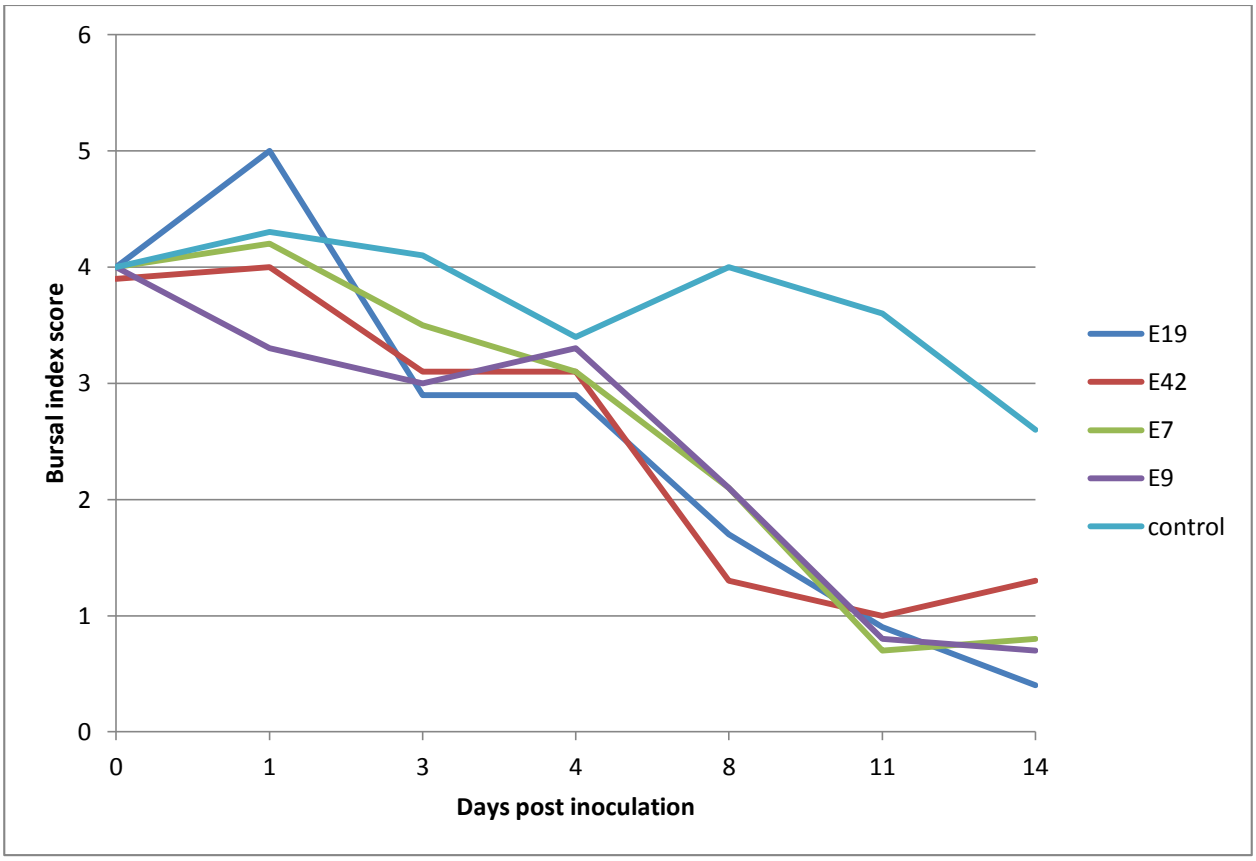


Figure 5.4: Variation of bursal index scores against days post inoculation with different isolates.

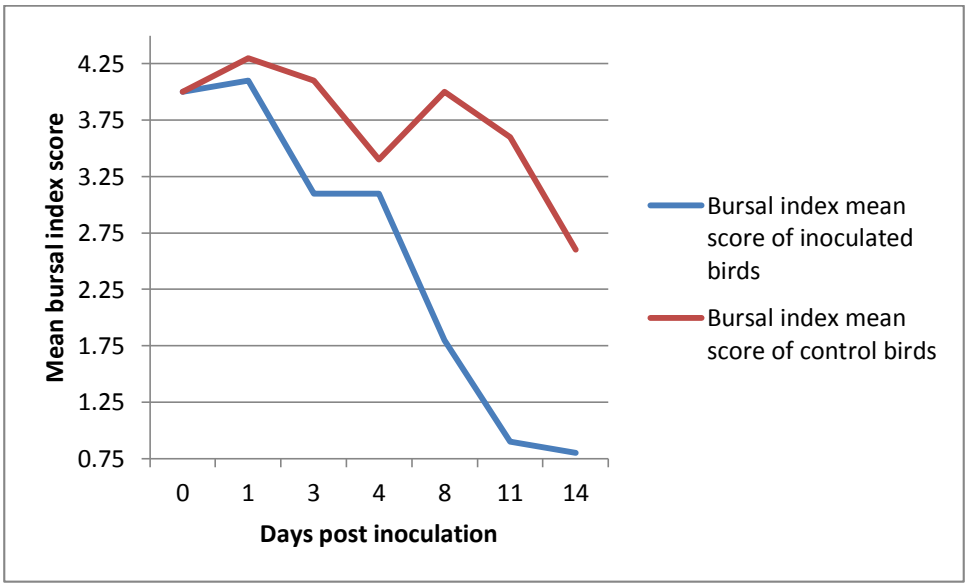


Figure 5.5: Mean bursal index scores of all inoculated birds against days post inoculation.

The spleen index tended to increase compared to control chickens **(Figure 5.6 and 5.7).**Results from ANOVA test showed that there was no statistically significant difference in mean splenic index between the isolates (P=0.338) but there were statistically significant differences between days post inoculation (P=0.001).The effect of number of days post inoculation on splenic index did not depend on type of isolate inoculated and there was no statistically significant interaction between the factors F (15, 51) =1.035, p=0.43.

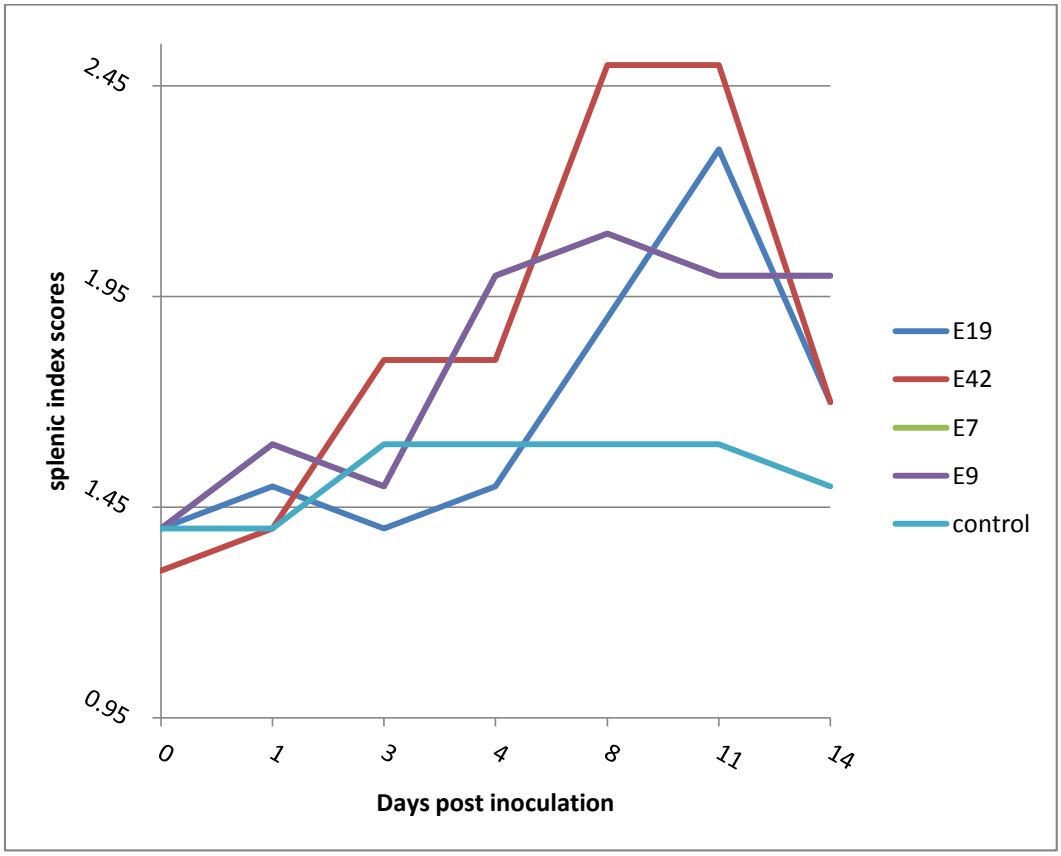


Figure 5.6: Variation of splenic index scores against days post inoculation with different isolates

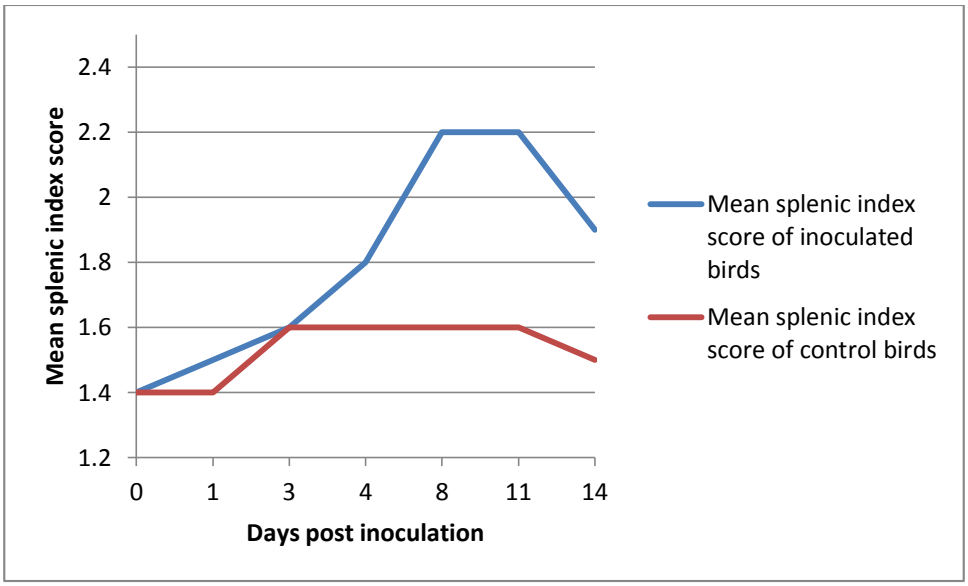


Figure 5.7: Mean splenic index scores of all inoculated birds against days post inoculation

Thymus gland index of inoculated birds increased one day post inoculation then started to decrease, continued to decrease until day 8, then started to increase again as compared to control**(Figure 5.8 and 5.9)**. Results from ANOVA test showed that there was no statistically significant difference in mean thymus gland index between the isolates (P=0.859) but there were statistically significant differences between days post inoculation (P<0.0005).There was no statistically significant interaction between isolate and number of days post inoculation on Thymus gland index score F (15, 51) =1.185, p=0.313. The effect of number of days post inoculation on Thymus gland index did not depend on type of isolate inoculated**.**

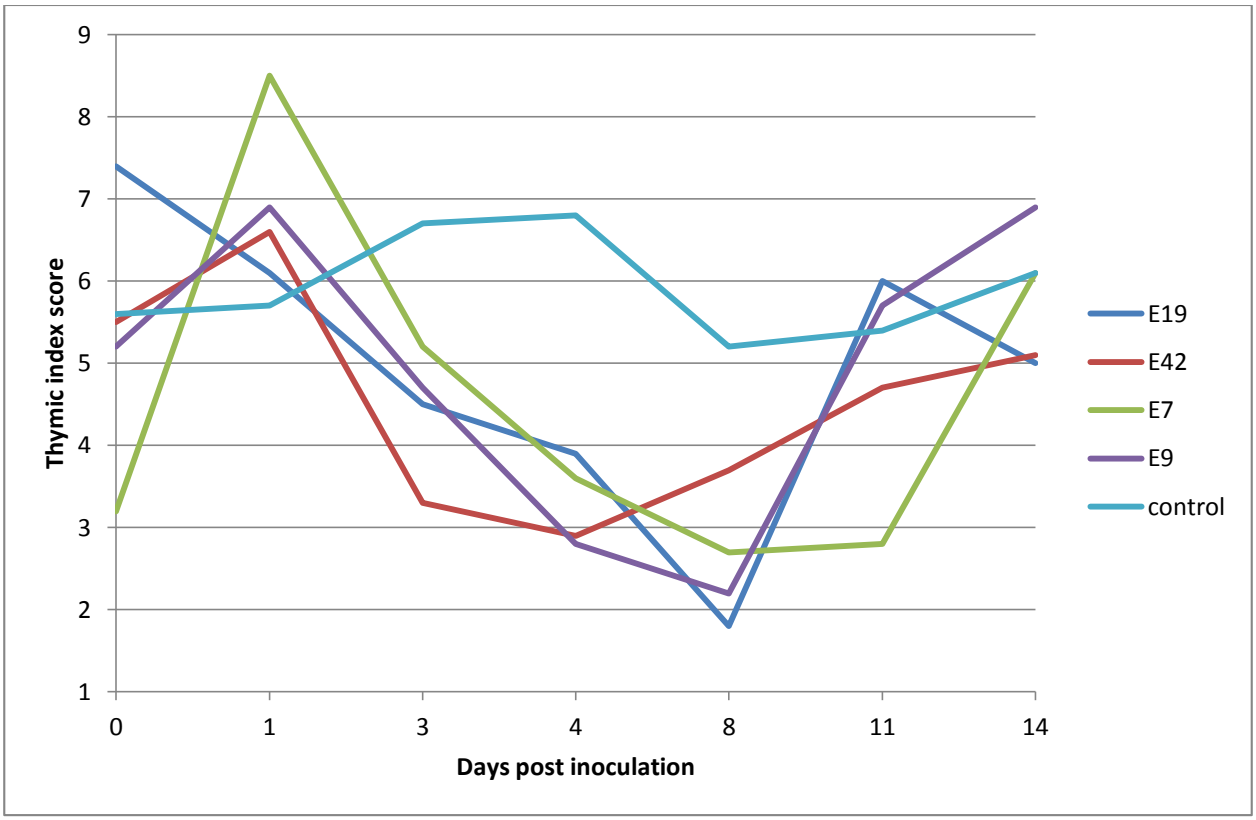


Figure 5.8: Variation of thymic index scores against days post inoculation with different isolates

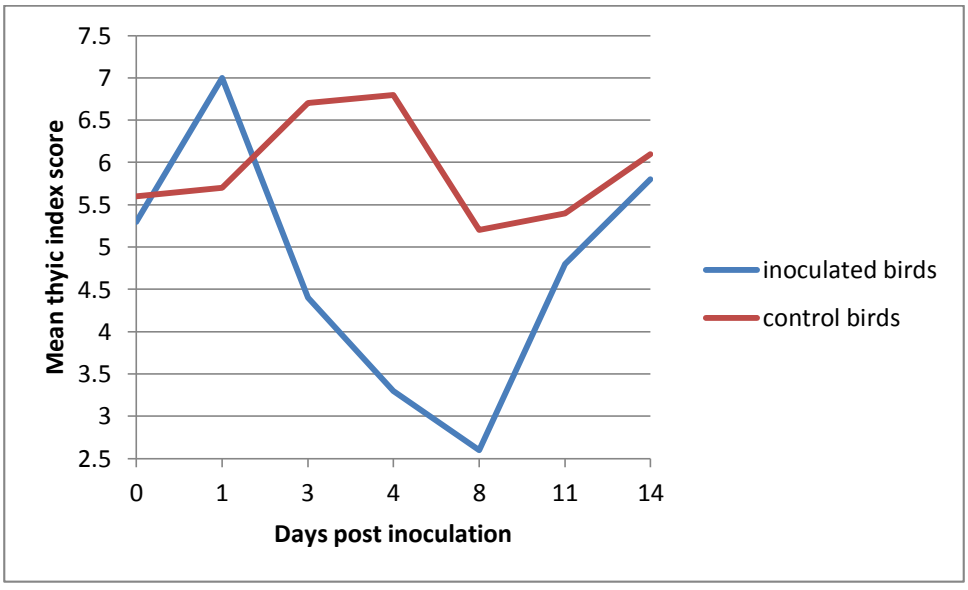


Figure 5.9: Mean thymic index scores of all inoculated birds against days post inoculation: note severe decline of thymic index of inoculated birds on days 1 to 8 then increase from days 8 to 14.

Caecal tonsils and Harderian gland indices of inoculated birds did not seem to vary when compared against controls although there was a general tendency to increase especially on days 1and 3 post inoculation **(Figures 5.10, 5.11, 5.12and 5.13).**Analysis of variance(ANOVA) test showed that there was no statistically significant difference in mean caecal tonsils index between the isolates (P=0.562) and also there was no statistically significant difference between days post inoculation (P=0.089).There was no statistically significant interaction between isolate and number of days post inoculation on caecal tonsils index score F (15, 51) =1.203, p=0.3.

Analysis of variance(ANOVA) test showed that there was no statistically significant difference in mean Harderian gland between the isolates (P=0.105) but there were statistically significant differences between days post inoculation (P=0.008).The effect of number of days post inoculation on caecal tonsils index did not depend on type of isolate inoculated. There was no statistically significant interaction between isolate and number of days post inoculation on Harderian gland index score F (15, 51) =0.796, p=0.677. The effect of number of days post inoculation on Harderian gland index did not depend on type of isolate inoculated.

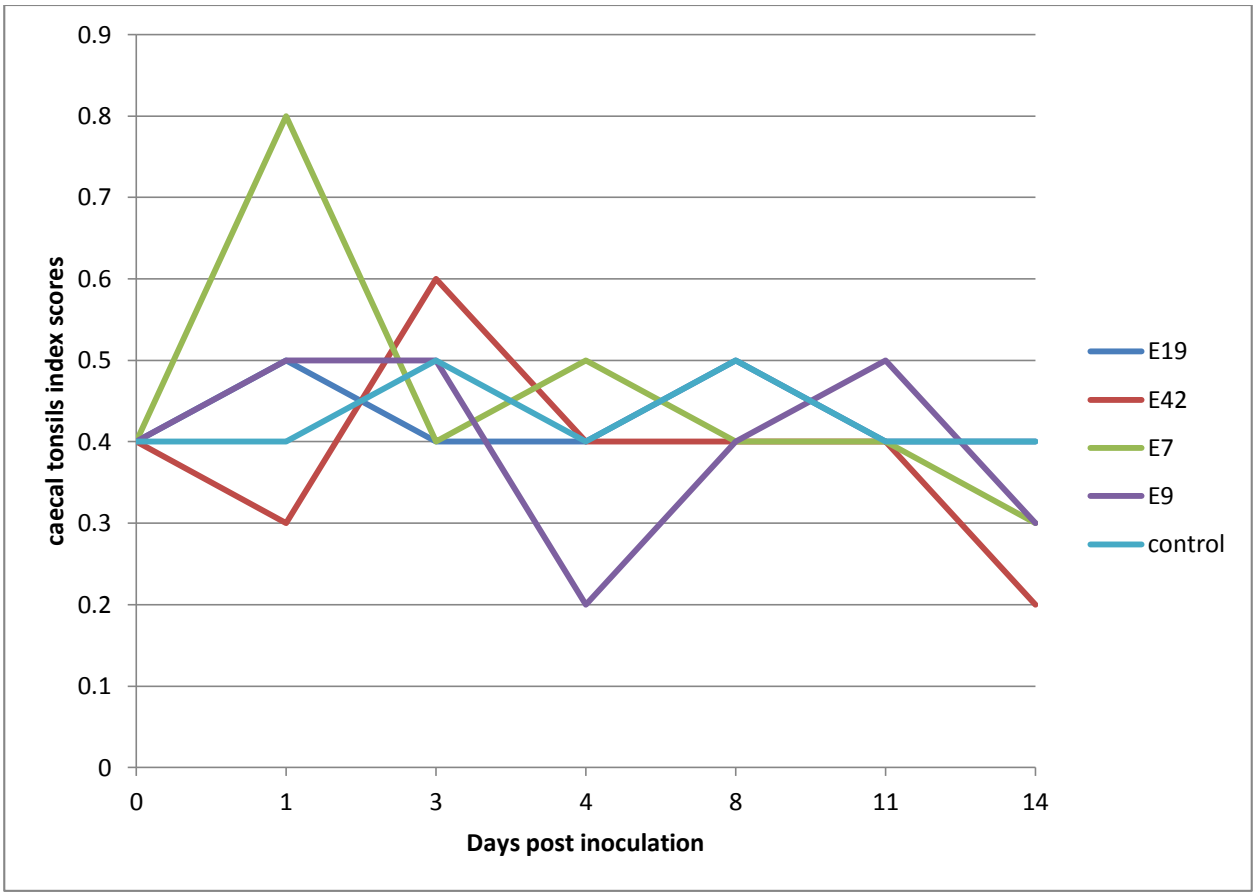


Figure 5.10: Showing variation of caecal tonsils index against days post inoculation with different isolates

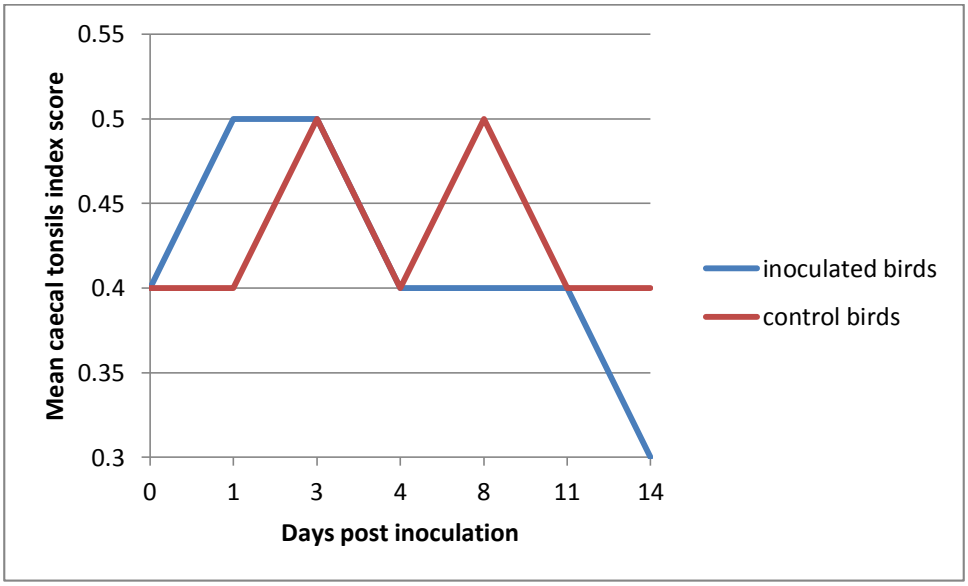


Figure 5.11: Mean caecal tonsils index of all isolates against days post inoculation

