EFFECT OF LEVAMISOLE ON HUMORAL IMMUNE RESPONSE TO NEWCASTLE DISEASE VACCINE IN INFECTIOUS BURSAL DISEASE VACCINATED BROILERS

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FEBRUARY, 2009

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**DEPARTMENT OF VETERINARY SURGERY AND MEDICINE,
FACULTY OF VETERINARY MEDICINE,
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FEBRUARY, 2009

DECLARATION

I declare that the work in the thesis entitled 'Effect of levamisole on humoral immune response to Newcastle disease vaccine in infectious bursal disease vaccinated broilers' has been performed by me in the Department of Veterinary Surgery and Medicine under the supervision of Prof. P.A. Abdu, Prof. E.O. Gyang and Dr. H.M. Kazeem.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any University.

Adamu, Jibril

Name of Student	**Signature**	**Date**

CERTIFICATION

This thesis entitled "EFFECT OF LEVAMISOLE ON HUMORAL IMMUNE RESPONSE TO NEWCASTLE DISEASE VACCINE IN INFECTIOUS BURSAL DISEASE VACCINATED BROILERS" by Adamu, Jibril meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to whoever believes in God and support the progress of his/her community by sharing knowledge and the little he/she has of resources, eagerly.

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ABSTRACT

Foreign infectious bursal disease (IBD) vaccines imported into Nigeria are usually live attenuated intermediate or intermediate plus IBD virus strains and often associated with immunosppression in vaccinated birds. The use of levamisole to potentiate humoral immune response to vaccination against Newcastle disease (ND) in Ross broilers vaccinated with an intermediate strain of IBD vaccine was evaluated. Levamisole at 2.5 and 5 mg/kg dose was injected into the crop of broilers every other day for one week at 16, 30 or 62 days of age when IBD and ND vaccines were administered at 16 and 30, 30 and 44 days of age or challenge with ND virus and not administered IBD or ND vaccines at 62 days of age respectively. Control birds were not injected with levamisole. Antibodies against ND and IBD viruses were determined weekly by hemagglutination inhibition (HI) and agar gel immunodifussion (AGID) tests respectively. Other parameters determined include, packed cell volume (PCV) and total protein levels at 62 days of age; body weight, organ to body weight ratios for the bursa, spleen and thymus which were also examined by histopathology at 69 days of age. All sera tested for precipitin antibodies, following IBD vaccination were negative. High, HI mean ND virus antibody titers above 3 $\log_2$ were detected by 14 days of age but were low or absent at 35 days of age irrespective of administration of levamisole, IBD and ND vaccines. High antibody titers were obtained with 5 mg/kg levamisole dose. Broilers administered 5 mg/kg levamisole and IBD vaccine at 16 days of age had the highest mean $\log_2$ antibody titers at 21 (1.74 ± 0.40), 35 (0.95 ± 0.62) and 49 (4.56 ± 0.66) days of age with statistical significant difference at 42 and 49 ($P< 0.001$) days of age. Flock immunity against ND was observed at 28 and 40 days post administration of La Sota and day-old ND vaccination respectively. Broilers challenged

with ND virus at 62 days of age were protected. Levamisole had no significant effect on body weight and total protein of broilers ($P > 0.05$). Broilers administered La Sota vaccine and levamisole had lower mean PCV than those not vaccinated with La Sota and the difference in PCV was statistically significant ($P < 0.001$). Stimulation of the bursa, spleen and thymus was observed with 5 mg/kg levamisole dose. In older broilers 2.5 mg/kg levamisole dose had a stronger immunopotentiative effect on the bursa and spleen. For effective management of immonosuppresive effect of intermediate or intermediate plus IBD vaccine, poultry farmers should administer 5 mg/kg levamisole in drinking water every other day for one week, following vaccination with IBD vaccine at two weeks. There is the need to investigate the mechanisms involved in the age related stimulation of the bursa and spleen as well as the decrease in PCV caused by levamisole. The antigenic relationship between the IBD vaccine virus of intermediate strain and the IBD field virus used in the experiment should be determined.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Vaccination

Vaccination for the control of Newcastle disease (ND) is routinely practiced throughout the world. Immunity against ND is usually associated with the presence of neutralizing and hemagglutination inhibiting (HI) antibodies. Humoral immunity is not the only mechanism conferring resistance, other responses are involved which are not completely defined (Seal *et al.,* 2000). Newcastle disease continues to be of economic significance in the production of poultry, requiring the development of better methods for its control, including the improvement of immunoprophylactic procedures (Huang *et al.*, 2003).