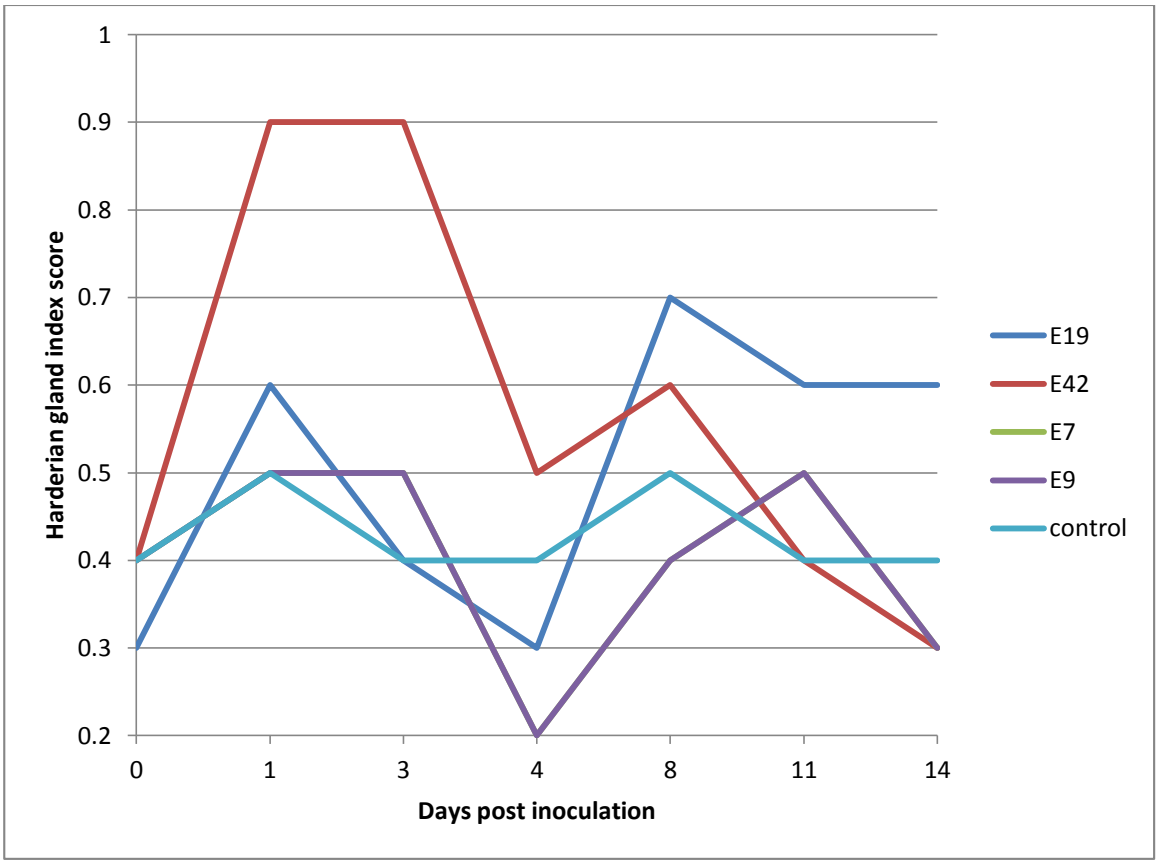


Figure 5.12: Variation of Harderian gland index against days post inoculation with different isolates

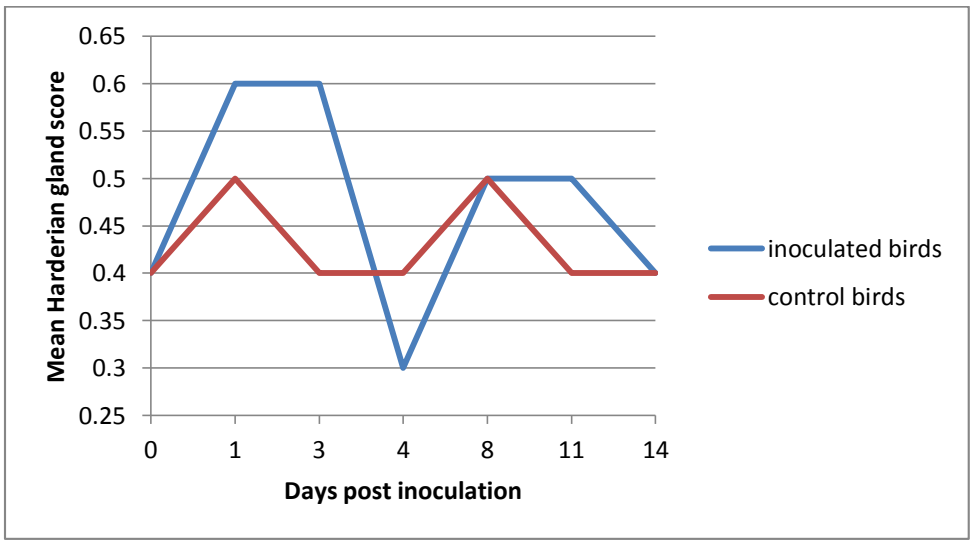
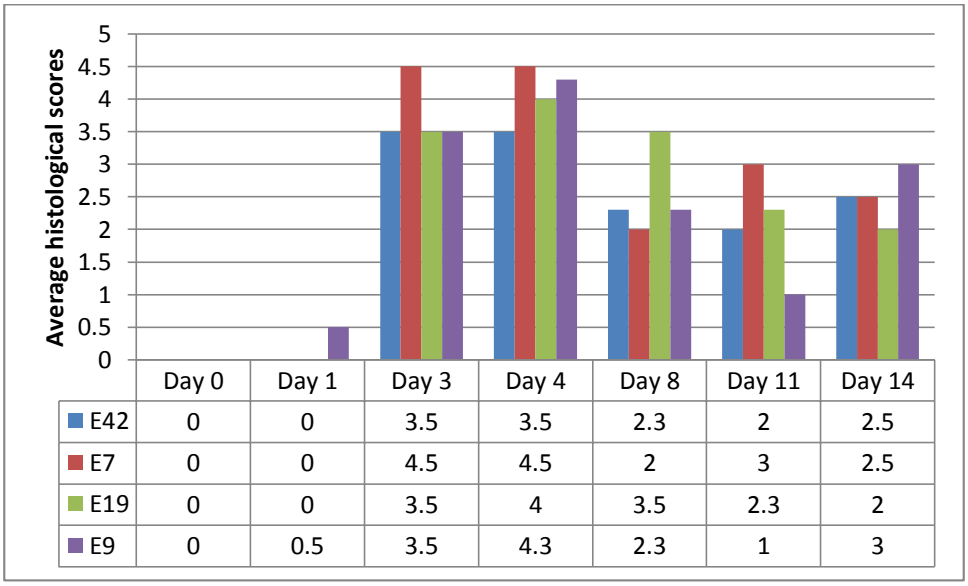


Figure 5.13: Mean Harderian gland index of all isolates against days post inoculation

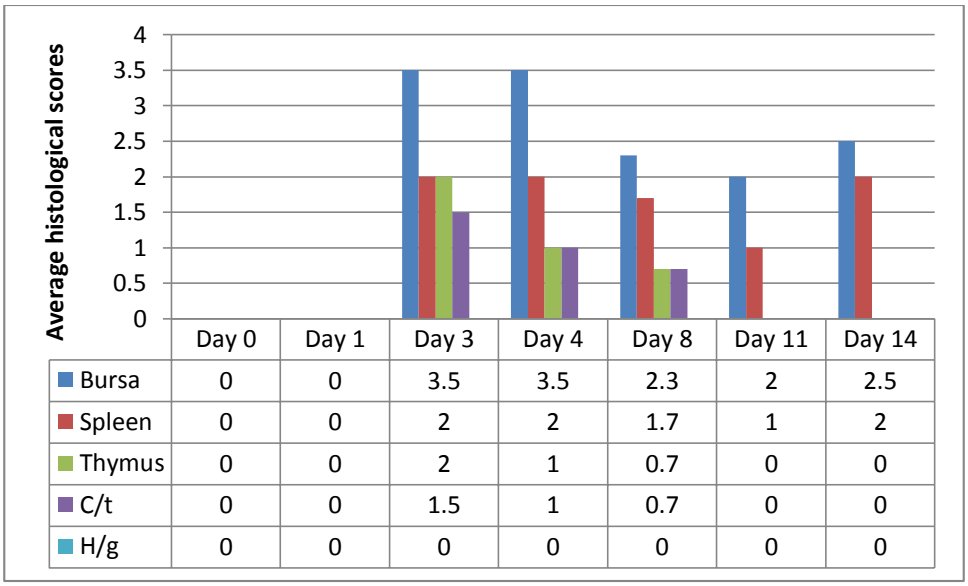
5.3.4 Effect of the isolates on histology of bursa of Fabricius

Patches of focal necrosis were noted on day 1 post inoculation in bursa of birds inoculated with isolate E9 while in birds inoculated with other isolates there was no appreciable change in the bursa until day 3. Bursa of Fabricius was the most damaged organ by days 3 and 4 post inoculation but some slight variation between isolates in severity of the damage was noticed **(Figure 5.14).** Isolate E7 had the severest damage to glandular architecture of BFs of inoculated birds with average score being 4.5 on days 3 and 4. Isolate E9 followed with BF average score being 3.5 on day 3 rising to 4.3 on day 4. This was followed by E19 at 3.5 and 4 on days 3 and 4 respectively and finally E42 at 3.5 on both days (**Figures 5.15, 5.16, 5.17 and 5.18**). Damaged bursas had haemorrhages, lymphocytic depletion, empty spaces, pyknotic lymphocytes and infiltration by granulocytes (**Figures 5.19 and 5.20**) as compared to normal glandular architecture observed in control bird B/F (**Figures5.21 and 5.22**). On days 8, 11 and14 post inoculation fibrocytes and repopulation with lymphocytes (**Figure 5.23**) were observed with variation in degree of restoration between isolates.



	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
E42	0	0	3.5	3.5	2.3	2	2.5
E7	0	0	4.5	4.5	2	3	2.5
E19	0	0	3.5	4	3.5	2.3	2
E9	0	0.5	3.5	4.3	2.3	1	3

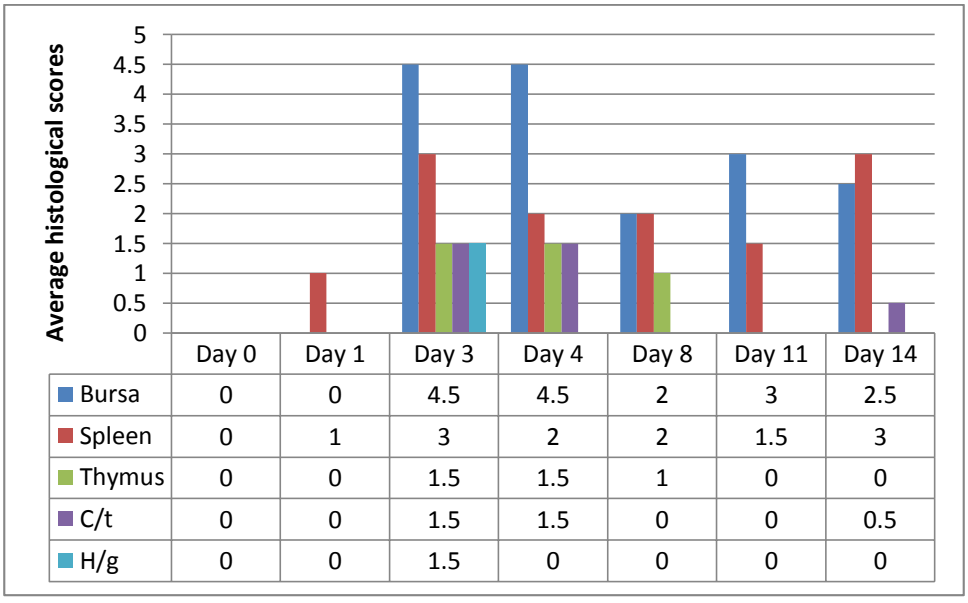
Figure 5.14: Average bursal histological scores on different days post inoculation with Infectious bursal disease virus isolates E42, E7, E19 and E9



	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0	0	3.5	3.5	2.3	2	2.5
Spleen	0	0	2	2	1.7	1	2
Thymus	0	0	2	1	0.7	0	0
C/t	0	0	1.5	1	0.7	0	0
H/g	0	0	0	0	0	0	0

Legend: C/t – caecal tonsils, H/g – Harderian gland

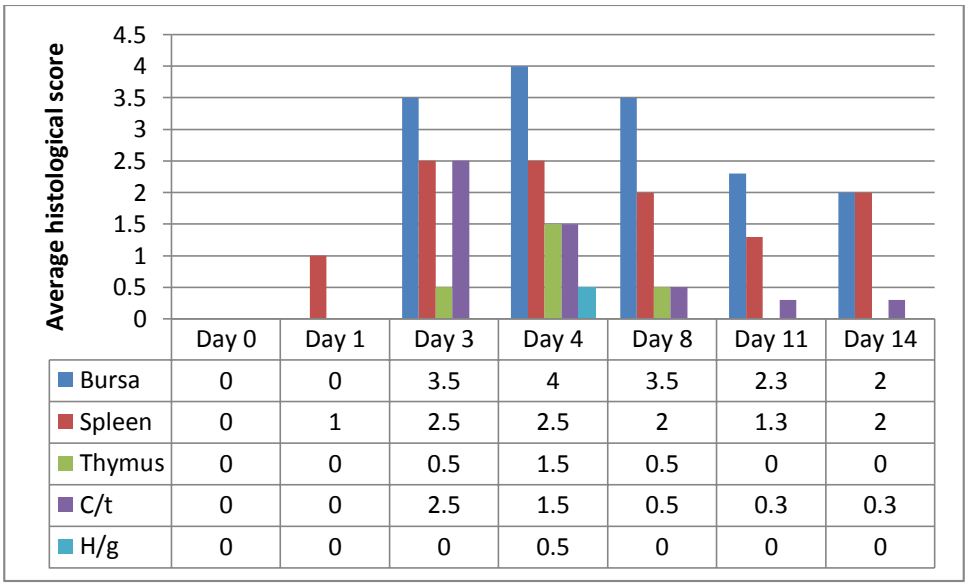
Figure 5.15: Average histological scores of organs on different days post inoculation with Infectious bursal disease virus isolate E42



	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0	0	4.5	4.5	2	3	2.5
Spleen	0	1	3	2	2	1.5	3
Thymus	0	0	1.5	1.5	1	0	0
C/t	0	0	1.5	1.5	0	0	0.5
H/g	0	0	1.5	0	0	0	0

Legend: C/t – caecal tonsils, H/g – Harderian gland

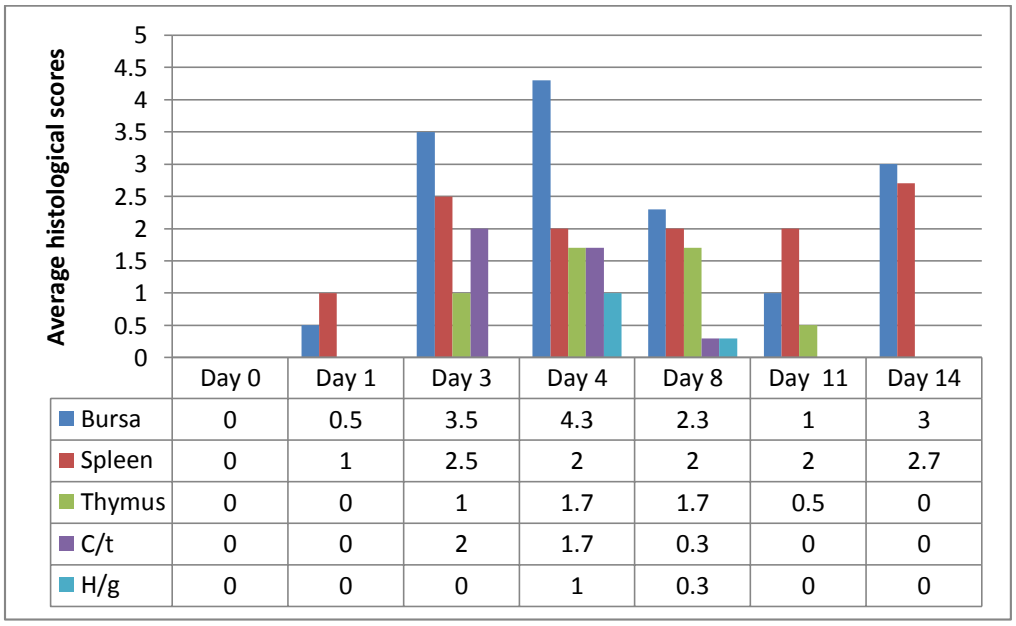
Figure 5.16 Average histological scores on different days post inoculation with Infectious bursal disease virusisolate E7



	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0	0	3.5	4	3.5	2.3	2
Spleen	0	1	2.5	2.5	2	1.3	2
Thymus	0	0	0.5	1.5	0.5	0	0
C/t	0	0	2.5	1.5	0.5	0.3	0.3
H/g	0	0	0	0.5	0	0	0

Legend: C/t – caecal tonsils, H/g – Harderian gland

Figure 5.17 Average histological scores on different days post inoculation with Infectious bursal disease virus isolate E19



	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0	0.5	3.5	4.3	2.3	1	3
Spleen	0	1	2.5	2	2	2	2.7
Thymus	0	0	1	1.7	1.7	0.5	0
C/t	0	0	2	1.7	0.3	0	0
H/g	0	0	0	1	0.3	0	0

Legend: C/t – caecal tonsils, H/g – Harderian gland

Figure 5.18 Average histological scores on different days post inoculation with Infectious bursal disease virus isolate E9

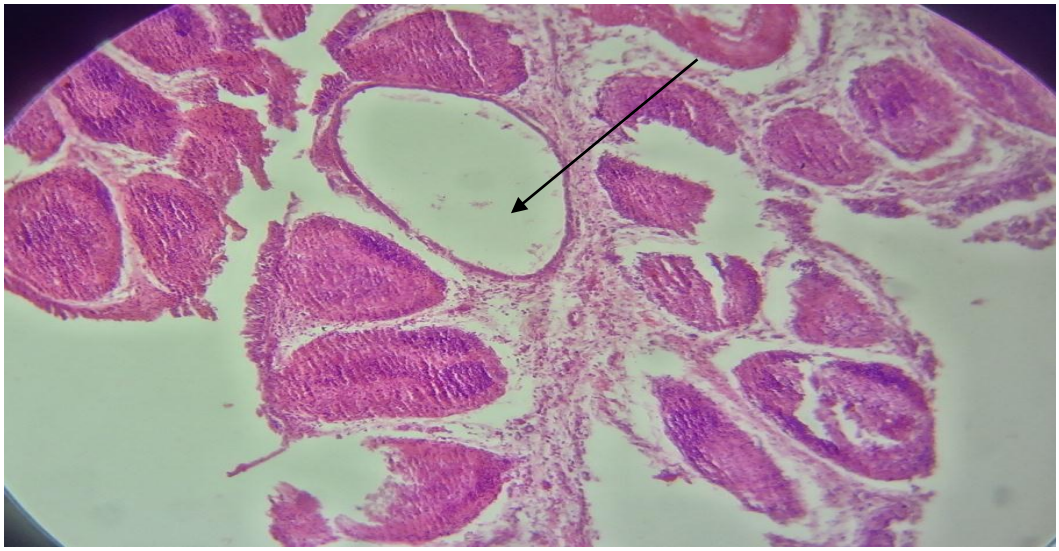


Figure: 5.19 Empty spaces (black arrow) in a bursa of Fabricius day 3 post inoculation with infectious bursal disease virus isolate E 42 (×5 H&E)].

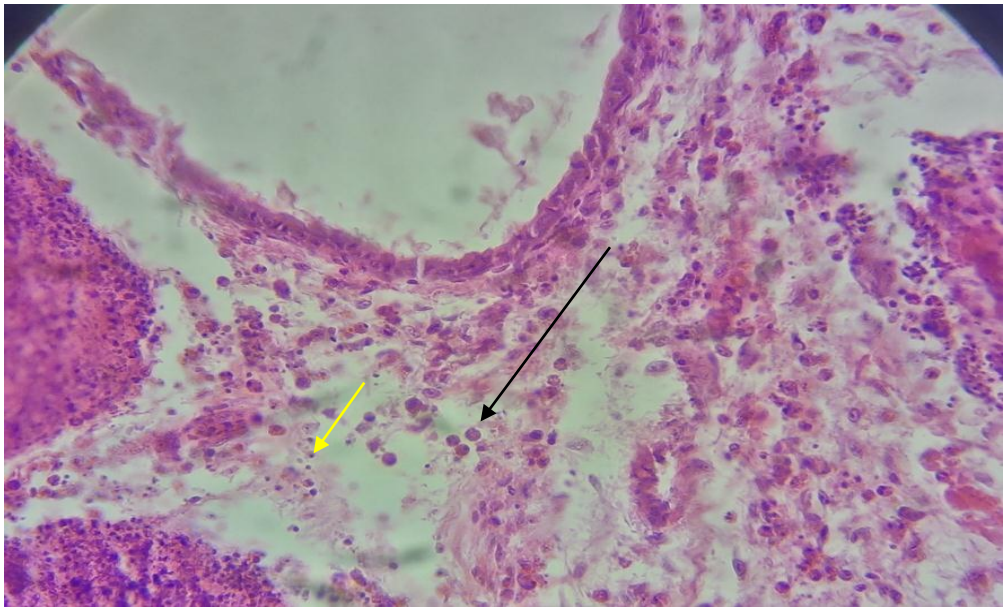


Figure: 5.20 A closer view of figure 5.18 showing granulocytes (long black arrow) and pyknotic lymphocyte (short yellow arrow) in a bursa day 3 post inoculation with Infectious bursal disease virus isolate E 42. [B (×50 H&E).

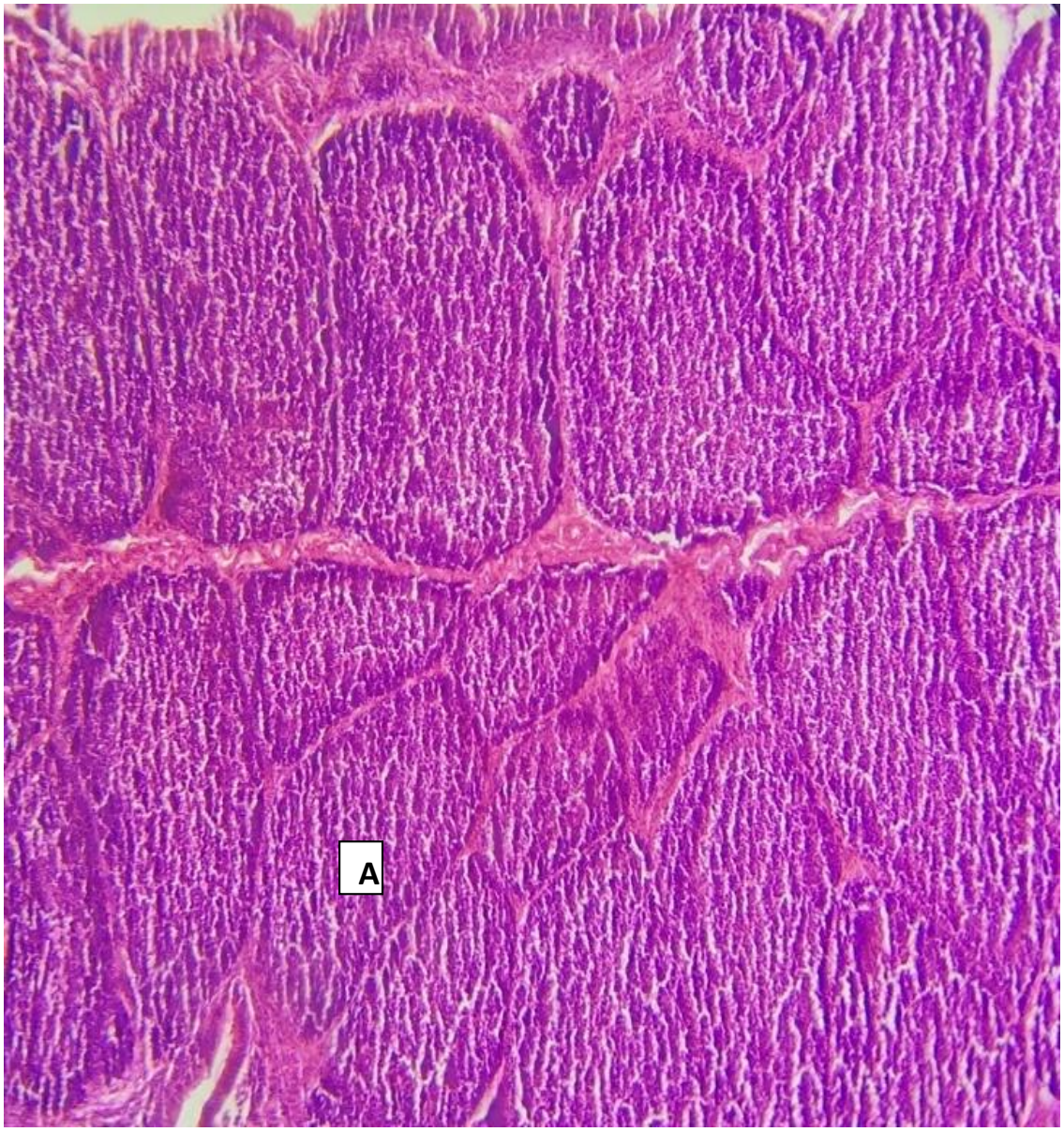


Figure: 5.21 A normal bursa of Fabricius from a control bird day 3 post inoculation showing normal glandular architecture characterized by high cell density (A) (×10 H&E).

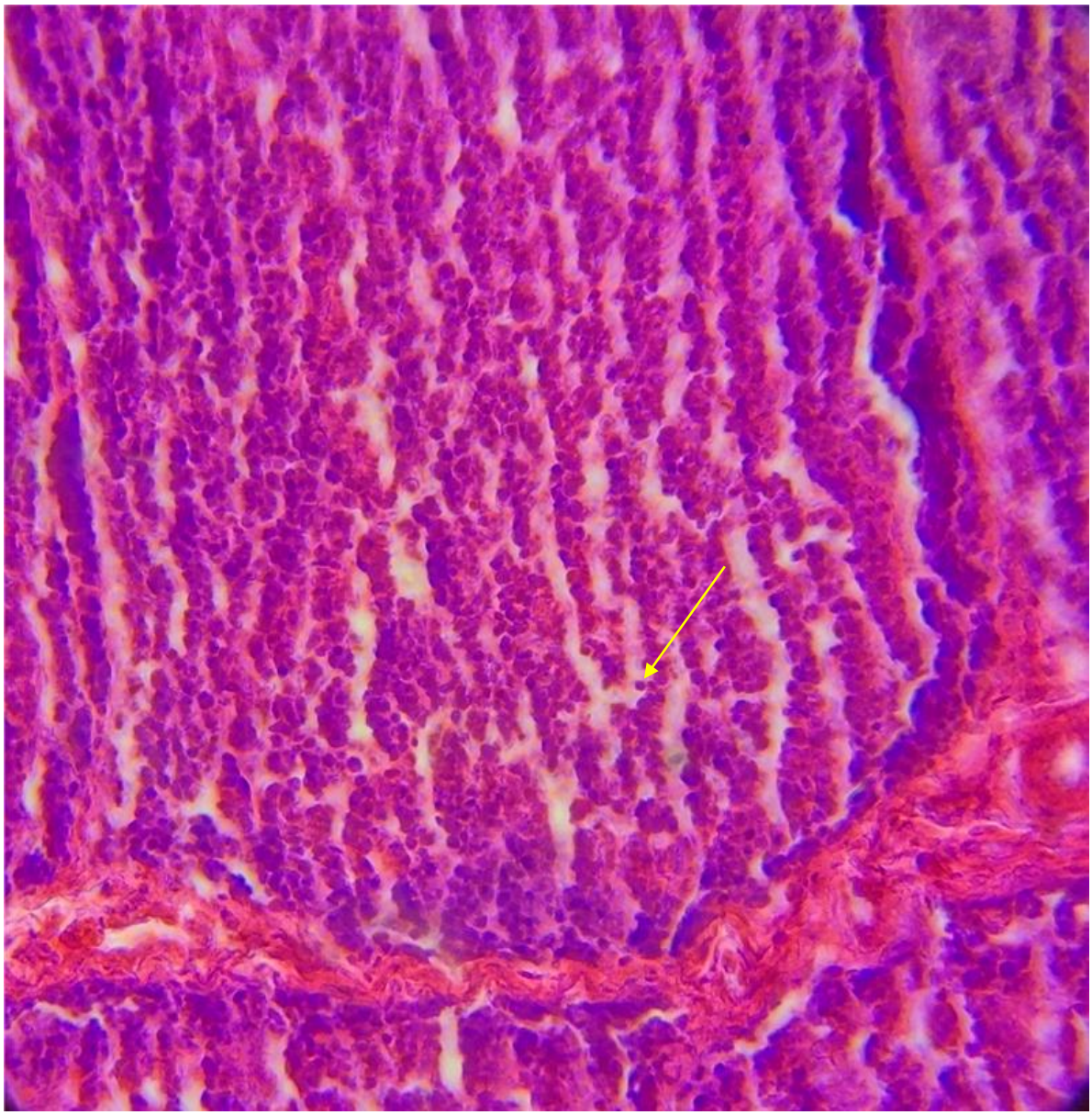


Figure: 5.22 A normal bursa of Fabricius from a control bird day 3 post inoculation showing follicles well populated with lymphocytes (yellow arrow) being a closer view of figure 5.20 (×100 H&E).

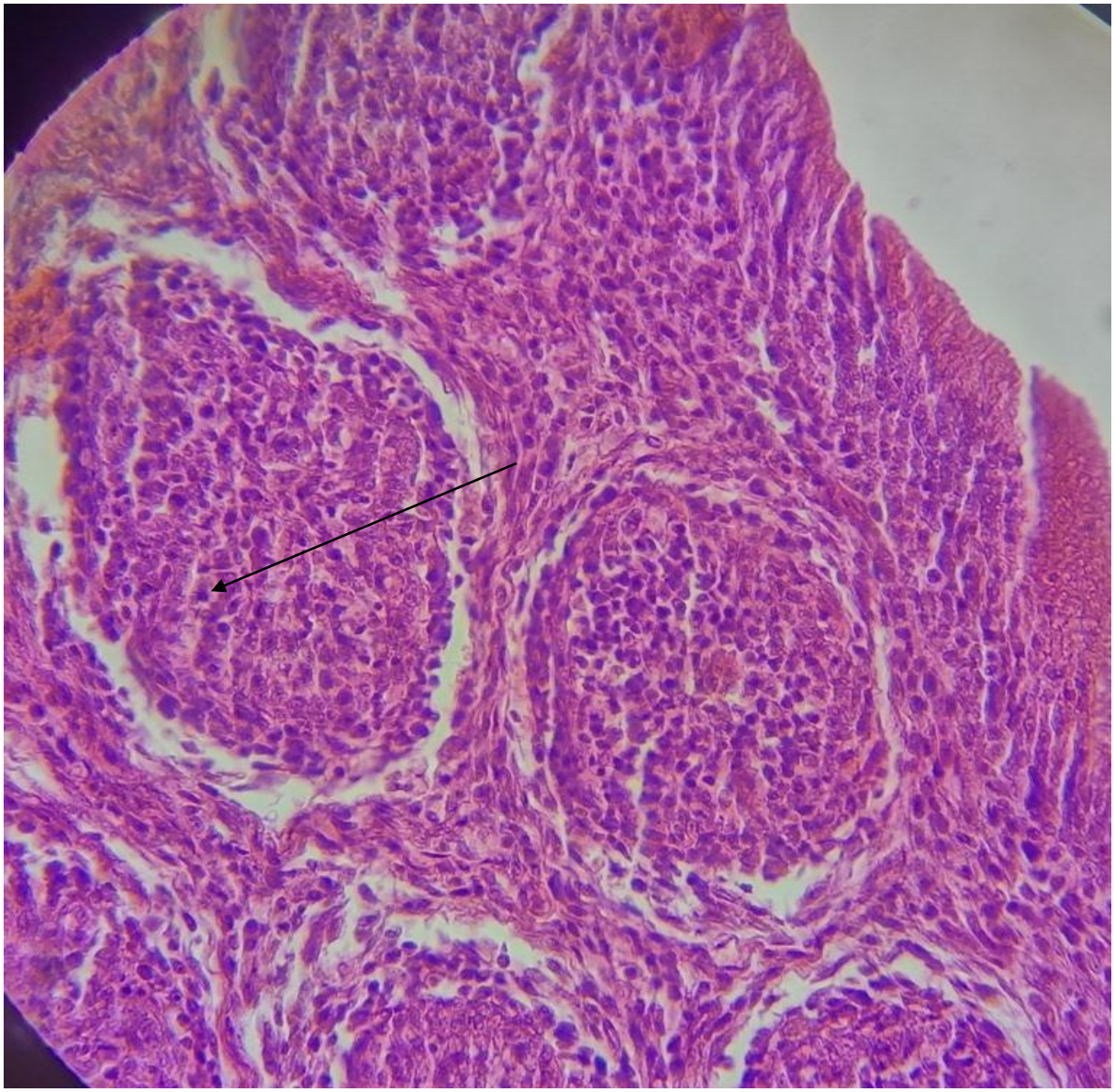


Figure 5.23: Glandular architecture being restored and repopulation with lymphocytes (black arrow) in bursa of Fabricius 8 days post inoculation with Infectious bursal disease virus isolate E42(×100 H&E).

5.3.5 Effect of the isolates on histology of spleen

All the isolates caused diffuse degeneration and necrosis of lymphoid cells **(Figure 5.24)** most evident in the peri-arterial sheath but with varying severity. There was appreciable pathology in

spleen of birds inoculated with isolate E7, E19 and E9 by end of first day post inoculation. The greatest pathology was observed on days 3 and 4 when scores were between 2 and 3 in all isolates **(Figures 5.15, 5.16, 5.17 and 5.18).** There was not much restoration to normal architecture in this organ on days 8, 11 and 14 unlike in the bursa where there was restoration. There was minimal variation between isolates on their effect on spleen.

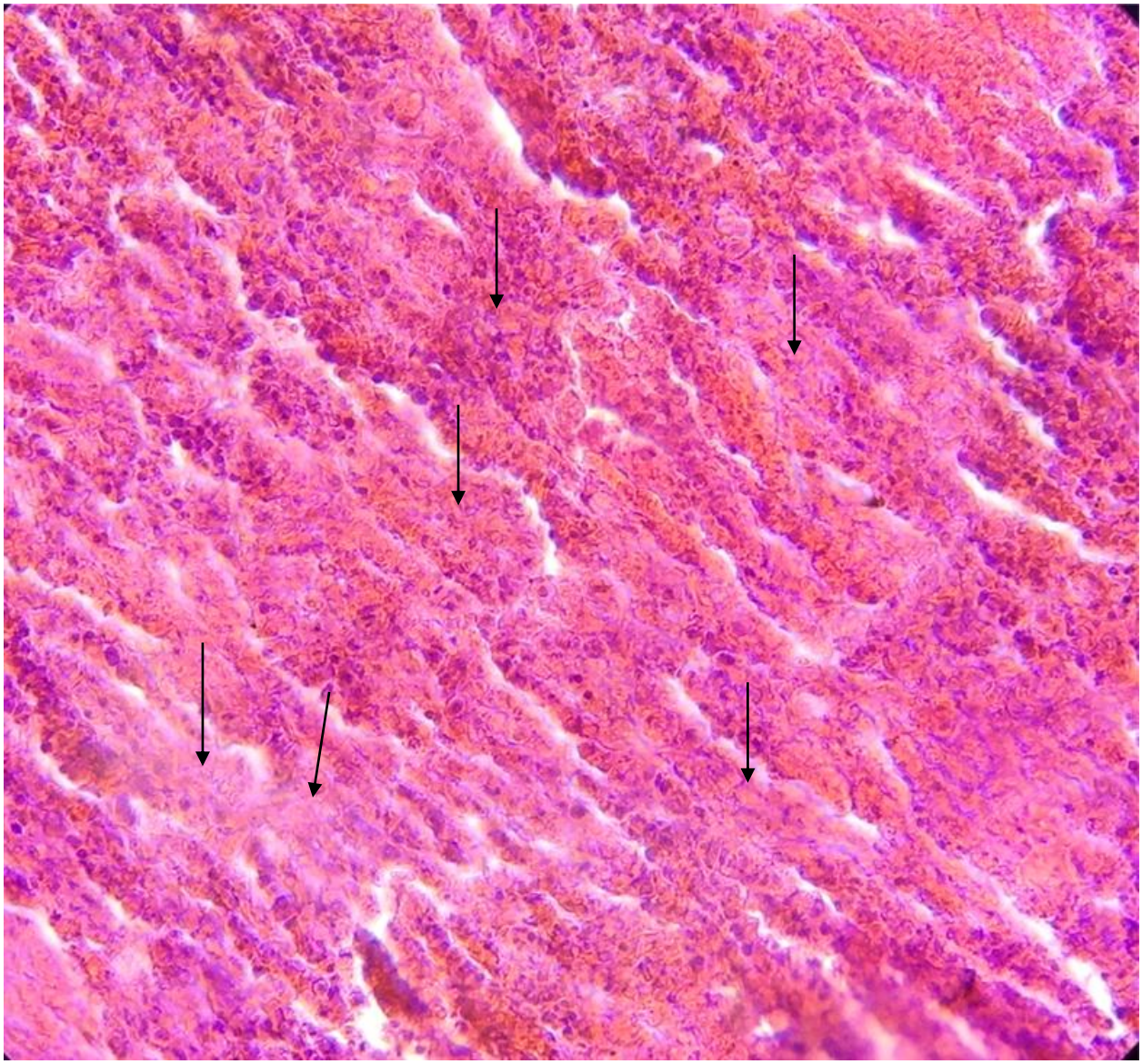


Figure 5.24 Diffuse necrosis (black arrows) in a spleen day 3 post inoculation with Infectious bursal disease virus isolate E9. (×50 H & E).

5.3.6 Effect of the isolates on histology of thymus gland

Significant lymphocytic depletion was observed in days 3, 4 and 8 with isolates E42, E7 and E19 after which the glandular architecture seemed to revert back to normal. Lymphocytic depletion with isolate E9 was observed on days 3, 4, 8 and 11. Highest average score was 2 on day 3 with isolate E42 followed by an average score of 1.7 on days 4 and 8 with isolate E9 **(Figures 5.15, 5.16, 5.17 and 5.18).**

5.3.7 Effect of the isolates on histology of caecal tonsils

Pyknosis, lymphocytic depletion **(Figure 5.25)** and infiltration with granulocytes were observed starting from day 3 with all isolates. Highest average score was 2.5 on day 3 isolate E19 followed by average score of 2 on same day with isolate E9 **(Figures 5.15, 5.16, 5.17 and 5.18).**

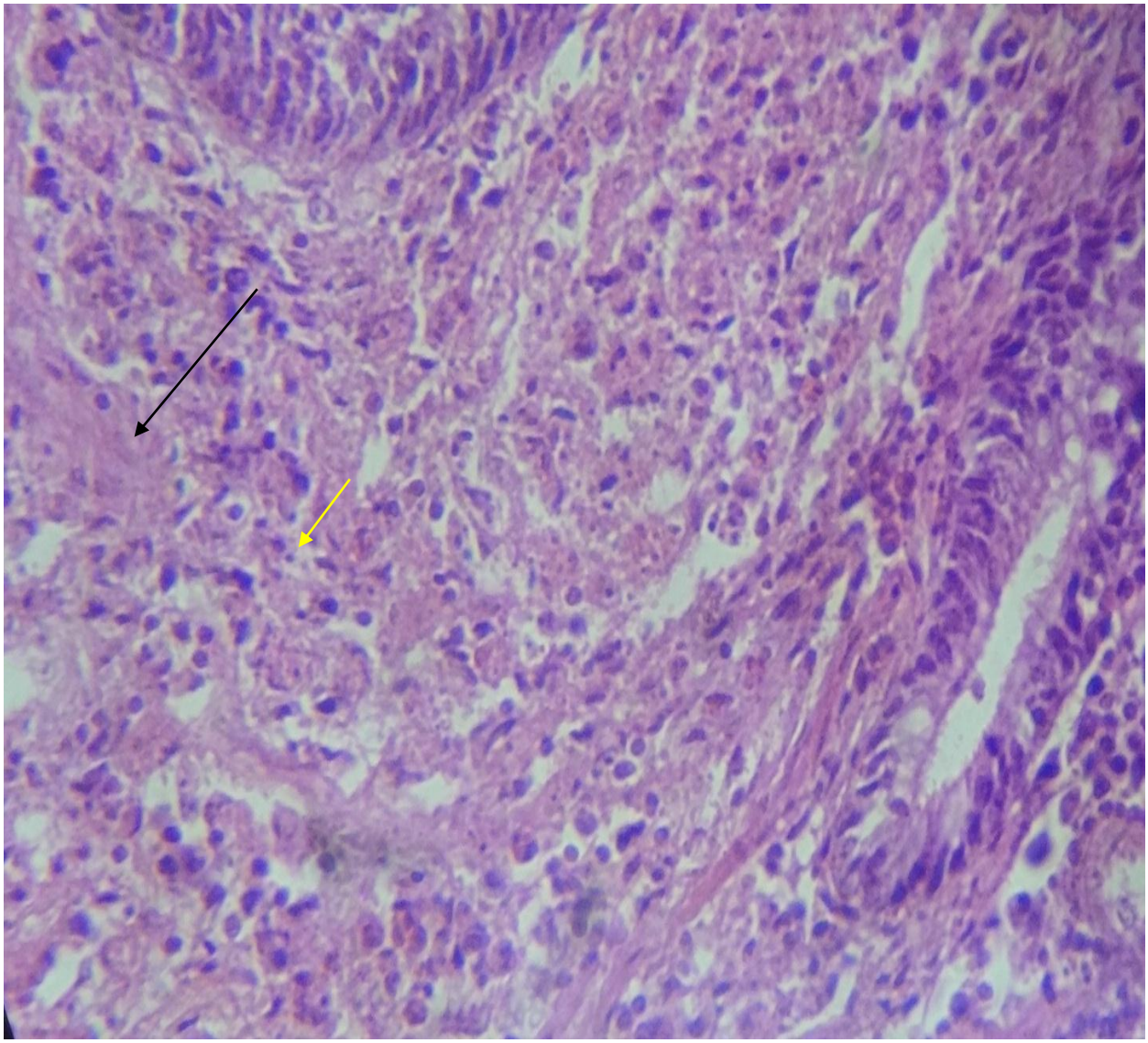


Figure 5.25: Pyknosis (short yellow arrow) and lymphocytic depletion (long black arrow) in a caecal tonsil day 3 post inoculation with Infectious bursal disease virus isolate E9. (×100 H & E).

5.3.8 Effect of the isolates on histology of Harderian gland

All the isolates had very minimal effect the Harderian gland and in most cases the organ was observed highly populated with plasma cells **(Figure 5.26).**Highest score was 1.5 recorded on day 3 pi with isolate E7 **(Figure 5.16),** followed by score of 1 day 4 isolate E9.Isolate E19 only yielded an average histological score of 0.5 on day 4 and lastly on day 8 post inoculation the score was 0.3 with isolate 9. On all other daysscores were 0 with all the other isolates. There was no noticeable

change in the Harderian gland in all the days of the experiment with isolate E42. **(Figures 5.15, 5.16, 5.17 and 5.18).**

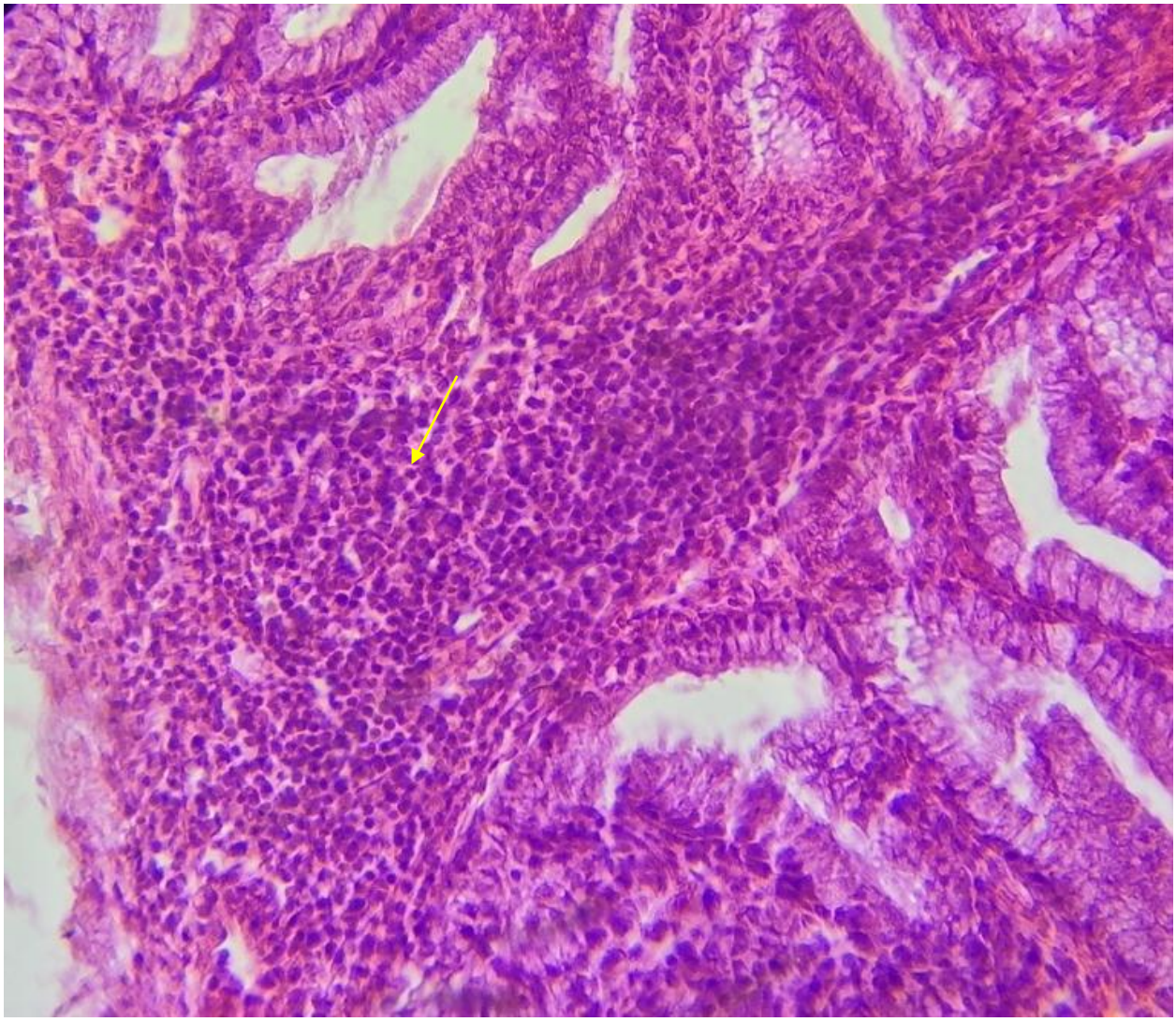


Figure 5.26: Harderian gland highly populated with plasma cells (yellow arrow) day 8 post inoculation with Infectious bursal disease virus isolate E42. (×50 H & E).

Isolate E9 had average score of 1 on day 4 and 0.3 on day 8 while on all the other days it was 0. Isolate E7 had the highest average score among the isolates and this was 1.5 on day 3 while the score was 0 on all the other days **(Figures 5.15, 5.16, 5.17 and 5.18).**

5.4 DISCUSSION

In this study, four IBDV isolates from outbreaks that occurred in different geographic areas of Kenya were characterized on basis of virulence in susceptible indigenous chickens after infection with a standardized dose. The results showed isolate E42 as the least virulent of all the isolates and yet it is the one that had caused 100% mortality rate in an outbreak in indigenous chicks from which it was isolated. A high mortality rate in field outbreaks of IBD may thus not always be a factor of the virulence of the virus. Other confounding factors (Jackwood and Sommer-Wagner, 2007) in an outbreak may increase mortality rate. Among such factors are the breed, age and immune status of the challenged chickens, the dose and route of inoculation of the challenge virus, and the possible presence of contaminating agents in the inoculum (OIE, 2008). Presence of other infectious agents and management practices in the flock influence the mortality rate recorded in an IBD outbreak (Jackwood and Sommer-Wagner, 2007).

There are two IBDV serotypes designated serotype 1 and 2; only serotype 1 viruses are pathogenic to chickens while serotype 2 viruses are apathogenic (Jackwood *et al.,* 1985). All the four isolates in this study were pathogenic to chickens and were therefore classified as serotype 1 virus. Several distinct pathotypes have been described within serotype 1 IBDV. These pathotypes vary from mild to very virulent (Jackwood *et al.,* 2011).

Serotype 1 IBDV belonging to the classical (also called standard or virulent) and vvIBDV pathotypes cause clinical disease, with lesions in B/Fs being mainly edema and hypertrophy accompanied by heterophil infiltration. This compares well with clinical signs of IBD and lesions in B/Fs that were observed with all the isolates in this experiment. Viruses termed as vvIBDV (or hypervirulent IBDV) cause very severe clinical disease and the severity of lesions in the other non-

bursal lymphoid organs is more intensive. All the isolates in this experiment produced severe clinical disease and severe lesions in the spleen and thymus gland and also significant lesions on the caecal tonsils and Harderian gland. In this study, effect of the viruses on thymus gland resulted in decrease of thymic index. Observations by other researchers found that greater decrease in the thymic index and more severe lesions in the cecal tonsil, thymus and spleen occurred in birds inoculated with vvIBDV (Tanimura *et al.,* 1995).

Antigenic variants of IBDV, commonly called variant pathotypes, cause subclinical infection characterized by absence of clinical signs but cause severe immunosuppression due to rapid bursal atrophy with minimal inflammatory response (Snyder, 1990). None of the isolates in this experiment caused subclinical infection.