1.2 Immunosuppression

The first published description of immunosuppressive effect of infectious bursal disease (IBD) virus in the chicken demonstrated a diminished antibody response to ND vaccination (Faragher *et al.,* 1972). Immunosuppression due to IBD had been discussed in reviews of the disease condition (Kibenge *et al.,* 1988; Lasher and Shane, 1994; Abdu *et al.,* 2001). More recently, there have been reports of immunosuppression due to IBD virus vaccines (Ali *et al*., 2004; Rautenschlein *et al.,* 2007).

1.3 Immunopotentiation

Levamisole, an anthelmintic used worldwide in man and animals, act as an immunomodulator in selected human and animal immunodeficiencies with related pathology (Desplenter, 1982). Levamisole has also been shown to potentiate immune responses in chickens infected with IBD virus (Suleiman, 2002) or IBD virus vaccine administration (Shadaksharappa *et al.,* 1998).

1.4 Research Problem

In Nigeria, there is a high demand for locally produced viral vaccines for poultry, especially IBD (Gumboro) and ND La Sota vaccines. National Veterinary Research Institute (NVRI) Vom is the only organ currently producing vaccines in Nigeria as such it is unable to produce enough vaccines to meet up with demand. Most poultry farmers have therefore resorted to buying imported foreign vaccines which are usually live attenuated, intermediate or intermediate plus IBD virus strains (Lasher and Shane, 1994). Foreign IBD vaccines strains are more prone to causing immunosuppression due to higher pathogenicity and are often associated with vaccination failures (Abdu, 1986).

Immunosuppression due to IBD virus infection or vaccination with IBD vaccines often compromise immune response to subsequent vaccination with La Sota vaccine. La Sota vaccine is usually administered after IBD vaccination in accordance with most vaccination schedules for chickens in Nigeria.

1.5 Justification

Effective vaccination is the practical means of controlling ND in Nigeria. Despite all the technological advances in vaccine development in the world, poultry farmers in Nigeria still rely on the use of live attenuated viral vaccines for the control of IBD and ND.

Most small scale poultry farmers are reluctant to administer oil adjuvant inactivated ND vaccine as a booster mainly due to high cost. Hence, there is the need to provide simple and affordable alternatives for potentiating immune response to ND vaccination. There is the need to determine the time frame within which levamisole should be administered

concurrently with IBD vaccination for it to be more effective in potentiating immune responses in the field.

1.6 Aim and Objectives

The aim of the study was to provide effective method for the use of levamisole to potentiate humoral immune response to ND vaccination in chickens, often compromised by vaccination with IBD vaccines.

The study had the following objectives:

1. To determine humoral immune response to ND vaccination in broilers concurrently administered IBD vaccine and different doses of levamisole.
2. To determine the age at which levamisole will be more effective at potentiating the immune response to ND vaccination in IBD vaccinated broilers.
3. To determine the effect of levamisole on lymphoid organs of broilers through organ to body weight ratios and histopathology.
4. To determine the effect of levamisole on packed cell volume (PCV) and total protein (TP).
5. To determine the influence of levamisole on ND virus challenge in broilers.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Infectious Bursal Disease (IBD)

2.1.1 General information

Infectious bursal disease in chicken is caused by a birnavirus that induces inflammation and necrosis of the bursa of Fabricius (bursa). Two serotypes have been recognized (Serotypes 1 and 2) and described (McFerran *et al.*, 1980). Serotype 1 IBD virus isolates specifically infect developing B-lymphoid cells in the bursa, resulting in bursal atrophy in approximately four days after infection, and also affect immature B cells in the spleen (Lasher and Shane, 1994). Serotype 1 isolates are subdivided into classical, antigenic variant (intermediate) and very virulent (intermediate plus) isolates (McFerran *et al.*, 1980).