Based on the signs and lesions observed in two lines of White Leghorn SPF chickens during acute experimental IBD following a 10^5 EID_{50} challenge, variant IBDVs induce little if any clinical signs and minimal or no mortality rate; classical IBDVs induce approximately 10 - 50% mortality rate; whereas vvIBDV strains induce approximately 50–100% mortality rate (OIE, 2008). By comparison, the results in this experiment were obtained after the birds were inoculated with a lower dose of 10^4 EID_{50}. It is apparent that isolates E7 and E9 which induced mortality rate of 61.1% and 66.7% respectively fall in the category of vvIBDV strains. Very virulent IBDV infections are characterized by severe clinical signs and high mortality (van den Berg, 2000). They produce disease with exacerbated acute phase and more severe clinical signs in the affected birds (van den Berg, 2000). All the isolates in this study produced an exacerbated acute phase. Although isolates E19 and E42 caused lower mortality rates, probably due to attenuation in the indigenous chicken embryos used in the isolation of the viruses, the clinical disease was severe according to the symptomatic index scores. The lesions too, in lymphoid organs of birds inoculated with E19

and E42were intense as is seen with vvIBDV pathotypes. In IBDV infections, bursa of Fabricius, being the principal diagnostic organ, becomes turgid, oedematous, and sometimes haemorrhagic and turns atrophic within 7 to 10 days of infection with vvIBDV. This atrophy may be more rapid in vvIBDV infections, even 3 to 4 days post inoculation (Tsukamoto *et al.,* 1992) as was observed with all the isolates in this study. In addition, in vvIBDV infections, severe depletion of lymphoid cells is observed not only in the bursa of Fabricius, but also in the non-bursal lymphoid tissues (van den Berg 2000) as was seen with all the isolates in this study. So in view of the above, the four isolates in this study can be classified as vvIBDV strains.

Chickens used in this study were not in-bred animals and did not have genetic homogeneity but have demonstrated that indigenous chickens in Kenya are highly susceptible to IBDV as has previously been reported (Mutinda, *et al.,* 2013). Standardization of pathogenicity trials might be difficult due to variation in the genetic backgrounds in a flock (van den Berg *et al.,* 2004). Therefore although some of the virulence variations observed in this study could be due to innate factors in the birds rather than the virus, groups of chickens of statistically significant size (number of chicken per group) were used for challenge. Molecular and immunological studies were recommended for further characterization of the isolates.

CHAPTER SIX: DETERMINATION OF IMMUNOGENICITY OF VIRUS ISOLATES IN INDIGENOUS CHICKENS

6.1 INTRODUCTION

Infectious bursal disease causes economic loss to poultry farmers due to death of chickens, growth retardation, immune-suppression, or rejection of chicken carcasses (van den Berg, 2000). The high resistance of IBDV to environmental exposure makes hygienic measures alone ineffective and vaccination is thus essential to control the disease. Infectious bursal disease virus has been shown to remain infectious for 122 days in a chicken house and for 52 days in feed and water (Benton *et al.,* 1967). Therefore, strict hygienic conditions within poultry farms have to be coupled with vaccination, using efficient vaccines to prevent IBD. The disease is of such high economic importance in both its clinical and sub-clinical manifestations that it warrants search for and the use of efficient vaccines (van den Berg, 2000). Satisfactory protection can be achieved by immunization with live or inactivated vaccines (Müller *et al.*, 2012). Classical live vaccines achieve lifelong and broad protection but possess residual pathogenicity and a proportional risk of reversion to virulence. Most commercially available conventional live IBDV vaccines are based on classical strains. Those classified as ‘‘mild’’ vaccines exhibit only poor efficacy in the presence of certain levels of maternally derived antibodies and against vvIBDV. ‘‘Intermediate’’ and ‘‘intermediate plus’’ or ‘‘hot’’ vaccines have a much better efficacy and may break through higher levels of maternally derived antibodies, but they can induce moderate to severe bursal lesions and, thus, cause corresponding levels of immuno-suppression. Inactivated vaccines, although costly, are used successfully (Box, 1989) but they must have a high antigenic content to stimulate an immune response that can protect against infection by IBDV strains (Müller *et al.,* 2012). The isolate that is selected for production of vaccine must be highly immunogenic among other factors.

Protection that is conferred to the vaccinated birds depends on the immune response elicited by the vaccine. This experiment was set up to determine immunogenicity of selected field isolates in indigenous chickens in Kenya.

6.2 MATERIALS AND METHODS

6.2.1 Viruses

Five of the 44 isolates obtained in Chapter 4 were selected. These were isolates E34, E39, E19, E3 and E42. They were bursal derived and prepared as described in **section 4.2.3.**

6.2.2 Titration

This was done as described in **section5.2.2** of this document.

6.2.3 Experimental birds

Day old indigenous chicks were obtained and reared as described in section **4.2.2.** At the age of 4weeks they were transferred to inoculation rooms. Feed and water were provided *ad-libitum.*

6.2.4 Experimental design

Six birds (4 weeks old) per isolate were inoculated with 0.3mls (intramuscular via the leg muscle) of 10^4EID_{50}inactivated virus. The chicks were pre-screened to confirm absence of IBDV antibodies before inoculation. Birds were inoculated on days 0, 14 and 21 while bleeding for serum was done on days 14, 21, 28 and 35 post first inoculations. On days 14 and 21 the birds were first bled before inoculation. Harvested serum was assayed for IBDV antibodies using AGPT and ELISA test.

6.2.5 Inactivation of viruses

The volume of virus (at 10^4 EID_{50}) required was 1.8mls rounded to 2mls; to inoculate each of the 6 birds with 0.3mls of viral suspension. Inactivation of the virus was achieved by adding 10µl of formalin (40%) to 1990µl of virus to make 2mls; to make a final concentration of 0.2%. This mixture was incubated in a dark place overnight (24hrs) at room temperature (Habib *et al.*, 2006). Fresh virus was prepared at each inoculation.

6.2.6 Procedure for Enzyme linked immuno-assay

An IBDV Enzyme linked Immunoassay test kit (IDEXX IBD-XR from IDEXX laboratories USA) was used to determine IBDV antibodies in serum according to the manufacturer's instructions. In brief, all the reagents and samples were allowed to attain room temperature. After mixing gently by inverting and swirling the reagent and sample, the antigen coated plates were removed and the sample position recorded. The serum samples were diluted five hundred fold (1:500) and 100µl of diluted serum dispensed into appropriate wells. The negative and positive controls were dispensed in duplicate. After incubating for 30minutes at room temperature, the liquid content of each well was aspirated and discarded. Each well was washed 5times with 350mls of distilled water after which 100µl of conjugate was added. The plate was then incubated for another 30minutes at room temperature, then washed again as previously described. After the last wash, 100µl of substrate provided was added into each well and after incubating for 15minutes to allow for reaction to occur, 100µl of stop solution was dispensed into each well. The absorbance values were then measured and recorded at 650nm, A (650). The relative level of antibody in the sample was determined by calculating the sample to positive (S/P) ratio (Briggs *et al.,* 1986) and the titer calculated relative to an end point titer at 1:500 dilution according to the formula given below:

Log_{10} Titer = 1.09(log_{10}S/P) +3.36.

This technique is significant because of the near linear relationship between the antibody titer and absorbance value at single working dilution (Snyder *et al.,* 1983).

6.2.7 Procedure for Agar gel precipitation test

This test was done as described in **section 3.2.6** of this document but adjusted to detect antibodies as described in OIE manual (2008). Instead of reference antibody, reference antigen was put in the central well and the test sera in peripheral wells. The reference antigen was used as a positive control.

6.3 RESULTS

6.3.1 Detection of antibodies by Agar gel precipitation test.

Antibodies were detected in all the birds by day 14 after first inoculation except in 2 of those inoculated with isolate E34.While all the sera from birds inoculated with isolates E39, E19, E3 and E42 yielded strongly visible precipitation lines **(Figure 6.1),** only two birds of the six inoculated with isolate E34 yielded strong precipitation lines by day 14. Out of the remaining 4birds of isolate E34, two yielded weak, barely visible lines while the remaining two yielded no visible precipitation lines between the antigen and test sera. On days 21, 28 and 34 post first inoculation, antibodies were detectable in all the inoculated birds yielding strong precipitation lines.

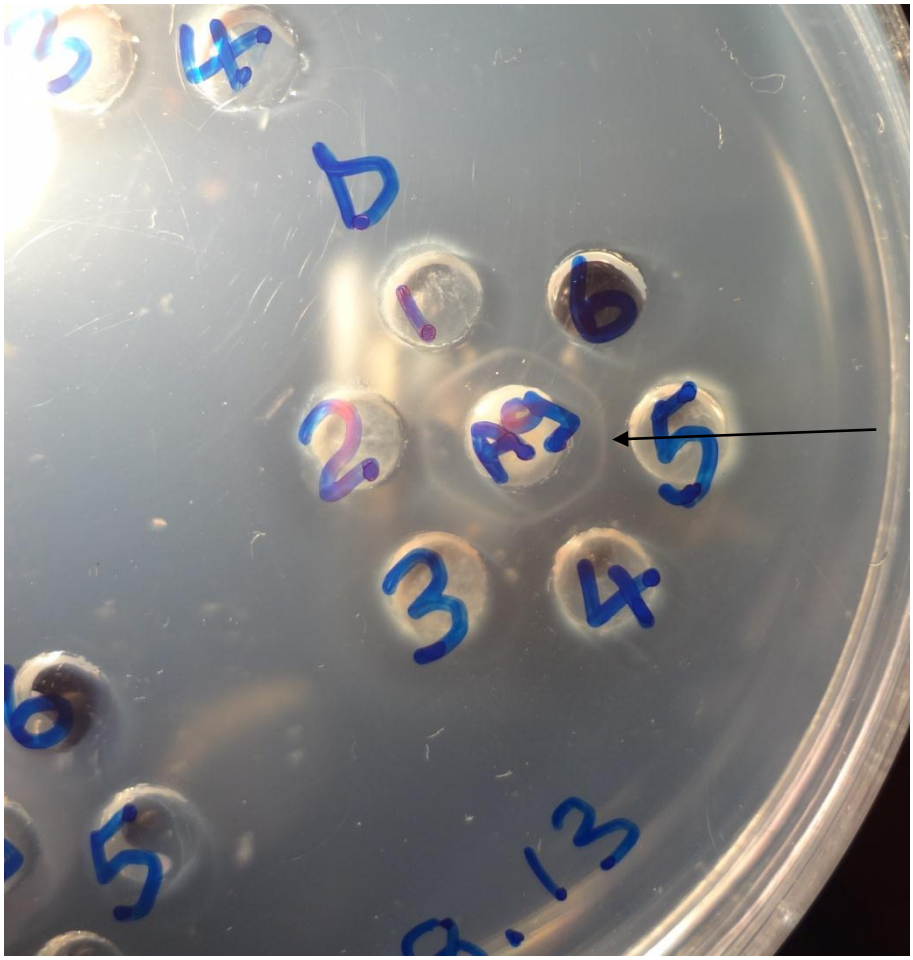
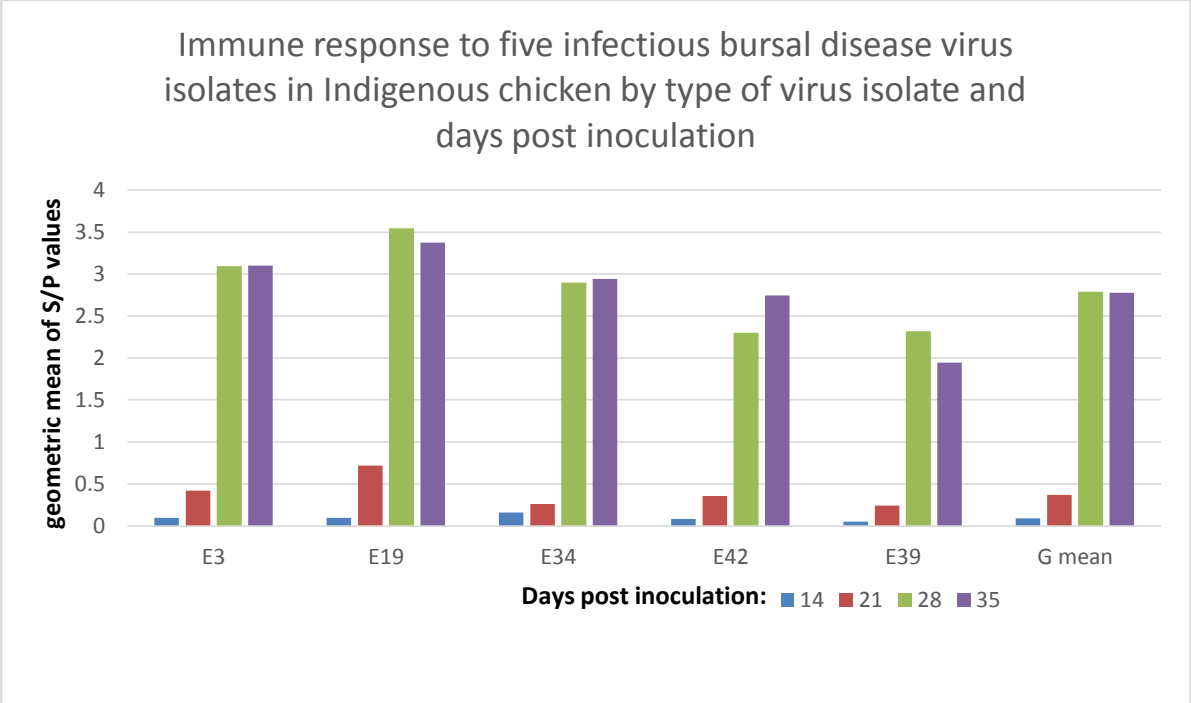


Figure 6.1: Agar gel precipitation test plate showing strong precipitation line (black arrow) between central antigen well and test sera (harvested on day 14) on peripheral wells (1 to 5). Well 6 had positive control serum

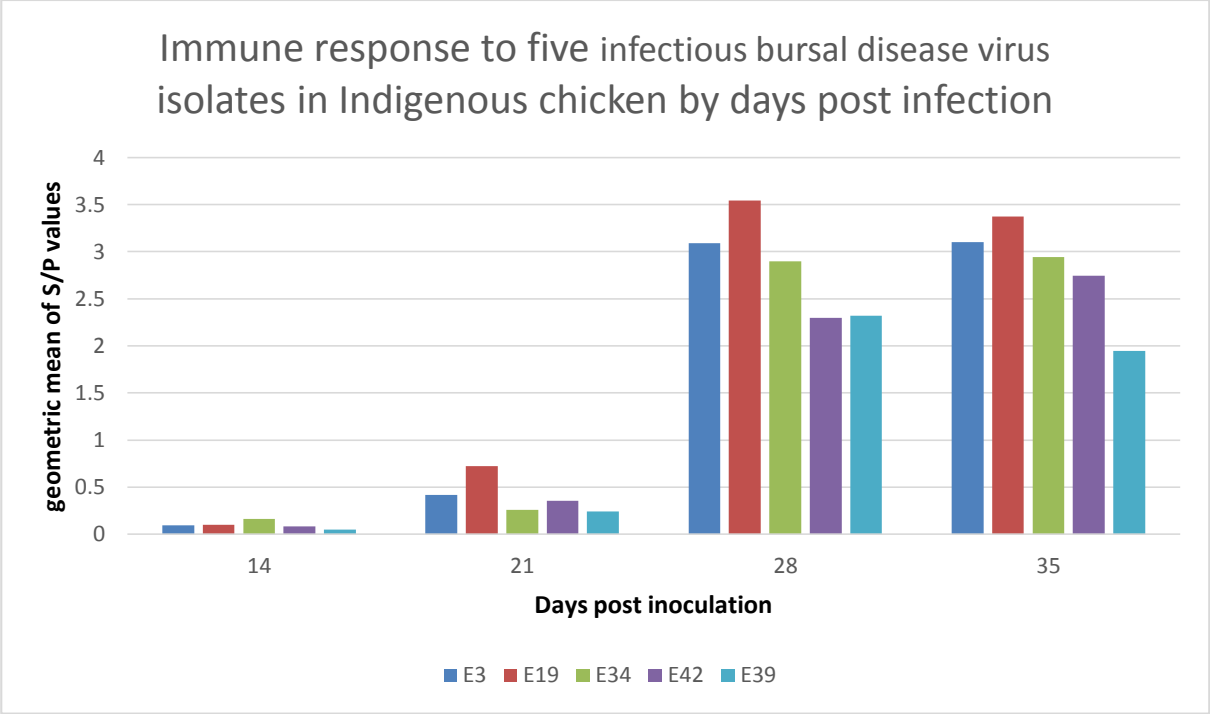
6.3.2 Detection and quantification of antibodies by enzyme linked immuno-assay test

All the samples had serum/positive (S/P) values increasing progressively post vaccine application in all the birds inoculated with various isolates. Serum collected on day 14 post 1st inoculation yielded the lowest values while those collected on days 28 and 35 yielded the highest values. Serum harvested on day 28 had the highest overall geometric mean of the S/P values as seen in **Figures 6.2, 6.3 and Table 6.1.**



Legend: S/P - sample to positive control ratio

Figure 6.2: Immune response (Serum/Positive ratio) to five infectious bursal disease virus isolates (E3, E19, E34, E42, E39) in indigenous chickens on days 14, 21, 28 and 35 post inoculation



Legend: S/P - sample to positive control ratio

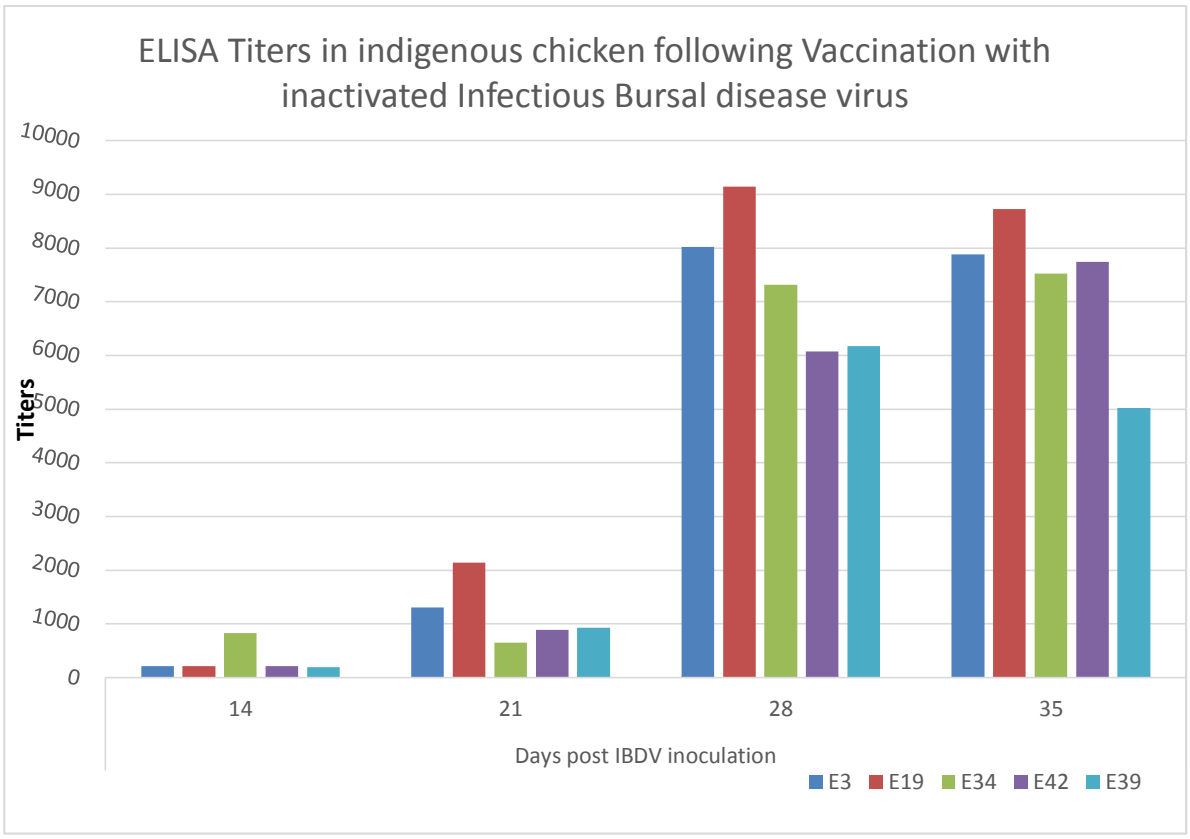
Figure: 6.3 Immune response (Serum/Positive ratio) to five infectious bursal disease virus isolates in indigenous chicken by days 14, 21, 28 and 35 post inoculation

Table 6.1: Antibody titers obtained by Enzyme immunoassay test of sera harvested on different days post first inoculation of indigenous chicks with different formalin killed viral isolates

Isolate	**Antibody titers on different days post first inoculation**			
	*Day 14	*Day 21	Day 28	Day 35
E3	208.7	1304.9▲	8023.3▲	7882.3▲
E19	209.0	2143.0▲	9140.0▲	8727.7▲
E34	831.9▲	653.4▲	7312.3▲	7529.0▲
E42	212.0	889.9▲	6069.3▲	7740.7▲
E39	189.0	928.6▲	6173.7▲	5023.0▲

Legend: * - Booster doses given on day 14 and 21, ▲- Protective titer (above 396)

The calculated ELISA titers showed that all titers posted on days 21, 28 and 35 were positive. A titer of 396 and above is deemed positive. On day 14 all the sera samples yielded titers below 396 except for isolate E34 **(Figure 6.4 and Table 6.1).** All the titers increased progressively from day 14 through day 28 then dipped on day 35 for isolate E3, E19 and E39 while a progressive increase to day 35 was noted in birds inoculated with isolate E42 and E34. The highest antibody titers were recorded in birds inoculated with isolate E19 followed by E3. These two isolates had consistently high titers throughout the experiment



Legend: ELISA: Enzyme linked immuno-sorbent assay

(Note: A titer above 396 is deemed protective)

Figure 6.4: ELISA titers in indigenous chicken following vaccination with inactivated infectious bursal disease virus isolates.

6.4 DISCUSSION

Generally, inactivated whole viruses, viral subunits or recombinant viral antigens, lack efficient immunogenicity unless they are combined with supporting adjuvants and administered in repeated injections or follow a suitable priming with a replicating antigen (Müller *et al.,* 2012). Inactivated vaccines are most efficiently used in a prime-boost regimen, using attenuated live IBDV as priming vaccine (Müller *et al.,* 2012). In this study it was demonstrated that an immune response was elicited when non primed indigenous chickens were inoculated with formalin killed IBDV local isolates without adjuvants and that response was detectable by AGPT and ELISA tests 14 days post inoculation, before a second dose was administered. Addition of adjuvant can further enhance the immune response and improve the efficiency of these vaccines and probably only one dose would be enough to immunize indigenous chickens. After repeating the dose, the titers were much higher than the 396 deemed positive (Ramadass *et al.,* 2008) and this agrees with work done by other researchers (Angani *et al.,* 2014). In Nigeria, Angani *et al.*(2014), while comparing efficacy of indigenous killed and live vaccines in Isa Brown chickens, demonstrated that an immune response elicited after administering a double dose of IBDV killed vaccine a week apart reached a titer of 893±458 on ELISA and protected the birds against challenge (Angani *et al.,* 2014). By comparison with the work of Angani *et al.* (2014), the titers obtained in this experiment on days 21, 28 and 35 were all protective. Although all the isolates were immunogenic and could be suitable vaccine candidates, isolates E19 and E3 were recommended as most suitable since they consistently yielded very high titers.

CHAPTER SEVEN: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 GENERAL DISCUSSION

This study showed that all three chicken types (layer, broiler and indigenous) in Kenya are susceptible to IBD and there is no difference in severity of outbreaks between them. This agrees with studies elsewhere in the world where indigenous chicken were also found to suffer severe outbreaks of IBD (Oluwayelu *et al.,* 2002; Chakraborty *et al.,* 2010). Findings in this study, that the highest average mortality rate was recorded in outbreaks in indigenous flocks, could be because indigenous chicks lack maternal antibodies. Indigenous village chickens are normally not immunized against IBDV presumably because they are thought to be hardy and resistant to many diseases as compared to exotic ones (Kitalyi, 1998).

Vaccination failures recorded in this study as well as in earlier studies in different parts of the world is a big challenge to the control of IBD(Islam *et al.,* 2005; Yahia *et al.,* 2008; Müller *et al.* 2012; Adamu *et al.,* 2013; Mutinda, *et al.*, 2014)). They have been attributed to different reasons, among them antigenic variation (Adamu *et al.*, 2013), as the virus has been evolving continuously in the field (Rautenschlein and Alkie1, 2016).Isolation of IBDV from local outbreaks is, thus, a step towards developing a local vaccine that would provide good protection against the local strains. Mutinda *et al.* (2015), an article published from this work, fulfilled Koch's postulate (Fredricks and Relman, 1996) on IBDV for the first time in Kenya; virus from outbreaks in chickens was isolated, repeatedly grown in embryos, and the final harvest of the virus used to induce the disease in susceptible chickens. The observation, in this study, that virus could not be isolated from 23 out of the 67 AGPT positive samples could be explained by the fact that AGPT

detects viral antigen even in samples where the virus has been inactivated (OIE 2008). Poor handling of field samples could also reduce chances of recovery of the virus even though it is known to be hardy and stable in the environment (Benton *et al.,* 1967).Variations in the embryo passage numbers leading to IBDV attenuation have been reported (Anjum *et al.,* 2010); however, in this study, all the 44 isolates were found to retain their high virulence even after 3 serial passages in indigenous chicken embryos. The selected isolates will, therefore, require further passages of, for example, between 8 and 43(Lazarus *et al.,* 2008; Yamaguchi *et al.,* 1996) to be attenuated; thus be candidates for a local vaccine.

Control of outbreaks caused by vvIBDV requires the use of highly efficacious vaccines coupled with strict bio-security measures (Müller *et al.,* 2012).Those classified as ‘‘mild’’ vaccines exhibit poor efficacy against vvIBDV; the four isolates tested for pathogenicity in this study were classified as vvIBDV strains. Emergence of vvIBDV strains is one of the reasons for vaccination failure in many parts of the world (Rautenschlein and Alkie1, 2016). In addition, Mutinda et al., (2016) showed that vaccinations done by poultry farmers in commercial broilers and breeding farms in Kenya did not yield protective antibody titers. Thus strict bio-security measures in poultry farms has to be used together with efficient live attenuated and / or inactivated vaccines to prevent IBD outbreaks in Kenya. Inactivated IBD vaccines, however, must have either a high or an optimized antigenic content in order to induce an immunity that offers protection from infection by IBDV strains (Müller *et al.,* 2012).Angani *et al.,* (2014) elicited a titer of 893±458 on ELISA in Isa Brown chickens after administering a double dose of IBDV killed vaccine a week apart; this protected the birds against challenge (Angani *et al.,* 2014). This compares well with the titers elicited by the killed vaccines in this study on days 21, 28 and 35; they were all protective. The

selected viruses were shown to be immunogenic and therefore suitable for further vaccine development.

7.2 CONCLUSIONS

1. Infectious bursal disease outbreaks occur in Kenya, causing high mortality rates in all chicken flock types: broilers, layers and indigenous chickens.
2. Outbreaks of IBD in indigenous chicken flocks tend to be more severe than in exotic flocks.
3. Outbreaks occur in vaccinated as well as in unvaccinated chicken flocks of all types; layers, broilers and indigenous.
4. Some AGPT IBDV antigen positive samples do not yield the virus at isolation, probably due to the fact that AGPT detects viral antigen even in samples where the virus has been inactivated.
5. Indigenous chicken embryos from SAN chickens support the growth of IBDV and can be used to propagate the virus, producing typical lesions. The isolates were still virulent to indigenous chickens after 3 serial passages in indigenous chicken embryos.
6. Hypervirulent pathotypes of serotype 1 IBDV are circulating in Kenya, causing disease even in vaccinated chicken flocks.
7. Four killed vaccines, developed from local strains isolated in this study elicited high antibody titers in SAN indigenous chickens and are considered to be of high efficacy.

7.3 RECOMMENDATIONS

1. Infectious bursal disease control strategies should be developed for layers, broilers and indigenous chicken flocks.

2. Vaccines and vaccination programmes should be developed targeting layers, broilers and indigenous chicken production systems in Kenya.
3. Even though IBDV virus is hardy, samples meant for virus isolation experiments must be handled cautiously to avoid inactivation of the virus.
4. Further passages of IBDV isolates in indigenous chicken embryos are recommended for development of a live attenuated vaccine from indigenous IBDV strains.
5. Molecular characterization and epidemiology of IBDV strains circulating in Kenya to determine how the virus is evolving is recommended.
6. Protection tests to determine the ability of the killed vaccines to protect chickens against IBD outbreaks in Kenya should be conducted.

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APPENDICES

APPENDIX I: CONFIRMED OUTBREAKS OF INFECTIOUS BURSAL DISEASE

COUNTY	AGE	FLOCK SIZE	MORTALITY RATE (%)
A1. VACCINATED LAYERS			
1. Nairobi	3weeks	500	20
2. Nairobi	7weeks	200	2.5
3. Nairobi	10weeks	1154	Over 80
4. Nairobi	6weeks	600	10
5. Meru	Not indicated	280	7.5
6. Kwale	4weeks	400	75
7.Kilifi	8weeks	100	60
8.Kilifi	7weeks	328	11
9. Kilifi	5weeks	350	86
10. Kiambu	3weeks	1000	20
11. Kiambu	Not indicated	500	4
12. Kiambu	4weeks	200	25
13. Kiambu	4weeks	250	60
14. Nakuru	3weeks	200	45
A2. UNVACCINATED LAYERS			
15. Nairobi	Not indicated	300	4.3
16. Taita Taveta	9weeks	300	86
17. Kericho	Not indicated	100	7
18. Nakuru	7weeks	200	35
19. Nakuru	4weeks	300	0.67
B1. VACCINATED BROILERS			
1. Kilifi	4weeks	200	7.5
2. Mombasa	6.5weeks	210	7.1
B2. UNVACCINATED BROILERS			
3. Nakuru	3weeks	500	0.4
4. Kajiado	5weeks	1100	18.9
5. Kilifi	7weeks	300	33.3
C1. VACCINATED INDIGENOUS CHICKENS			
1. Kiambu	Not indicated	180	5.5
2. Kilifi	3weeks	500	64
3. Kilifi	14weeks	100	100
4. Nakuru	6weeks	30	33.3
5. Nakuru	7weeks	37	Not indicated
6. Nakuru	4weeks	28	32.1
C2. UNVACCINATED INDIGENOUS CHICKENS			
7. Nakuru	6weeks	120	3.3

8. Nakuru	5weeks	35	Not indicated
9. Nakuru	8weeks	40	2.5
10. Nakuru	4weeks	150	1.3
11. Nakuru	4weeks	29	3.4
12. Baringo	4weeks	273	25.6
13. Kilifi	4weeks	300	58.3
14. Kwale	4weeks	8	100
15. Nairobi	7weeks	5	80

APPENDIX II: PREPARATION OF AGAR GELS AND PLATES

Eighty grams of sodium chloride and 5gms of phenol were measured and dissolved in 1 litre of distilled water. Then 12.5gms of agar was measured and added into the mixture. The whole mixture was then steamed till the agar dissolved. While still hot it was dispensed into 20ml volumes in universal bottles and refrigerated until required. To prepare agar plates the contents of one universal bottle were dissolved and poured into a Petri dish on a level surface. When set the plates were refrigerated overnight before use.

Four sets of hexagonally arranged wells were cut on the agar plate using a template and tubular cutter. Each set was composed of a central well with six peripherally arranged wells. The agar plugs from cut wells were removed using a hooked needle. The wells were 3mm in diameter and up to 6mm apart.

APPENDIX III: TITRATION OF ISOLATES USING REED AND MUENCH FORMULAE

Embryo death occurring within first 2 days after inoculation was considered non-specific and was not tallied. Those that died after day 2 were opened and lesions recorded. The total number of specific deaths in each dilution was noted and used to calculate the virus titer / infectivity by using Reed and Muench formula (1938).

Titration of isolate E19

Dilution	No. of embryos inoculated	No. infected	No. healthy	Cumulative infected	Cumulative healthy	Ratio	% mortality
Neat	5	4/4	0	23	0	23/23	100
10^{-1}	5	4/4	0	19	0	19/19	100
10^{-2}	5	5/5	0	15	0	15/15	100
10^{-3}	5	5/5	0	10	0	10/10	100
10^{-4}	5	3/4	1	5	1	5/6	83.3
10^{-5}	5	2/4	2	2	3	2/5	40
10^{-6}	5	0/5	5	0	8	0/8	0

Proportionate distance = $\frac{\text{\% mortality 50 – Next below 50}}{\text{\% mortality above 50 – Next below 50}}$

$= \frac{50 - 40}{83.3 - 40}$

$= 0.23$

EID50 = (PD X Log of dilution factor) + Log of dilution with more than 50% mortality

$= (0.23 \times 1) + 4$

$= 4.23$

$= 10^{4.23}$ /0.2mls =16982/0.2mls

Titration E34

Dilution	Embryos	Number of infected	No. of healthy	Cumulative no. of infected	Cumulative no. of healthy	Ratio	% mortality
Neat	5	5/5	0	19	0	19/19	100
10^{-1}	5	5/5	0	14	0	14/14	100
10^{-2}	5	5/5	0	9	0	9/9	100
10^{-3}	5	2/4	2	4	2	4/6	66.7
10^{-4}	5	1/3	2	2	4	2/6	33.3
10^{-5}	5	¼	3	1	7	1/8	13
10^{-6}	5	0/4	4	0	11	0/11	0

Proportionate distance = $\frac{\text{\% mortality 50 – Next below 50}}{\text{\% mortality above 50 – Next below 50}}$

$= \frac{50 - 33.3}{66.7 - 33.3}$

$=0.5$

EID50 = (PD X Log of dilution factor) + Log of dilution with more than 50% mortality

$= (0.5 \times 1) + 3$

$= 3.5$

$= 10^{3.5}$ /0.2mls = 3162/0.2mls

Titration of E3

Dilution	Embryos	Infected	No. of healthy	Cumulative no. of infected	Cumulative no. of healthy	Ratio	% mortality
Neat	5	2/2	0	14	0	14/14	100
10^{-1}	5	4/4	0	12	0	12/12	100
10^{-2}	5	4/4	0	8	0	8/8	100
10^{-3}	5	1/3	2	4	2	4/6	66.7
10^{-4}	5	2/5	3	3	5	5/8	62.5
10^{-5}	5	¼	3	1	8	1/9	11.1
10^{-6}	5	0	5	0	13	0/13	0
Total		14	13				

Proportionate distance = $\frac{\text{\% mortality 50 – Next below 50}}{\text{\% mortality above 50 – Next below 50}}$

$= \frac{50 - 11.1}{62.5 - 11.1}$

$= 0.75$

EID50 = (PD X Log of dilution factor) + Log of dilution with more than 50% mortality

$= (0.75 \times 1) + 4$

$= 4.75$

$= 10^{4.75}$ /0.2mls = 56234/0.2mls

Titration E39

Dilution	Embryos	Infected	No. of healthy	Cumulative no. of infected	Cumulative no. of healthy	Ratio	% mortality
Neat	5	3/3	0	18	0	18/18	100
10^{-1}	5	4/4	0	15	0	15/15	100
10^{-2}	5	3/4	1	11	1	11/12	91.7
10^{-3}	5	4/5	1	8	2	8/10	80
10^{-4}	5	3/5	2	4	4	4/8	50
10^{-5}	5	1/4	3	1	7	1/8	12.5
10^{-6}	5	0/5	5	0	12	0/12	0

Proportionate distance = $\frac{\text{\% mortality 50 – Next below 50}}{\text{\% mortality above 50 – Next below 50}}$

$= \frac{50 - 12.5}{80 - 12.5}$

$=0.55$

EID50 = (PD X Log of dilution factor) + Log of dilution with more than 50% mortality

$= (0.55 \times 1) + 3$

$= 3.55$

$= 10^{3.55}$ / 0.2mls = 3548/0.2mls

Titration of E42

Dilution	Embryos	Infected	No. of healthy	Cumulative no. of infected	Cumulative no. of healthy	Ratio	% mortality
Neat	5	4/4	0	17	0	17/17	100
10^{-1}	5	4/4	0	13	0	13/13	100
10^{-2}	5	4/5	1	9	1	9/10	90
10^{-3}	5	3/4	1	5	2	5/7	71
10^{-4}	5	1/2	1	2	3	2/5	40
10^{-5}	5	1/3	2	1	5	1/6	17
10^{-6}	5	0/3	3	0	8	0/8	0

Proportionate distance = $\frac{\text{\% mortality 50 – Next below 50}}{\text{\% mortality above 50 – Next below 50}}$

$= \frac{50 - 40}{71 - 40}$

$=0.32$

EID50 = (PD X Log of dilution factor) + Log of dilution with more than 50% mortality

$= (0.32x1) + 3$

$= 3.32$

$= 10^{3.32}$ / 0.2mls = 2089/ 0.2mls

Titration of E7

Dilution	embryos	Infected	No. of healthy	Cumulative no. of infected	Cumulative no. of healthy	Ratio	% Mortality
Neat	5	5/5	0	23	0	23/23	100
10-1	5	4/4	0	18	0	18/18	100
10-2	5	4/4	0	14	0	14/14	100
10-3	5	4/5	1	10	1	10/11	90.9
10-4	5	3/4	1	6	2	6/8	75
10-5	5	2/5	3	3	5	3/8	37.5
10-6	5	1/5	4	1	9	1/10	10

Proportionate distance = $\frac{\text{\% mortality 50 – Next below 50}}{\text{\% mortality above 50 – Next below 50}}$

$= \frac{50-37.5}{75-37.5} = \frac{12.5}{37.5}$

$= 0.33$

EID50 = (PD X Log of dilution factor) + Log of dilution with more than 50% mortality

$= (0.33 \times 1) + 4$

$= 4.33$

$\mathbf{= 10^{4.33}}$

Titration of E9

Dilution	Embryos	Infected	No. of healthy	Cumulative no. of infected	Cumulative no. of healthy	Ratio	% Mortality
Neat	5	4/4	0	21	0	21/21	100
10-1	5	2/2	0	17	0	17/17	100
10-2	5	5/5	0	15	0	15/15	100
10-3	5	4/5	1	10	1	10/11	90.9
10-4	5	4/5	1	6	2	6/8	75
10-5	5	1/5	4	2	6	2/8	25
10-6	5	1/5	4	1	10	1/11	9.1

Proportionate distance = $\frac{\text{\% mortality 50 – Next below 50}}{\text{\% mortality above 50 – Next below 50}}$

$= \frac{50-25}{75-25}$

$= 0.5$

EID50 = (PD X Log of dilution factor) + Log of dilution with more than 50% mortality

$= (0.5 \times 1) + 4$

$= 4.5$

$= \mathbf{10^{4.5}}$

SUMMARY OF THE TITRES FOR EACH ISOLATE

Isolate	Titer(EID_{50}per 0.2mls)
E19	$10^{4.23}$
E34	$10^{3.5}$
E3	$10^{4.75}$
E39	$10^{3.55}$
E42	$10^{3.32}$
E7	$10^{4.33}$
E9	$10^{4.5}$

APPENDIX IV: CLINICAL SIGNS SCORE AND SYMPTOMATIC INDEX FOLLOWING INOCULATION OF CHICKS

Clinical signs were quantified by symptomatic index as previously described by Le Nouën *et al* (2012) with minor modifications. Scores ranged from 0 to 3 with increasing severity, as shown in below:-

Scores	Description of clinical signs
0	lack of signs
1	typical IBD signs (ruffled feathers) conspicuous in quiet bird only, the bird stimulated by a sudden change in environment (light, noise, or vicinity of experiment observer) appears normal, motility is not reduced
2	typical IBD signs conspicuous even when bird is stimulated, dehydration is apparent, diarrhea, dullness evident and motility may be slightly reduced
3	typical severe IBD signs with prostration or death

The mean symptomatic index (MSI) scores of the surviving chickens were calculated daily and the following values obtained for each isolate:-

IBDV isolate E7 clinical signs and mortality observations

Date	Day	Score 0	Score 1	Score 2	Score 3	No. dead	Total	SYMPTOMATIC INDEX SCORE
21.8.2014	0	Inoculation						
22.8.2014	1	18				0	18	0
23.8.2014	2	0	15	3		0	18	1.2
24.8.2014	3	0	2	13	3	0	18	2.1
25.8.2014	4	0	4	6	8	6	18	2.2
26.8.2014	5	0	5	2	5	4	12	2
27.8.2014	6	0	7	0	1	1	8	1.3
28.8.2014	7	7				0	7	0
29.8.2014	8	7				0	7	0
30.8.2014	9	7				0	7	0
31.8.2014	10	7				0	7	0
1.9.2014	11	7				0	7	0
2.9.2014	12	7				0	7	0
3.9.2014	13	7				0	7	0
4.9.2014	14	7				0	7	0

Mortality rate =61.1%

IBDV isolate E19 clinical signs and mortality observations

Date	Day	Score 0	Score 1	Score 2	Score 3	No. dead	Total	SYMPTOMATIC INDEX
22.4.2014	0	Inoculation						
23.4.2014	1	18				0	18	0
24.4.2014	2	3	12	3		0	18	1
25.4.2014	3			18		0	18	2
26.4.2014	4		1	12	5	5	18	2.2
27.4.2014	5		7	6		0	13	1.5
28.4.2014	6		13			0	13	0.1
29.4.2014	7	9	4			0	13	0.3
30.4.2014	8	13				0	13	0
1.5.2014	9	13				0	13	0
2.5.2014	10	13				0	13	0
3.5.2014	11	13				0	13	0
4.5.2014	12	13				0	13	0
5.5.2014	13	13				0	13	0
6.5.2014	14	13				0	13	0

Mortality rate = 27.8%

IBDV isolate E 42 clinical signs and mortality observations

Date	Day	Score 0	Score 1	Score 2	Score 3	No. dead	Total	SYMPTOMATIC INDEX
1.7.2014	0	Inoculation						
2.7.2014	1	18				0	18	0
3.7.2014	2		18			0	18	1
4.7.2014	3		8	10		0	18	1.6
5.7.2014	4		14	1	3	3	18	1.4
6.7.2014	5	7	7	1		0	15	0.6
7.7.2014	6	14	1			0	15	0.1
8.7.2014	7	15				0	15	0
9.7.2014	8	15				0	15	0
10.7.2014	9	15				0	15	0
11.7.2014	10	15				0	15	0
12.7.2014	11	15				0	15	0
13.7.2014	12	15				0	15	0
14.7.2014	13	15				0	15	0
15.7.2014	14	15				0	15	0

Mortality rate = 16.7%

IBDV isolate E9 clinical signs and mortality observations

Date	Day	Score 0	Score 1	Score 2	Score 3	No. dead	Total	SYMPTOMATIC INDEX
21.8.2014	0	Inoculation						
22.8.2014	1	18				0	18	0
23.8.2014	2	18				0	18	0
24.8.2014	3		4	9	5	3	18	2.1
25.8.2014	4		4	4	8	7	15	2.4
26.8.2014	5		6	1	1	1	8	1.4
27.8.2014	6	1	6			0	7	0.9
28.8.2014	7	6	1			0	7	0.1
29.8.2014	8	6			1	0	7	0.4
30.8.2014	9	6			1	1	7	0.4
31.8.2014	10	6				0	6	0
1.9.2014	11	6				0	6	0
2.9.2014	12	6				0	6	0
3.9.2014	13	6				0	6	0
4.9.2014	14	6				0	6	0

Mortality rate = 66.7%

APPENDIX V: BODY WEIGHTS AND ORGAN INDEX FOR VARIOUS ORGANS FOR BIRDS INOCULATED WITH VARIOUS ISOLATES

Birds were weighed before slaughter and sampled lymphoid organs weighed immediately after slaughter. The lymphoid organs sampled (bursa, spleen, thymus, caecal tonsils and Harderian glad) were weighed separately. The weight of each organ was expressed relative to body weight of the bird for comparison and uniformity. Each organ/body weight ratio was determined by the following formula: (organ weight in grams / body weight of individual bird in grams) × 1000 (Tanimura *et al.,* 1995). Below are the average values obtained for each isolate:-

Average organ index in birds inoculated with isolate E19

	Body weight (g)	**Bursa index**	**Spleen index**	**Thymus index**	**C/T index**	**H/gland index**
Pre-testing	423	4.0	1.4	7.4	0.4	0.3
Day 1						
Control	419	5.5	1.7	7.6	0.3	0.4
Inoculated	438	5.0	1.5	6.1	0.5	0.6
Day 3						
Control	449	4.0	2.1	5.7	0.5	0.3
Inoculated	340	2.9	1.4	4.5	0.4	0.4
Day 4						
Control	465	2.8	0.8	5.9	0.5	0.7
Inoculated	361	2.9	1.5	3.9	05	0.3
Day 8						

Control	508	4.1	1.3	5.7	0.3	0.9
Inoculated	274	1.7	1.9	1.8	0.5	0.7
Day 11						
Control	510	4.8	1.9	6.8	0.5	0.4
Inoculated	529	0.9	2.3	6.0	0.4	0.6
Day14						
Control	666	3.3	1.3	5.5	0.4	0.4
Inoculated	520	0.4	1.7	5.1	0.4	0.6

Average organ index in birds inocuated with isolate E42

	Body weight (g)	**Bursa index**	**Spleen index**	**Thymus index**	**C/T index**	**H/gland index**
Pre-testing	302	3.9	1.3	5.5	0.4	0.4
Day 1						
Control	330	4	1.3	5.5	0.4	0.5
Inoculated	369	4	1.4	6.6	0.3	0.9
Day 3						
Control	369	4.8	1.8	6.9	0.6	0.5
Inoculated	319	3.1	1.8	3.3	0.6	0.9
Day 4						
Control	412	3.4	1.4	7.1	0.6	0.4
Inoculated	357	3.1	1.8	2.9	0.4	0.5

Day 8						
Control	455	3.9	1.7	6.5	0.5	0.4
Inoculated	381	1.3	2.5	3.7	0.4	0.6
Day11						
Control	391	3.4	1.8	5.6	0.4	0.2
Inoculated	412	1.0	2.5	4.7	0.4	0.4
Day 14						
Control	513	2.4	1.5	8.7	0.5	0.3
Inoculated	498	1.3	1.7	5.1	0.2	0.3

Average organ index in birds inoculated with isolate E7

	Body weight (g)	**Bursa index**	**Spleen index**	**Thymus index**	**C/T index**	**H/gland index**
Pre-testing	303	4.0	1.4	5.2	0.4	0.4
Day 1						
Control	301	3.8	1.2	4.9	0.4	0.5
Inoculated	362	4.2	1.5	8.5	0.8	0.7
Day 3						
Control	489	3.7	1.2	7.0	0.4	0.4
Inoculated	333	3.5	2.0	5.2	0.4	0.6
Day 4						
Control	516	3.7	0.8	7.1	0.4	0.3

Inoculated	329	3.1	2.4	3.6	0.5	0.4
Day 8						
Control	449	4.0	1.6	4.3	0.6	0.4
Inoculated	328	2.1	2.2	2.7	0.4	0.6
Day11						
Control	432	2.6	1.3	4.6	0.3	0.5
Inoculated	430	0.7	1.9	2.8	0.4	0.5
Day 14						
Control	516	2.3	1.5	5.1	0.3	0.5
Inoculated	436	0.8	2.2	6.1	0.3	0.4

Average organ index in birds inoculated with isolate E9

	Body weight (g)	**Bursa index**	**Spleen index**	**Thymus index**	**C/T index**	**H/gland index**
Pre-testing	303	4.0	1.4	5.2	0.4	0.4
Day 1						
Control	301	3.8	1.2	4.9	0.4	0.5
Inoculated	382	3.3	1.6	6.9	0.4	0.5
Day 3						
Control	489	3.7	1.2	7.0	0.4	0.4
Inoculated	343	3.0	1.5	4.7	0.5	0.5
Day 4						
Control	516	3.7	0.8	7.1	0.4	0.3
Inoculated	303	3.3	2.0	2.8	0.4	0.2

Day 8						
Control	449	4.0	1.6	4.3	0.6	0.4
Inoculated	379	2.1	2.1	2.2	0.3	0.4
Day11						
Control	432	2.6	1.3	4.6	0.3	0.5
Inoculated	424	0.8	2.0	5.7	0.5	0.5
Day 14						
Control	516	2.3	1.5	5.1	0.3	0.5
Inoculated	510	0.7	2.0	6.9	0.3	0.3

A summary of pre-testing values of control birds (average)

Body weight (g)	Bursal index	Splenic index	Thymic index	C/t index	H/g index
330	4.0	1.4	5.6	0.4	0.4

A summary of average body weights (g) across the days against control

Days	**Isolate E9**		**Isolate E7**		**Isolate E19**		**Isolate E42**	
	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated
1	301	382	301	362	419	438	330	369
3	489	342	489	333	449	340	369	319
4	516	303	516	329	465	361	412	357
8	449	379	449	328	508	274	455	381
11	432	424	432	430	510	529	391	412
14	516	510	516	436	666	520	513	498