Broilers and layers which serve as the natural host of IBD virus are most susceptible to IBD among chickens (Abdu *et al.*, 2001). Genetic background had been shown to affect the level of resistance to IBD virus infection in young broilers this was related to their histocompatibility complex molecules link with immune response to vaccination against IBD (Juul-Madsen *et al.*, 2002).

In a fully susceptible chicken flock (i.e. between three to six weeks of age), the clinical IBD is responsible for losses due to impaired growth and death. Susceptible chickens less than three weeks of age do not exhibit clinical signs, but have a subclinical infection characterized by microscopic lesions in the bursa and immunosuppression (Kibenge *et al.*, 1988).

2.1.2 Pathogenesis

Depending on the virulence of the IBD virus strain and age of infected bird, the destruction of the bursa can be permanent or temporary. Bursal architecture and immunity may recover after IBD virus infection (Okoye and Uzoukwu, 1984; Kim *et al.*, 1999; Kim *et al.*, 2000). The duration of suppression of the primary antibody response to antigens in chickens exposed to different strain of IBD virus was between six to seven weeks of the IBD virus exposure. Subsequently, the antibody response to IBD virus returned to near normal levels. This recovery from the acute effects of the virus may be facilitated by strong virus induced T cell responses (Kim *et al.*, 1999). T cells had been shown to accumulate in the bursa and cecal tonsils following extensive viral replication (Tanimura and Sharma, 1997). Intra bursal T cells and T cell mediated responses may be important in viral clearance and promoting recovery from infection (Kim *et al.*, 2000). The immunosuppression against many antigens by IBD virus infection occurs simultaneously with stimulation of very high antibody levels to the virus itself and immunity, in part develops in the spleen and other lymphoid organs (Kibenge *et al.*, 1988).

Variant strains of IBD virus may be present in commercial broilers longer than previously thought. Cecal tonsils and bone marrow may serve as non bursal lymphoid tissues, supporting virus replication at later time points post infection. This longer period of viral presence may explain in part, the vaccine breaks and immunosuppression that are noticed in commercial broilers and may also aid in the spread of the virus to successive crops (Elankumaran *et al.*, 2002).

Apart from causing follicular lymphoid necrosis and depletion in the bursa, more virulent strains of IBD virus can cause severe atrophy in the thymus and cortical lymphocyte

depletion through virus induced apoptosis (Inoue *et al.*, 1994). It was hypothesized that IBD virus infection induces a change from lymphocyte proliferation to apoptosis (Ojeda *et al.*, 1997).

The effect of IBD virus infection on cellular protein expression is essential for viral pathogenesis. This is because majority of protein expression changes appeared between 48 and 96 hours after IBD virus infection and analysis of these changes will characterize the cellular response to IBD virus infection. Fifty one altered cellular proteins were demonstrated including 13 up-regulated proteins and 38 down regulated proteins between 12 to 96 hours post infection (Zheng *et al.*, 2008). This useful dynamic protein-related information will facilitate further investigation of the underlying mechanism of IBD virus infection and pathogenesis.

2.1.3 Vaccines

An ideal live attenuated IBD vaccine should not cause disease, bursal lesions, immunosuppression, or be excreted. The IBD vaccine should stimulate a long-lasting immunity and protect chickens from being infected by classical and variant IBD virus strains. However, such a vaccine is not available (Amakye-Anim *et al.*, 2000).

Current attenuated IBD virus vaccines vary in their safety and efficacy (Abdu *et al.*, 2001). They are generally classified as 'mild' highly attenuated vaccine, intermediate vaccines of moderate virulence and intermediate plus or hot vaccines (Kibenge *et al.*, 1988; Okoye, 2005). The production of IBD virus vaccine involves adaptation of wild-type virus by

propagation in chicken embryo or tissue cultures. Adapted wild-type IBD virus always has reduced virulence (attenuation), but the embryo derived IBD virus has a high frequency of virus re-isolation that could be obtained from bursal, spleen and thymic samples collected from birds inoculated with embryo-derived IBD virus (Rodriguez-Chavez *et al.*, 2002). Some intermediate vaccines were almost as pathogenic as a known virulent (Edgar strain) virus with respect to bursal atrophy and histologic lesions. Also the most pathogenic intermediate vaccines could be disseminated laterally from vaccinates, and virulence could be enhanced by bird-to-bird passage (Lasher and Shane, 1994).