APPENDIX VI: HISTOLOGY SCORES OF LYMPHOID ORGANS OF INOCULATED BIRDS PER ISOLATE

Isolate E42

Organ	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0,0,0	0,0,	3,4	4,3,	2,4,1	2,1,3	3,2,
Spleen	0,0,0	0,0,	2,2,	2,2,	3,2,0	2,1,0	0,2,
Thymus	0,0,0	0,0,	2,0,	1,	0,2,0	0,0,0	0,0
C/t	0,0,0	0,0,	1,2,	0,1,	0,2,0	0,0,0	0,0,
H/g	0,0,0	0,0,	0,0	0,0	0,0,0	0,0,0	0,0,

Averages E42

Organ	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0	0	3.5	3.5	2.3	2	2.5
Spleen	0	0	2	2	1.7	1	2
Thymus	0	0	2	1	0.7	0	0
C/t	0	0	1.5	1	0.7	0	0
H/g	0	0	0	0	0	0	0

Isolate DV 13 (E7)

Organ	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0,0,0	0	5,4,	4,5,	2,	5,1,	3,2,
Spleen	0,0,0	1	3,3,	3,1,	2,	3,0,	3,3,
Thymus	0,0,0	0	1,2,	2,1,	1,	0,0,	0,0,
C/t	0,0,0	0	2,1,	2,1,	0,	0,0,	1,0,
H/g	0,0,0	0	2,1,	0,0,	0,	0,0,	0,0,

Averages E7

Organ	Day 0	Day 1	Day 2	Day 4	Day 8	Day 11	Day 14
Bursa	0	0	4.5	4.5	2	3	2.5
Spleen	0	1	3	2	2	1.5	3
Thymus	0	0	1.5	1.5	1	0	0
C/t	0	0	1.5	1.5	0	0	0.5
H/g	0	0	1.5	0	0	0	0

Isolate E19

Organ	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0,0,0	0,0	3,4,	3,5,	3,4	1,3,3	0,3,3
Spleen	0,0,0	0,2,	3,2,	3,2,	3,1	1,2,1	3,2,1
Thymus	0,0,0	0,0,	0,1,	1,2,	0,1	0,0,0	0,0,0
C/t	0,0,0	0,0,	2,3,	2,1,	0,1	0,1,0	0,1,0
H/g	0,0,0	0,0,	0,0,	1,0	0,0	0,0,0	0,0,0

Averages E19

Organs	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0	0	3.5	4	3.5	2.3	2
Spleen	0	1	2.5	2.5	2	1.3	2
Thymus	0	0	0.5	1.5	0.5	0	0
C/t	0	0	2.5	1.5	0.5	0.3	0.3
H/g	0	0	0	0.5	0	0	0

MK 7 (E9)

Organ	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0,0,0	1,0	5,2	5,5,3	3,2,2	1,1,	3,2,4
Spleen	0,0,0	1,1	3,2	2,2,2	2,2,2	2,2,	2,3,3
Thymus	0,0,0	0,0	2,0	2,2,1	2,1,2	1,0,	0,0,0
C/t	0,0,0	0,0	3,1	3,1,1	1,0,0	0,0,	0,0,0
H/g	0,0,0	0,0	0,0	0,2,1	1,0,0	0,0,	0,0,0

Averages E9

Organ	Day 0	Day 1	Day 3	Day 4	Day 8	Day 11	Day 14
Bursa	0	0.5	3.5	4.3	2.3	1	3
Spleen	0	1	2.5	2	2	2	2.7
Thymus	0	0	1	1.7	1.7	0.5	0
C/t	0	0	2	1.7	0.3	0	0
H/g	0	0	0	1	0.3	0	0

APPENDIX VII: ELISA RESULTS FOR FORMALIN KILLED IBDV IMMUNISED BIRDS

After running ELISA and taking absorbance values at 650nm, A(650), The relative level of antibody in the sample was determined by calculating the sample to positive (S/P) ratio (Briggs et al., 1986) and the titer calculated relative to an end point titer at 1:500 dilution according to the formula given; Log_{10} Titer = $1.09(\log_{10}S/P) + 3.36$.

$$S/P = \frac{\text{Sample mean - Negative control mean}}{\text{Positive control mean - Negative control mean}}$$

Below are the S/P ratios obtained:-

ISOLATE	**BIRD NO.**	**DATE OF BLEEDING FROM 1ST INOCULATION S/P RATIO**			
		Day 35	Day 28	Day 21	Day 14
E3	1945	3.23	-	0.34	0.09
	1155	3.19	-	0.34	0.20
	1217	2.90	-	0.38	0.08
	1316	-	2.71	0.22	0.03
	1133	-	4.23	1.89	0.13
	1284	-	3.21	0.29	0.13
	1284	-	2.48		
Geometric mean		**3.103109263**	**3.090769**	**0.417514**	**0.094892**
E19	1378	3.78	-	2.55	0.32
	1352	3.76	-	0.45	0.11
	1400	2.70	-	0.66	0.03
	1795	-	3.60	0.53	0.08
	1386	-	3.96	0.37	0.11
	1362	-	3.12	0.95	0.10
Geometric mean		**3.372985**	**3.543106**	**0.721522**	**0.098785**
E34	1348	3.06	-	0.12	0.08
	1430	3.46	-	0.46	1.57
	1918	2.41	-	0.59	0.29
	1791	-	3.01	0.15	0.06
	1590	-	3.00	0.39	0.16
	1306	-	2.69	0.16	0.05
Geometric mean		**2.9440026**	**2.896099**	**0.259425**	**0.161103**
E42	30	2.23	-	0.11	0.04
	1353	3.09	-	0.46	0.04
	1942	3.06	-	0.54	0.07

	30	2.69	-	0.18	0.07
	1114	-	1.45	0.51	0.13
	28	-	3.33	0.67	0.31
	355	-	2.52	0.44	0.11
Geometric mean		**2.744317**	**2.300052**	**0.357028**	**0.085987**
E39	1390	1.22	-	0.10	0.04
	1303	2.61	-	0.33	0.02
	1513	2.31	-	0.23	0.00
	1349	-	2.44	0.16	0.02
	1414	-	1.45	0.1	0.05
	1353	-	3.52	1.59	0.33
Geometric mean		**1.944781**	**2.317929**	**0.240414**	**0.048342**
Overall Geomean		2.772760823	2.808007393	0.365126721	0.092974274

The S/P means for each isolate were:-

Isolates	**Days post inoculation**			
	14	**21**	**28**	**35**
E3	0.094892	0.417514	3.090769	3.103109263
E19	0.098785	0.721522	3.543106	3.372985
E34	0.161103	0.259425	2.896099	2.9440026
E42	0.085987	0.357028	2.300052	2.744317
E39	0.048342	0.240414	2.317929	1.944781
Overall geometric mean (of summary)	0.091108	0.367552	2.790104	2.774667
Overall Geometric mean (of gross data)	**0.092974274**	**0.365126721**	**2.808007393**	**2.772760823**

APPENDIX VIII: SPSS TWO WAY ANALYSIS OF VARIANCE (ANOVA) OUTPUT

1. ANALYSIS OF VARIANCE ON BODY WEIGHT: INTERACTION BETWEEN ISOLATE AND DAYS POST INOCULATION

```
UNIANOVA weight BY days isolate
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /SAVE=PRED RESID SRESID
  /PLOT=PROFILE(days*isolate isolate*days)
  /EMMEANS=TABLES(days*isolate)
  /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE
  /CRITERIA=ALPHA(.05)
  /DESIGN=days isolate days*isolate.
```

Univariate Analysis of Variance

Notes

Output Created		20-JAN-2016 21:32:58
Input	Data	C:\Users\Mrs Dickson\Documents\phd varables for data on isolate vs weight.sav
	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	75
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics are based on all cases with valid data for all variables in the model.
Syntax		UNIANOVA weight BY days isolate /METHOD=SSTYPE(3) /INTERCEPT=INCLUDE /SAVE=PRED RESID SRESID /PLOT=PROFILE(days*isolate isolate*days) /EMMEANS=TABLES(days*isolate) /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE /CRITERIA=ALPHA(.05) /DESIGN=days isolate days*isolate.
Resources	Processor Time	00:00:01.31
	Elapsed Time	00:00:00.53
Variables Created or Modified	PRE_2	Predicted Value for weight
	RES_2	Residual for weight
	SRE_2	Studentized Residual for weight

Between-Subject Factors

		N
days post inoculation	1	10
	3	14
	4	20
	8	11
	11	10
	14	10
name code of inoculated virus	E19	23
	E42	21
	E7	14
	E9	17

Descriptive Statistics

Dependent Variable: body weight of bird

days post inoculation	name code of inoculated virus	Mean	Std. Deviation	N
1	E19	438.0000	104.79027	3
	E42	369.3333	59.50070	3
	E7	363.0000	21.21320	2
	E9	381.5000	71.41778	2
	Total	391.1000	70.21942	10
3	E19	343.0000	58.02586	3
	E42	319.0000	112.34322	3
	E7	333.0000	72.79423	3
	E9	342.6000	126.22718	5
	Total	335.5714	90.96213	14
4	E19	365.6250	50.62449	8
	E42	357.3333	40.33691	6
	E7	354.6667	31.53305	3
	E9	342.3333	62.78004	3
	Total	358.0000	44.23859	20
8	E19	274.3333	30.89229	3
	E42	381.0000	50.23943	3
	E7	327.5000	60.10408	2
	E9	379.0000	15.13275	3
	Total	341.6364	58.18294	11
11	E19	529.0000	50.26927	3
	E42	412.3333	31.78574	3
	E7	429.5000	16.26346	2
	E9	424.0000	66.46804	2
	Total	453.1000	63.97300	10
14	E19	519.6667	109.55516	3
	E42	497.6667	115.41808	3
	E7	449.5000	19.09188	2
	E9	485.5000	70.00357	2
	Total	492.2000	83.00040	10
Total	E19	401.6087	104.73679	23
	E42	384.8571	81.52808	21
	E7	371.5714	59.15262	14
	E9	379.9412	87.99252	17
	Total	386.4000	86.43073	75

Levene's Test of Equality of Error Variances[a]

Dependent Variable: body weight of bird

F	df1	df2	Sig.
1.884	23	51	.030

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + days + isolate + days * isolate

Tests of Between-Subjects Effects

Dependent Variable: body weight of bird

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	297256.425[a]	23	12924.192	2.579	.002	.538
Intercept	10245170.539	1	10245170.539	2044.676	.000	.976
days	211389.717	5	42277.943	8.438	.000	.453
isolate	10949.210	3	3649.737	.728	.540	.041
days * isolate	56880.556	15	3792.037	.757	.716	.182
Error	255543.575	51	5010.658			
Total	11750672.000	75				
Corrected Total	552800.000	74				

a. R Squared = .538 (Adjusted R Squared = .329)

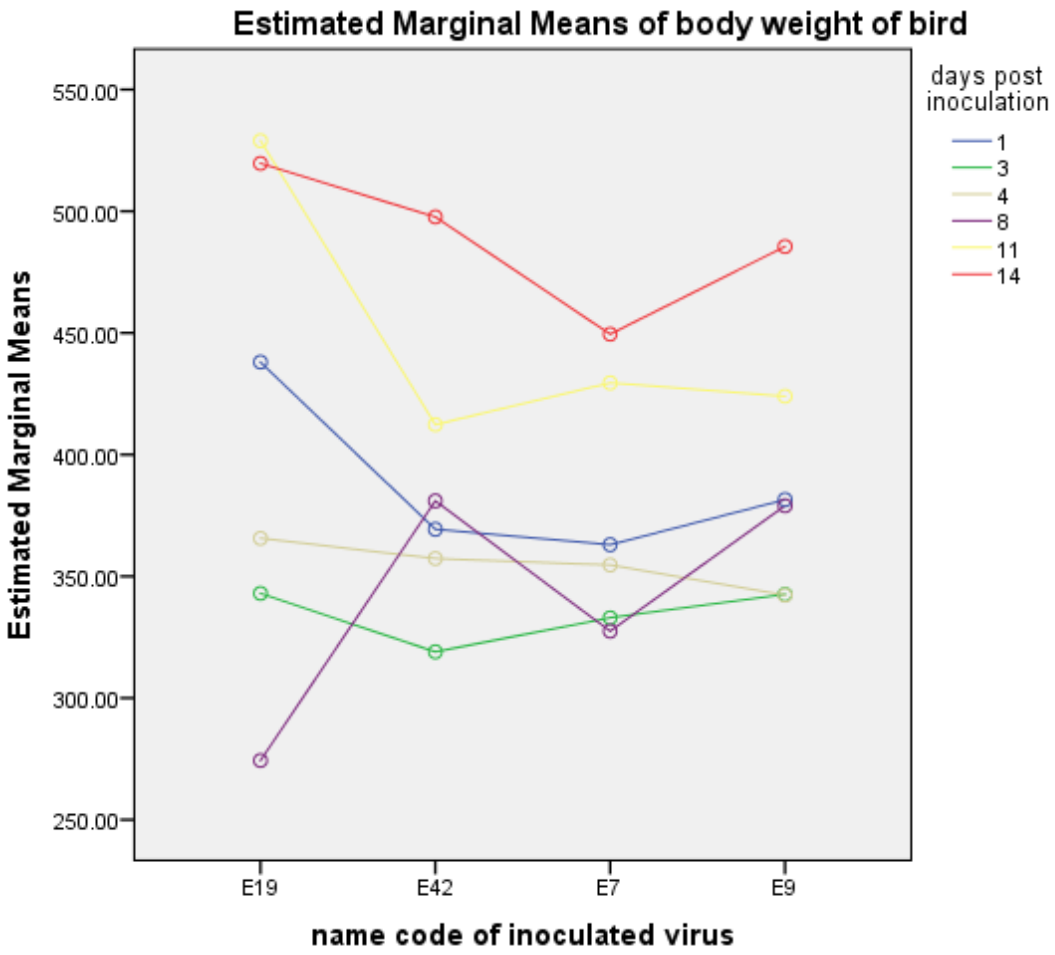
Estimated Marginal Means

days post inoculation * name code of inoculated virus

Dependent Variable: body weight of bird

days post inoculation	name code of inoculated virus	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
1	E19	438.000	40.868	355.953	520.047
	E42	369.333	40.868	287.287	451.380
	E7	363.000	50.053	262.514	463.486
	E9	381.500	50.053	281.014	481.986
3	E19	343.000	40.868	260.953	425.047
	E42	319.000	40.868	236.953	401.047
	E7	333.000	40.868	250.953	415.047
	E9	342.600	31.656	279.047	406.153
4	E19	365.625	25.027	315.382	415.868
	E42	357.333	28.898	299.318	415.349
	E7	354.667	40.868	272.620	436.713
	E9	342.333	40.868	260.287	424.380
8	E19	274.333	40.868	192.287	356.380
	E42	381.000	40.868	298.953	463.047
	E7	327.500	50.053	227.014	427.986
	E9	379.000	40.868	296.953	461.047
11	E19	529.000	40.868	446.953	611.047
	E42	412.333	40.868	330.287	494.380
	E7	429.500	50.053	329.014	529.986
	E9	424.000	50.053	323.514	524.486
14	E19	519.667	40.868	437.620	601.713
	E42	497.667	40.868	415.620	579.713
	E7	449.500	50.053	349.014	549.986
	E9	485.500	50.053	385.014	585.986

Profile Plots




```
Your license will expire in 9 days.


Your license will expire in 8 days.
```

2. ANALYSIS OF VARIANCE ON BURSAL INDEX: INTERACTION BETWEEN ISOLATE AND DAYS POST INOCULATION

```
GET
  FILE='C:\Users\Mrs Dickson\Documents\IBM\Untitled1.sav'.
DATASET NAME DataSet1 WINDOW=FRONT.
DATASET ACTIVATE DataSet1.

SAVE OUTFILE='C:\Users\Mrs Dickson\Documents\IBM\Untitled1.sav'
  /COMPRESSED.
```

```
Your temporary usage period for IBM SPSS Statistics will expire in 7 days.

GET
  FILE='C:\Users\Mrs Dickson\Documents\IBM\Untitled1.sav'.
Your license will expire in 7 days.
DATASET NAME DataSet1 WINDOW=FRONT.
UNIANOVA bf BY isolate days
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /SAVE=PRED ZRESID SRESID
  /PLOT=PROFILE(isolate*days days*isolate)
  /EMMEANS=TABLES(isolate*days)
  /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE
  /CRITERIA=ALPHA(.05)
  /DESIGN=isolate days isolate*days.
```

Univariate Analysis of Variance

Notes

Output Created		23-JAN-2016 10:25:59
Input	Data	C:\Users\Mrs Dickson\Documents\IBM\Untitled1.sav
	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	78
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics are based on all cases with valid data for all variables in the model.
Syntax		UNIANOVA bf BY isolate days /METHOD=SSTYPE(3) /INTERCEPT=INCLUDE /SAVE=PRED ZRESID SRESID /PLOT=PROFILE(isolate*days days*isolate) /EMMEANS=TABLES(isolate*days) /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE /CRITERIA=ALPHA(.05) /DESIGN=isolate days isolate*days.
Resources	Processor Time	00:00:02.50
	Elapsed Time	00:00:01.44
Variables Created or Modified	PRE_1	Predicted Value for bf
	ZRE_1	Standardized Residual for bf
	SRE_1	Studentized Residual for bf

[DataSet1] C:\Users\Mrs Dickson\Documents\IBM\Untitled1.sav

Between-Subjects Factors

		N
name code of inoculated virus	E19	24
	E42	21
	E7	16
	E9	17
days post inoculation	1	10
	3	14
	4	23
	8	11
	11	10
	14	10

Descriptive Statistics

Dependent Variable: bursal index score

name code of inoculated virus	days post inoculation	Mean	Std. Deviation	N
E19	1	5.0000	1.04403	3
	3	2.9333	.66583	3
	4	2.9222	.87146	9
	8	1.7000	.10000	3
	11	3.5667	4.71840	3
	14	.4333	.20817	3
	Total	2.8000	1.98560	24
E42	1	4.0000	.17321	3
	3	3.1000	1.30000	3
	4	3.0833	.51153	6
	8	1.2667	.45092	3
	11	1.0333	.20817	3
	14	.8667	.05774	3
	Total	2.3476	1.29716	21
E7	1	4.2000	.70711	2
	3	3.4667	.58595	3
	4	2.7600	.49800	5
	8	2.1000	.42426	2
	11	.7000	.14142	2
	14	.8500	.21213	2
	Total	2.4938	1.25404	16
E9	1	3.2500	.91924	2
	3	3.0000	.48477	5
	4	3.1000	.91652	3
	8	2.1000	.80000	3
	11	.8000	.14142	2
	14	1.2000	1.13137	2
	Total	2.4176	1.09043	17
Total	1	4.1900	.90854	10
	3	3.1071	.70325	14
	4	2.9522	.68813	23
	8	1.7636	.56617	11
	11	1.6800	2.58319	10
	14	.8000	.48990	10
	Total	2.5321	1.48425	78

Levene's Test of Equality of Error Variances[a]

Dependent Variable: bursal index score

F	df1	df2	Sig.
9.747	23	54	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + isolate + days + isolate * days

Tests of Between-Subjects Effects

Dependent Variable: bursal index score

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	102.137[a]	23	4.441	3.553	.000	.602
Intercept	387.548	1	387.548	310.072	.000	.852
isolate	3.631	3	1.210	.968	.414	.051
days	75.472	5	15.094	12.077	.000	.528
isolate * days	19.935	15	1.329	1.063	.410	.228
Error	67.493	54	1.250			
Total	669.710	78				
Corrected Total	169.630	77				

a. R Squared = .602 (Adjusted R Squared = .433)

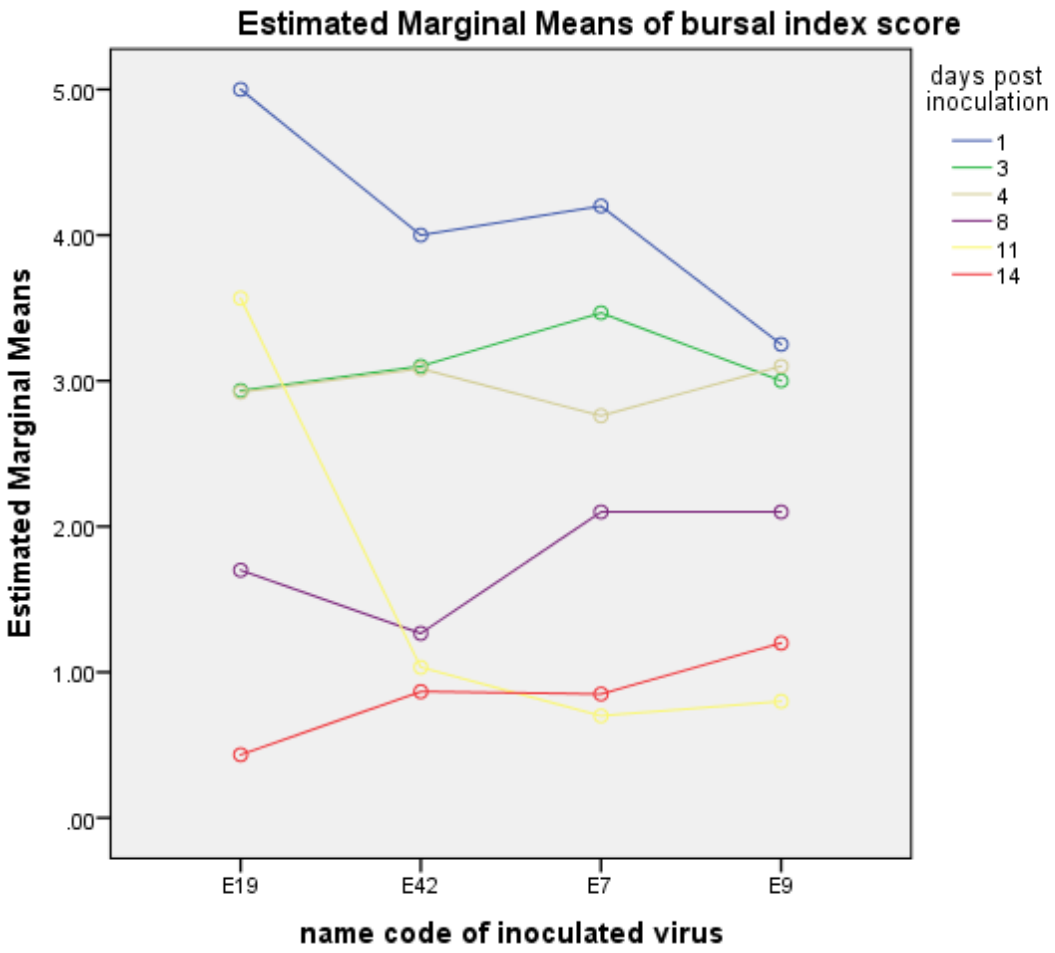
Estimated Marginal Means

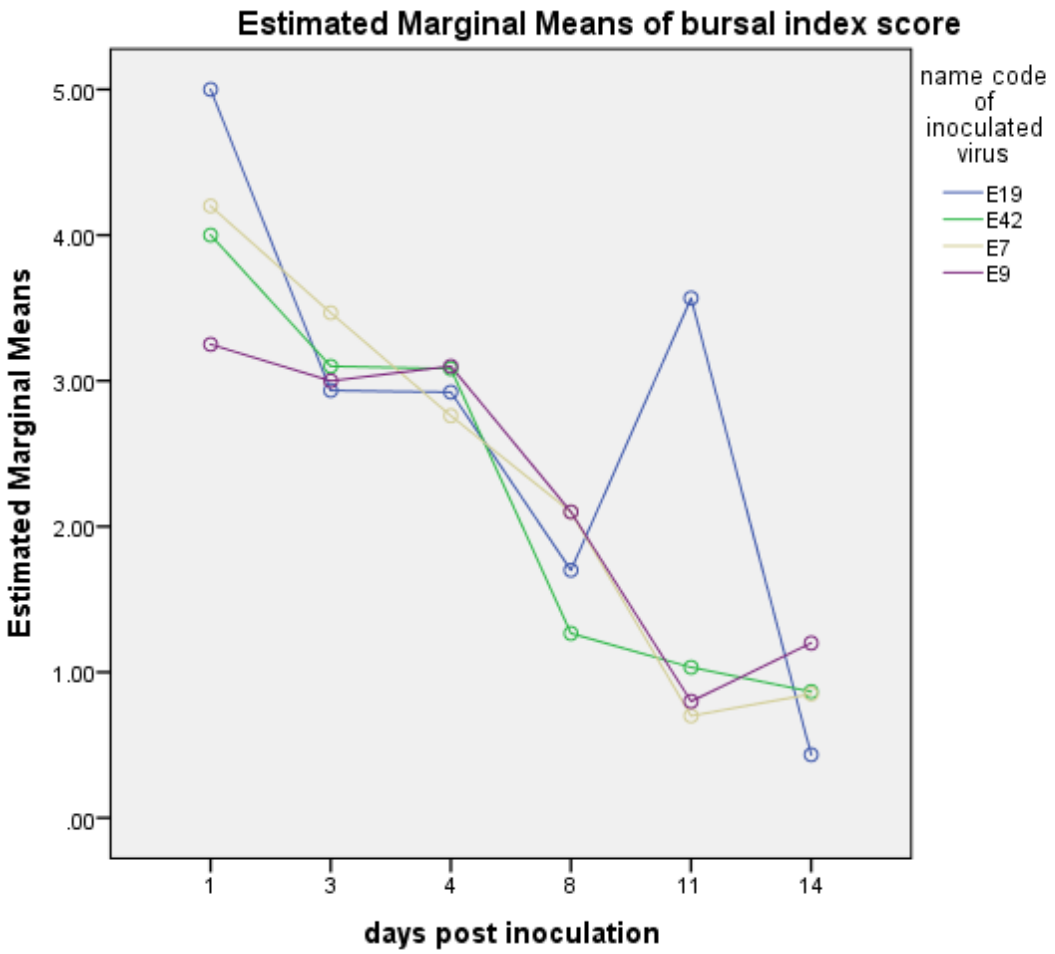
name code of inoculated virus * days post inoculation

Dependent Variable: bursal index score

name code of inoculated virus	days post inoculation	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
E19	1	5.000	.645	3.706	6.294
	3	2.933	.645	1.639	4.227
	4	2.922	.373	2.175	3.669
	8	1.700	.645	.406	2.994
	11	3.567	.645	2.273	4.861
	14	.433	.645	-.861	1.727
E42	1	4.000	.645	2.706	5.294
	3	3.100	.645	1.806	4.394
	4	3.083	.456	2.168	3.998
	8	1.267	.645	-.027	2.561
	11	1.033	.645	-.261	2.327
	14	.867	.645	-.427	2.161
E7	1	4.200	.791	2.615	5.785
	3	3.467	.645	2.173	4.761
	4	2.760	.500	1.758	3.762
	8	2.100	.791	.515	3.685
	11	.700	.791	-.885	2.285
	14	.850	.791	-.735	2.435
E9	1	3.250	.791	1.665	4.835
	3	3.000	.500	1.998	4.002
	4	3.100	.645	1.806	4.394
	8	2.100	.645	.806	3.394
	11	.800	.791	-.785	2.385
	14	1.200	.791	-.385	2.785

Profile Plots






```
Your license will expire in 7 days.
```

3. ANALYSIS OF VARIANCE ON SPLENIC INDEX: INTERACTION BETWEEN ISOLATE AND DAYS POST INOCULATION

```
GET
  FILE='C:\Users\aofu063373\Documents\SPSS SPLEEN INDEX.sav'.
DATASET NAME DataSet1 WINDOW=FRONT.
DATASET ACTIVATE DataSet1.

SAVE OUTFILE='C:\Users\aofu063373\Documents\SPSS SPLEEN INDEX.sav'
  /COMPRESSED.
UNIANOVA si BY isolate days
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /SAVE=PRED RESID SRESID
```

```
/PLOT=PROFILE(days*isolate isolate*days)
/EMMEANS=TABLES(isolate*days)
/PRINT=ETASQ HOMOGENEITY DESCRIPTIVE
/CRITERIA=ALPHA(.05)
/DESIGN=isolate days isolate*days.
```

Univariate Analysis of Variance

Notes

Output Created		04-MAR-2016 22:53:07
Comments		
Input	Data	C:\Users\aofu063373\Documents\SPSS SPLEEN INDEX.sav
	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	75
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics are based on all cases with valid data for all variables in the model.
Syntax		UNIANOVA si BY isolate days /METHOD=SSTYPE(3) /INTERCEPT=INCLUDE /SAVE=PRED RESID SRESID /PLOT=PROFILE(days*isolate isolate*days) /EMMEANS=TABLES(isolate*days) /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE /CRITERIA=ALPHA(.05) /DESIGN=isolate days isolate*days.
Resources	Processor Time	00:00:02.31
	Elapsed Time	00:00:01.17
Variables Created or Modified	PRE_1	Predicted Value for si
	RES_1	Residual for si
	SRE_1	Studentized Residual for si

[DataSet1] C:\Users\aofu063373\Documents\SPSS SPLEEN INDEX.sav

Between-Subjects Factors

		N
name code of inoculated virus	E19	23
	E42	21
	E7	14
	E9	17
number of days post inoculation	1	10
	3	14
	4	20
	8	11
	11	10
	14	10

Descriptive Statistics

Dependent Variable: splenic index

name code of inoculated virus	number of days post inoculation	Mean	Std. Deviation	N
E19	1	1.5333	.70946	3
	3	1.4667	.37859	3
	4	1.7000	.34226	8
	8	1.9333	.80208	3
	11	2.3000	.78102	3
	14	1.7333	.20817	3
	Total	1.7609	.52720	23
E42	1	1.3667	.55076	3
	3	1.8333	.11547	3
	4	1.7833	.31252	6
	8	2.4667	.70238	3
	11	2.5333	.49329	3
	14	1.7333	.37859	3
	Total	1.9286	.55058	21
E7	1	1.5000	.56569	2
	3	1.9667	.15275	3
	4	2.4333	.65064	3
	8	2.1500	.77782	2
	11	1.8500	.63640	2
	14	1.4500	.07071	2
	Total	1.9357	.54998	14
E9	1	1.6000	.00000	2
	3	1.5200	.30332	5
	4	2.0333	.11547	3
	8	2.1000	.69282	3
	11	1.9500	.07071	2
	14	.8000	.84853	2
	Total	1.6882	.54873	17
Total	1	1.4900	.47246	10
	3	1.6714	.31727	14
	4	1.8850	.42831	20
	8	2.1636	.65463	11
	11	2.2100	.56263	10
	14	1.4900	.51737	10
	Total	1.8240	.54198	75

Levene's Test of Equality of Error Variances[a]

Dependent Variable: splenic index

F	df1	df2	Sig.
2.229	23	51	.009

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + isolate + days + isolate * days

Tests of Between-Subjects Effects

Dependent Variable: splenic index

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	9.840[a]	23	.428	1.834	.036	.453
Intercept	220.931	1	220.931	947.140	.000	.949
isolate	.804	3	.268	1.149	.338	.063
days	5.449	5	1.090	4.672	.001	.314
isolate * days	3.621	15	.241	1.035	.437	.233
Error	11.896	51	.233			
Total	271.260	75				
Corrected Total	21.737	74				

a. R Squared = .453 (Adjusted R Squared = .206)

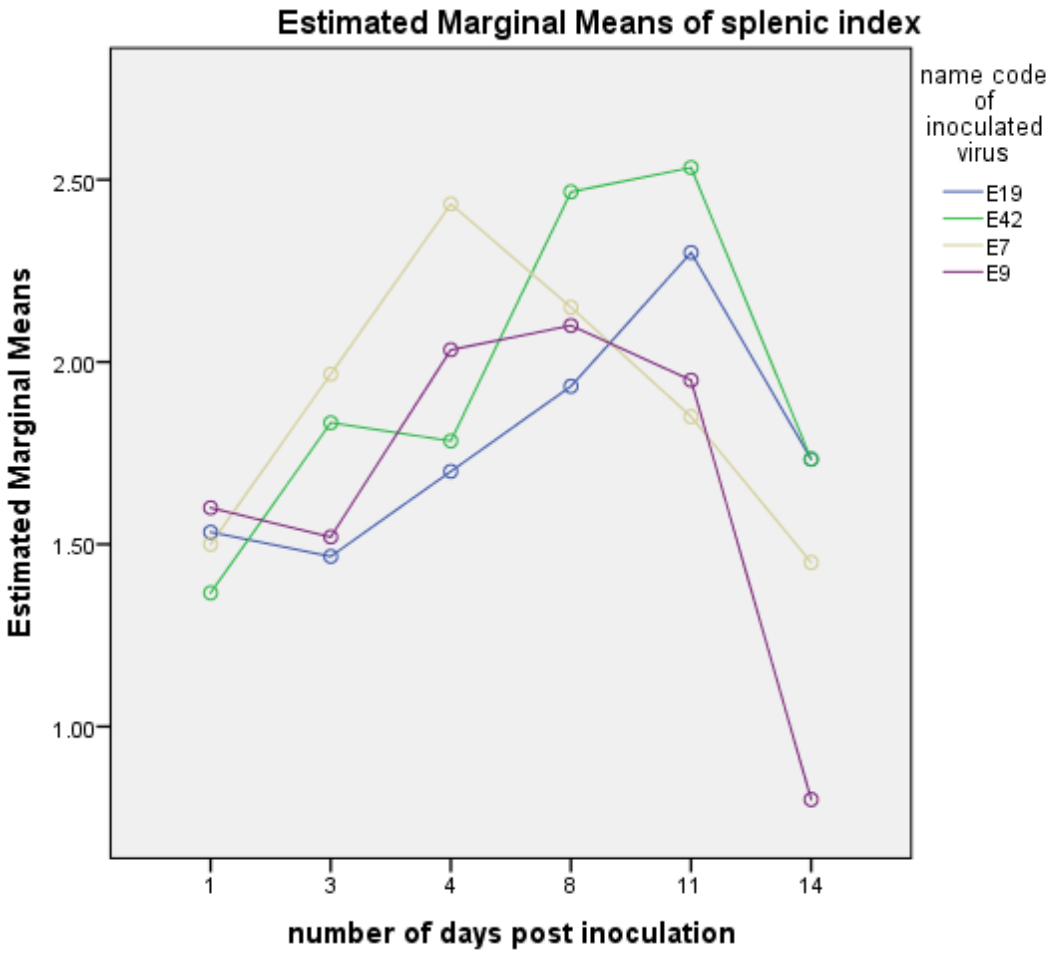
Estimated Marginal Means

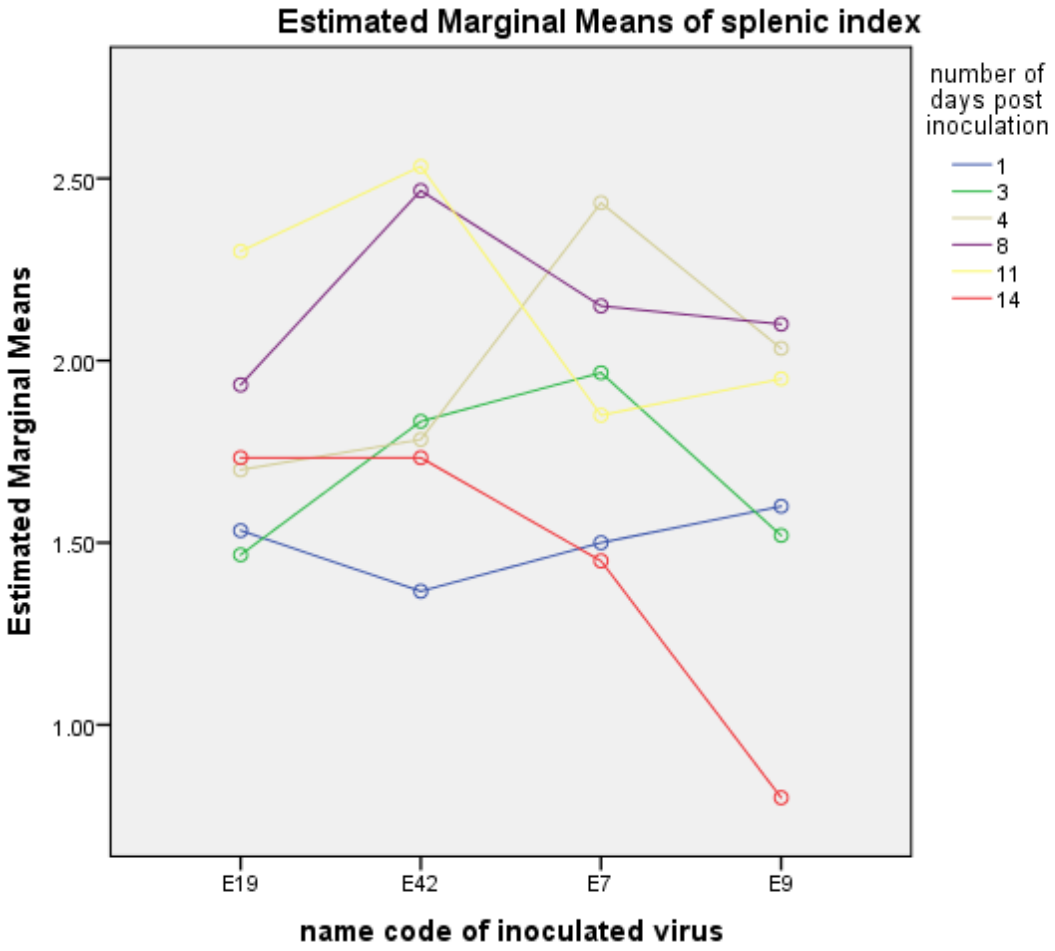
name code of inoculated virus * number of days post inoculation

Dependent Variable: splenic index

name code of inoculated virus	number of days post inoculation	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
E19	1	1.533	.279	.974	2.093
	3	1.467	.279	.907	2.026
	4	1.700	.171	1.357	2.043
	8	1.933	.279	1.374	2.493
	11	2.300	.279	1.740	2.860
	14	1.733	.279	1.174	2.293
E42	1	1.367	.279	.807	1.926
	3	1.833	.279	1.274	2.393
	4	1.783	.197	1.387	2.179
	8	2.467	.279	1.907	3.026
	11	2.533	.279	1.974	3.093
	14	1.733	.279	1.174	2.293
E7	1	1.500	.342	.814	2.186
	3	1.967	.279	1.407	2.526
	4	2.433	.279	1.874	2.993
	8	2.150	.342	1.464	2.836
	11	1.850	.342	1.164	2.536
	14	1.450	.342	.764	2.136
E9	1	1.600	.342	.914	2.286
	3	1.520	.216	1.086	1.954
	4	2.033	.279	1.474	2.593
	8	2.100	.279	1.540	2.660
	11	1.950	.342	1.264	2.636
	14	.800	.342	.114	1.486

Profile Plots





4. ANALYSIS OF VARIANCE ON THYMIC INDEX: INTERACTION BETWEEN ISOLATE AND DAYS POST INOCULATION

```
UNIANOVA ti BY isolate days
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /SAVE=PRED RESID SRESID
  /PLOT=PROFILE(days*isolate isolate*days)
  /EMMEANS=TABLES(isolate*days)
  /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE
  /CRITERIA=ALPHA(.05)
  /DESIGN=isolate days isolate*days.
```

Univariate Analysis of Variance

Notes

Output Created		04-MAR-2016 23:07:24
Comments		
Input	Data	C:\Users\aofu063373\Documents\SPSS SPLEEN INDEX.sav
	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	75
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics are based on all cases with valid data for all variables in the model.
Syntax		UNIANOVA ti BY isolate days /METHOD=SSTYPE(3) /INTERCEPT=INCLUDE /SAVE=PRED RESID SRESID /PLOT=PROFILE(days*isolate isolate*days) /EMMEANS=TABLES(isolate*days) /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE /CRITERIA=ALPHA(.05) /DESIGN=isolate days isolate*days.
Resources	Processor Time	00:00:01.22
	Elapsed Time	00:00:00.32
Variables Created or Modified	PRE_2	Predicted Value for ti
	RES_2	Residual for ti
	SRE_2	Studentized Residual for ti

Between-Subjects Factors

		N
name code of inoculated virus	E19	23
	E42	21
	E7	14
	E9	17
number of days post inoculation	1	10
	3	14
	4	20
	8	11
	11	10
	14	10

Descriptive Statistics

Dependent Variable: thymic index

name code of inoculated virus	number of days post inoculation	Mean	Std. Deviation	N
E19	1	6.1000	1.27671	3
	3	4.5333	.85049	3
	4	4.1125	1.26879	8
	8	1.7667	.05774	3
	11	6.0333	3.65285	3
	14	5.1000	.62450	3
	Total	4.5000	1.94095	23
E42	1	6.6000	2.00749	3
	3	3.2667	.55076	3
	4	2.9000	.74027	6
	8	3.6667	1.78979	3
	11	4.7333	1.24231	3
	14	5.0667	.32146	3
	Total	4.1619	1.65936	21
E7	1	8.4500	2.89914	2
	3	5.2333	1.20554	3
	4	3.5667	.49329	3
	8	2.6500	.07071	2
	11	2.8000	.28284	2
	14	6.1000	.42426	2
	Total	4.7429	2.22597	14
E9	1	6.0000	3.53553	2
	3	4.7200	1.51228	5
	4	2.7667	.65064	3
	8	2.2333	.20817	3
	11	5.6500	2.19203	2
	14	5.3000	3.11127	2
	Total	4.2647	2.07995	17
Total	1	6.7000	2.11975	10
	3	4.4786	1.25648	14
	4	3.4650	1.07863	20
	8	2.5727	1.11363	11
	11	4.9200	2.32226	10
	14	5.3300	1.17384	10
	Total	4.3973	1.92761	75

Levene's Test of Equality of Error Variances[a]

Dependent Variable: thymic index

F	df1	df2	Sig.
3.582	23	51	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + isolate + days + isolate * days

Tests of Between-Subjects Effects

Dependent Variable: thymic index

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	161.274[a]	23	7.012	3.146	.000	.587
Intercept	1381.009	1	1381.009	619.531	.000	.924
isolate	1.694	3	.565	.253	.859	.015
days	119.998	5	24.000	10.766	.000	.514
isolate * days	39.613	15	2.641	1.185	.313	.258
Error	113.685	51	2.229			
Total	1725.200	75				
Corrected Total	274.959	74				

a. R Squared = .587 (Adjusted R Squared = .400)

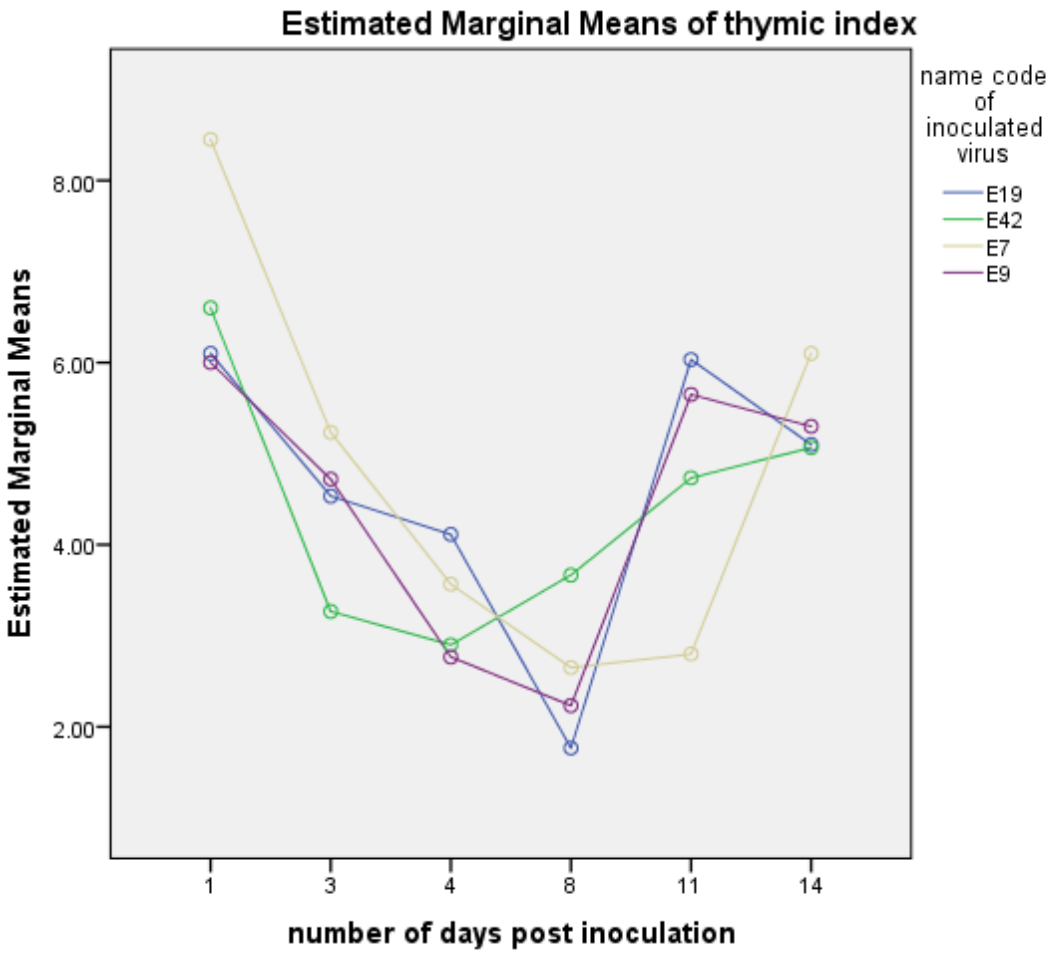
Estimated Marginal Means

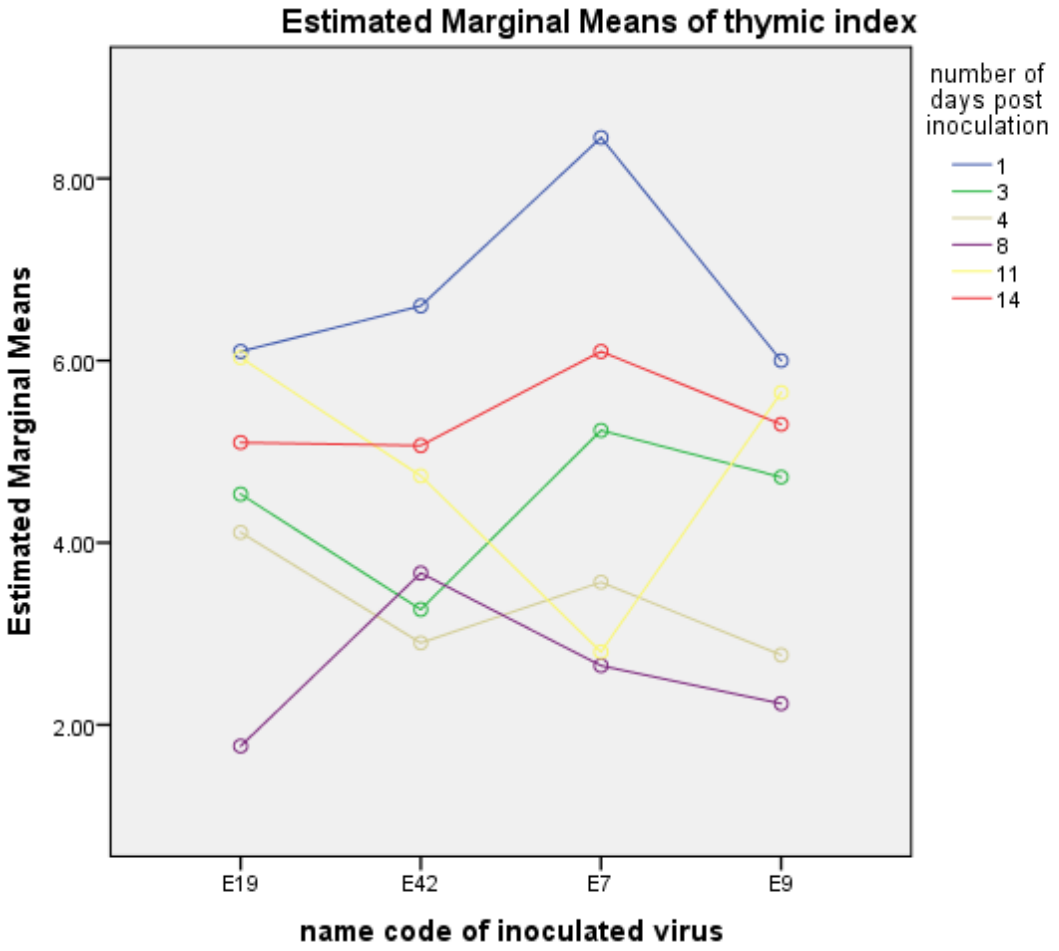
name code of inoculated virus * number of days post inoculation

Dependent Variable: thymic index

name code of inoculated virus	number of days post inoculation	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
E19	1	6.100	.862	4.369	7.831
	3	4.533	.862	2.803	6.264
	4	4.112	.528	3.053	5.172
	8	1.767	.862	.036	3.497
	11	6.033	.862	4.303	7.764
	14	5.100	.862	3.369	6.831
E42	1	6.600	.862	4.869	8.331
	3	3.267	.862	1.536	4.997
	4	2.900	.610	1.676	4.124
	8	3.667	.862	1.936	5.397
	11	4.733	.862	3.003	6.464
	14	5.067	.862	3.336	6.797
E7	1	8.450	1.056	6.331	10.569
	3	5.233	.862	3.503	6.964
	4	3.567	.862	1.836	5.297
	8	2.650	1.056	.531	4.769
	11	2.800	1.056	.681	4.919
	14	6.100	1.056	3.981	8.219
E9	1	6.000	1.056	3.881	8.119
	3	4.720	.668	3.380	6.060
	4	2.767	.862	1.036	4.497
	8	2.233	.862	.503	3.964
	11	5.650	1.056	3.531	7.769
	14	5.300	1.056	3.181	7.419

Profile Plots





5. ANALYSIS OF VARIANCE ON CAECAL TONSILS INDEX: INTERACTION BETWEEN ISOLATE AND DAYS POST INOCULATION

```
GET
  FILE='C:\Users\aofu063373\Documents\SPSS SPLEEN INDEX.sav'.
DATASET NAME DataSet1 WINDOW=FRONT.
UNIANOVA ci BY isolate days
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /SAVE=PRED RESID SRESID
  /PLOT=PROFILE(days*isolate isolate*days)
  /EMMEANS=TABLES(isolate*days)
  /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE
  /CRITERIA=ALPHA(.05)
  /DESIGN=isolate days isolate*days.
```

Univariate Analysis of Variance

Notes

Output Created		05-MAR-2016 08:34:04
Comments		
Input	Data	C:\Users\aofu063373\Documents\SPSS SPLEEN INDEX.sav
	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	75
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics are based on all cases with valid data for all variables in the model.
Syntax		UNIANOVA ci BY isolate days /METHOD=SSTYPE(3) /INTERCEPT=INCLUDE /SAVE=PRED RESID SRESID /PLOT=PROFILE(days*isolate isolate*days) /EMMEANS=TABLES(isolate*days) /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE /CRITERIA=ALPHA(.05) /DESIGN=isolate days isolate*days.
Resources	Processor Time	00:00:02.22
	Elapsed Time	00:00:01.13
Variables Created or Modified	PRE_3	Predicted Value for ci
	RES_3	Residual for ci
	SRE_3	Studentized Residual for ci

[DataSet1] C:\Users\aofu063373\Documents\SPSS SPLEEN INDEX.sav

Between-Subjects Factors

		N
name code of inoculated virus	E19	23
	E42	21
	E7	14
	E9	17
number of days post inoculation	1	10
	3	14
	4	20
	8	11
	11	10
	14	10

Descriptive Statistics

Dependent Variable: caecal tonsils index

name code of inoculated virus	number of days post inoculation	Mean	Std. Deviation	N
E19	1	.5000	.17321	3
	3	.4333	.05774	3
	4	.4625	.20659	8
	8	.5000	.10000	3
	11	.4000	.00000	3
	14	.4000	.17321	3
	Total	.4522	.14731	23
E42	1	.3333	.05774	3
	3	.6333	.15275	3
	4	.4000	.16733	6
	8	.4000	.10000	3
	11	.3667	.11547	3
	14	.2333	.11547	3
	Total	.3952	.16272	21
E7	1	.7500	.49497	2
	3	.4333	.05774	3
	4	.5000	.10000	3
	8	.4000	.14142	2
	11	.3500	.07071	2
	14	.3000	.14142	2
	Total	.4571	.21018	14
E9	1	.4000	.14142	2
	3	.4800	.13038	5
	4	.4000	.00000	3
	8	.3333	.05774	3
	11	.5000	.00000	2
	14	.3500	.07071	2
	Total	.4176	.10146	17
Total	1	.4800	.24855	10
	3	.4929	.12688	14
	4	.4400	.16026	20
	8	.4091	.10445	11
	11	.4000	.08165	10
	14	.3200	.13166	10
	Total	.4293	.15577	75

Levene's Test of Equality of Error Variances[a]

Dependent Variable: caecal tonsils index

F	df1	df2	Sig.
2.973	23	51	.001

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + isolate + days + isolate * days

Tests of Between-Subjects Effects

Dependent Variable: caecal tonsils index

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.667[a]	23	.029	1.311	.208	.372
Intercept	12.156	1	12.156	549.402	.000	.915
isolate	.046	3	.015	.691	.562	.039
days	.225	5	.045	2.037	.089	.166
isolate * days	.399	15	.027	1.203	.300	.261
Error	1.128	51	.022			
Total	15.620	75				
Corrected Total	1.795	74				

a. R Squared = .372 (Adjusted R Squared = .088)

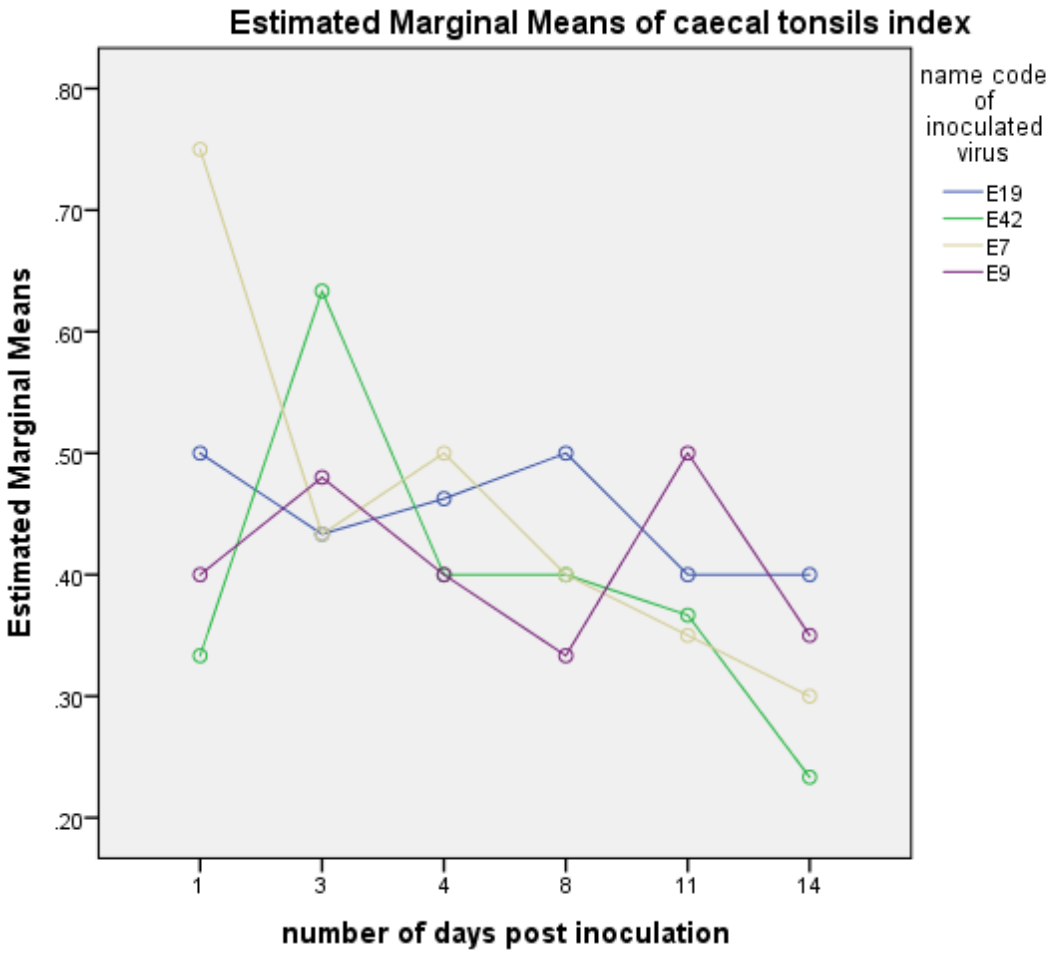
Estimated Marginal Means

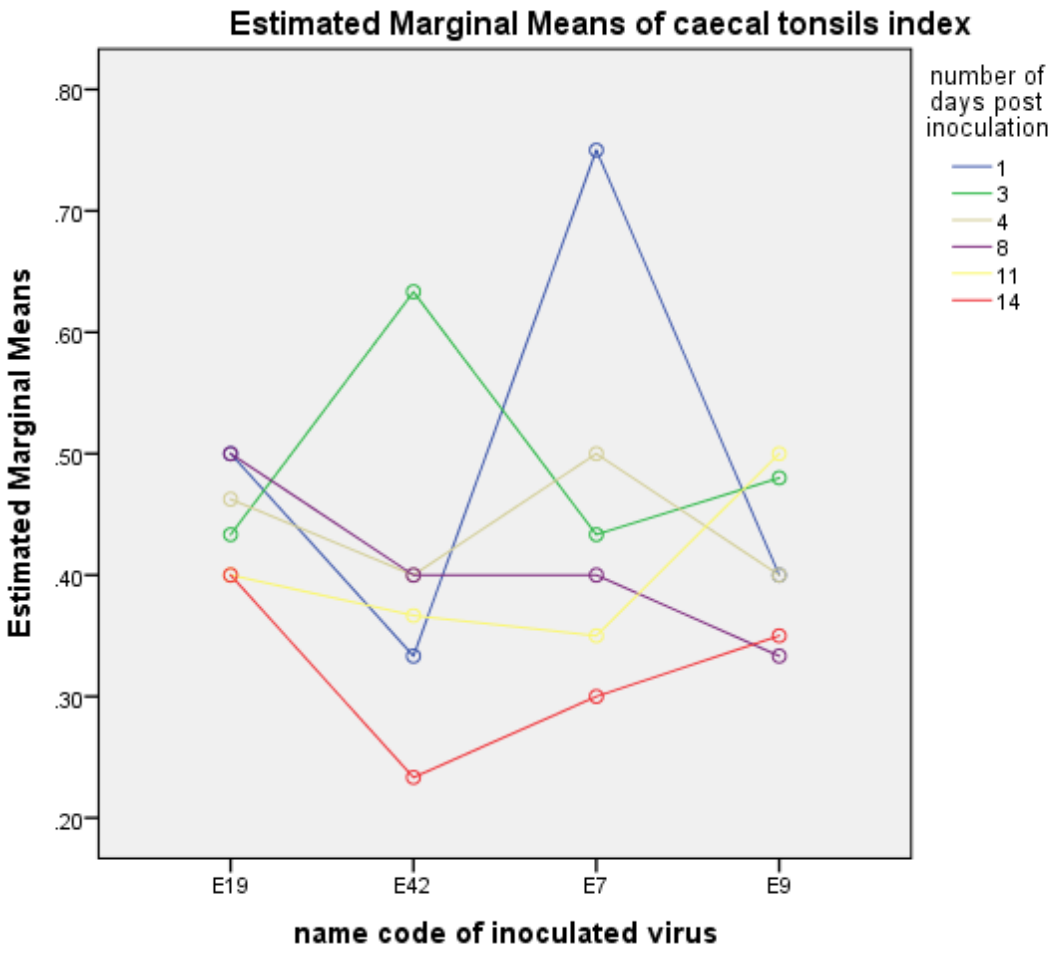
name code of inoculated virus * number of days post inoculation

Dependent Variable: caecal tonsils index

name code of inoculated virus	number of days post inoculation	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
E19	1	.500	.086	.328	.672
	3	.433	.086	.261	.606
	4	.462	.053	.357	.568
	8	.500	.086	.328	.672
	11	.400	.086	.228	.572
	14	.400	.086	.228	.572
E42	1	.333	.086	.161	.506
	3	.633	.086	.461	.806
	4	.400	.061	.278	.522
	8	.400	.086	.228	.572
	11	.367	.086	.194	.539
	14	.233	.086	.061	.406
E7	1	.750	.105	.539	.961
	3	.433	.086	.261	.606
	4	.500	.086	.328	.672
	8	.400	.105	.189	.611
	11	.350	.105	.139	.561
	14	.300	.105	.089	.511
E9	1	.400	.105	.189	.611
	3	.480	.067	.346	.614
	4	.400	.086	.228	.572
	8	.333	.086	.161	.506
	11	.500	.105	.289	.711
	14	.350	.105	.139	.561

Profile Plots





1. ANALYSIS OF VARIANCE ON HARDERIAN GLAND INDEX: INTERACTION BETWEEN ISOLATE AND DAYS POST INOCULATION

```
UNIANOVA hi BY isolate days
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /SAVE=PRED RESID SRESID
  /PLOT=PROFILE(days*isolate isolate*days)
  /EMMEANS=TABLES(isolate*days)
  /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE
  /CRITERIA=ALPHA(.05)
  /DESIGN=isolate days isolate*days.
```

Univariate Analysis of Variance

Notes

Output Created		05-MAR-2016 08:44:44
Comments		
Input	Data	C:\Users\aofu063373\Documents\SPSS SPLEEN INDEX.sav
	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	75
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics are based on all cases with valid data for all variables in the model.
Syntax		UNIANOVA hi BY isolate days /METHOD=SSTYPE(3) /INTERCEPT=INCLUDE /SAVE=PRED RESID SRESID /PLOT=PROFILE(days*isolate isolate*days) /EMMEANS=TABLES(isolate*days) /PRINT=ETASQ HOMOGENEITY DESCRIPTIVE /CRITERIA=ALPHA(.05) /DESIGN=isolate days isolate*days.
Resources	Processor Time	00:00:01.09
	Elapsed Time	00:00:00.31
Variables Created or Modified	PRE_4	Predicted Value for hi
	RES_4	Residual for hi
	SRE_4	Studentized Residual for hi

Between-Subjects Factors

		N
name code of inoculated virus	E19	23
	E42	21
	E7	14
	E9	17
number of days post inoculation	1	10
	3	14
	4	20
	8	11
	11	10
	14	10

Descriptive Statistics

Dependent Variable: Harderian gland index

name code of inoculated virus	number of days post inoculation	Mean	Std. Deviation	N
E19	1	.5667	.15275	3
	3	.4333	.11547	3
	4	.3750	.12817	8
	8	.6667	.30551	3
	11	.3667	.05774	3
	14	.5667	.20817	3
	Total	.4696	.18448	23
E42	1	.9000	.60828	3
	3	.6667	.15275	3
	4	.4833	.31252	6
	8	.5667	.30551	3
	11	.4333	.05774	3
	14	.3000	.10000	3
	Total	.5476	.32805	21
E7	1	.7000	.00000	2
	3	.6333	.11547	3
	4	.3667	.05774	3
	8	.5500	.07071	2
	11	.4500	.07071	2
	14	.3500	.07071	2
	Total	.5071	.14917	14
E9	1	.4500	.07071	2
	3	.5200	.13038	5
	4	.2333	.05774	3
	8	.3667	.28868	3
	11	.4500	.07071	2
	14	.2500	.07071	2
	Total	.3941	.17128	17
Total	1	.6700	.34657	10
	3	.5571	.14525	14
	4	.3850	.19808	20
	8	.5364	.26181	11
	11	.4200	.06325	10
	14	.3800	.17512	10
	Total	.4813	.22939	75

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Harderian gland index

F	df1	df2	Sig.
2.801	23	51	.001

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + isolate + days + isolate * days

Tests of Between-Subjects Effects

Dependent Variable: Harderian gland index

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1.633[a]	23	.071	1.601	.082	.419
Intercept	15.662	1	15.662	353.224	.000	.874
isolate	.287	3	.096	2.154	.105	.112
days	.794	5	.159	3.579	.008	.260
isolate * days	.529	15	.035	.796	.677	.190
Error	2.261	51	.044			
Total	21.270	75				
Corrected Total	3.894	74				

a. R Squared = .419 (Adjusted R Squared = .157)

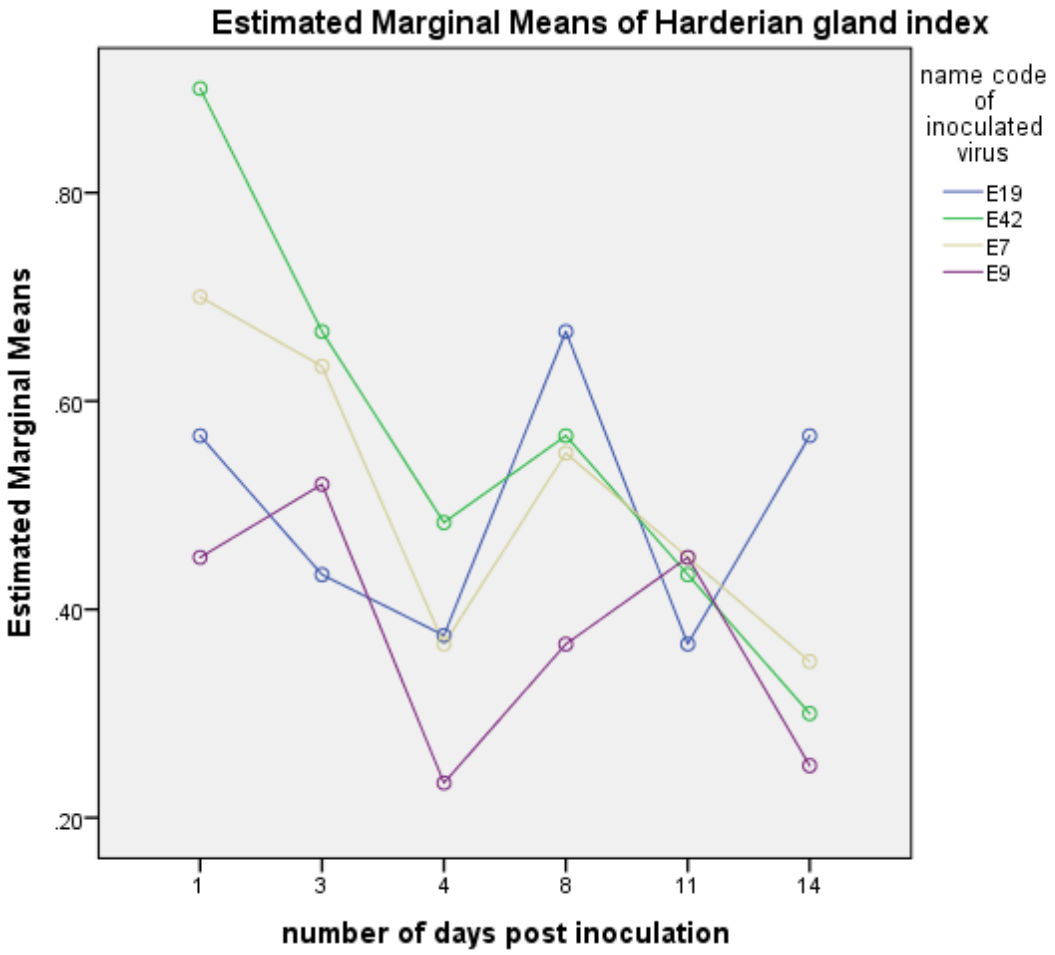
Estimated Marginal Means

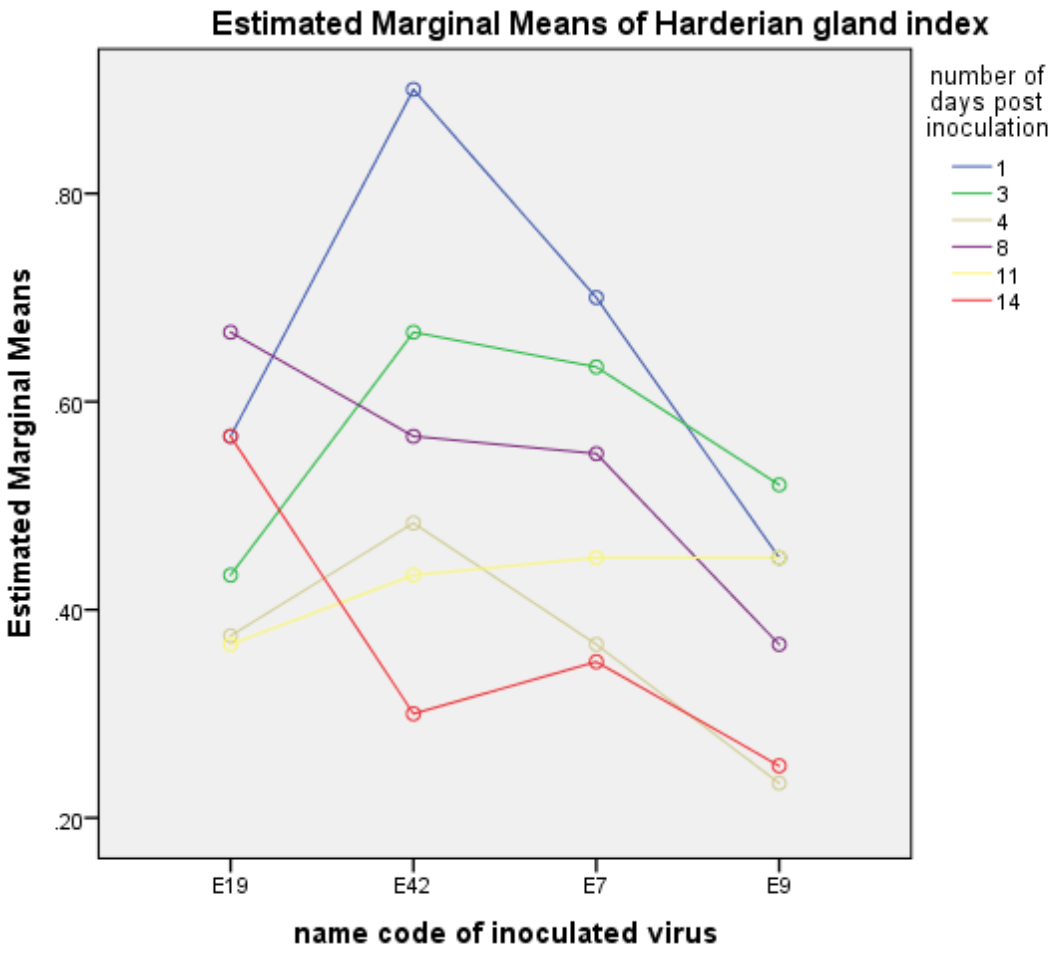
name code of inoculated virus * number of days post inoculation

Dependent Variable: Harderian gland index

name code of inoculated virus	number of days post inoculation	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
E19	1	.567	.122	.323	.811
	3	.433	.122	.189	.677
	4	.375	.074	.226	.524
	8	.667	.122	.423	.911
	11	.367	.122	.123	.611
	14	.567	.122	.323	.811
E42	1	.900	.122	.656	1.144
	3	.667	.122	.423	.911
	4	.483	.086	.311	.656
	8	.567	.122	.323	.811
	11	.433	.122	.189	.677
	14	.300	.122	.056	.544
E7	1	.700	.149	.401	.999
	3	.633	.122	.389	.877
	4	.367	.122	.123	.611
	8	.550	.149	.251	.849
	11	.450	.149	.151	.749
	14	.350	.149	.051	.649
E9	1	.450	.149	.151	.749
	3	.520	.094	.331	.709
	4	.233	.122	-.011	.477
	8	.367	.122	.123	.611
	11	.450	.149	.151	.749
	14	.250	.149	-.049	.549

Profile Plots




Estimated Marginal Means of Harderian gland index
Estimated Marginal Means
.80
.60
.40
.20
E19
E42
E7
E9
name code of inoculated virus
number of days post inoculation
1
3
4
8
11
14

APPENDIX IX: PUBLISHED ARTICLES

Two articles have been published out of the work done in this study.

1. Isolation of Infectious Bursal Disease Virus Using Indigenous Chicken Embryos in Kenya by Mutinda, W. U. , Njagi, L. W., Nyaga, P. N., Bebora, L. C., Mbuthia, P. G.,Kemboi, D. Githinji, J. W. K., Muriuki, A. in International Scholarly Research Notices. Volume 2015 (2015), Article ID 464376, 7 pages. http://dx.doi.org/10.1155/2015/464376

2. Gumboro Disease Outbreaks Cause High Mortality Rates in Indigenous Chickens in Kenya by Mutinda, W U., Nyaga, P N Njagi, L W Bebora, L C., Mbuthia P .G.. in Bulletin of Animal Health and production. Volume 61, No 4 (2013) pages 571-578