2.1.4 Immunosuppression

Immunosuppressed flocks perform poorly and show reduced economic return. Despite widely used vaccination programs, IBD is one of the major economically important diseases, especially in broiler production (Lasher and Shane, 1994). Even sub clinical infection of IBD was shown to be the probable causative factor of marked immunosuppression of ND vaccine in young chicks (Almassy and Kakuk, 1976).

Two IBD virus strains isolated from broilers were tested for immunosuppressiveness in susceptible chickens and were found to suppress humoral antibodies response to ND and one of the strains suppressed macrophage activity at the third day post inoculation (Genova, 2000). The general findings with regards to immunosuppression in IBD is that both humoral and cell mediated immune responses were affected. The depletion of B lymphocyte in the bursa is the major cause of depression of the humoral immune response,

but cell mediated immunosuppression was not associated with altered helper or cytotoxic subpopulations of T lymphocytes (Rodenberg *et al.*, 1994; Corley and Giambrone, 2002).

The effect of IBD virus on cell mediated immune response is transient and less obvious than its effect on humoral immune response. Suppressor T cells and macrophages have been implicated in cell mediated immune depression following infection with IBD virus (Kim *et al*., 1999; Corley and Giambrone, 2002). The duration of immunosuppression varies depending on the virulence of the IBD virus and in one report it varied with type of the bird affected (Rautenschlein *et al*., 2007).

Several IBD virus vaccines or vaccine strains have been shown to be safe without causing immunosuppression in the affected birds (Okoye, 1985; Genova, 2000; Abdu *et al.*, 2001; Corley and Giambrone, 2002).

Other research findings showed pronounced immunosuppression following IBD virus vaccine administration (Ezeokoli *et al.*, 1990; Ali *et al.*, 2004; Rautenschlein *et al.*, 2007). It was also suggested that the destruction of the bursal B cells might not be the only factor interfering with ND antibody development. Suppression of T-helper cell activity might contribute to the reduced ND antibodies response in IBD virus inoculated chickens (Rautenschlein *et al.*, 2007).

2.2 Newcastle Disease

2.2.1 General background

Newcastle disease virus causes a highly contagious and fatal disease affecting different species of birds. The disease can vary from sub clinical to highly virulent forms, depending on the virus strain and the host species. The disease has a worldwide distribution and remains a constant major threat to commercial poultry production (Alexander, 1997). Newcastle disease virus is a member of the genus Avulavirus in the sub family Paramyxovirinae and family Paramyxoviridae (Mayo, 2002). Newcastle disease virus is also synonymous with avian paramyxovirus type 1.

Newcastle disease virus is an enveloped virus with a negative sense single stranded RNA genome of 15,186kb which codes an RNA directed RNA polymerase, hemagglutinin-neuraminidase (HN) protein, fusion protein, matrix protein, phosphoprotein and nucleoprotein in the 5´ to 3´ direction (Seal *et al.*, 2000).

Isolates of ND virus are characterized into three main pathotypes, depending on the severity of disease. Lentogenic isolates are of low virulence, mesogenic isolates are intermediate, while velogenic isolates are highly virulent viruses that cause high mortality in birds and are termed neurotropic or viscerotropic velogenic (Seal *et al.*, 2000). Velogenic ND viruses are among List A pathogens that require reporting to OIE and outbreaks result in strict embargoes.

2.2.2 Host range

Newcastle disease virus (NDV) has a wide host range with most orders of birds reported to have been infected (Seal *et al.*, 2000). Among poultry, chickens are the most susceptible, whereas ducks and geese are the least susceptible. Virus shedding is usually of short duration, one to two weeks in gallinaceous birds and some species of song birds, but can last several weeks to many months in psittacines. Long lasting shedding of NDV might be observed in columbiformes (pigeons and doves) and passerifomes in cases where there is chronic infection of the kidney. Pigeons may carry highly virulent NDV without overt signs of clinical disease (Wakamatsu *et al.*, 2006).

Newcastle disease virus V protein was shown to play an important role in host range restriction. It was suggested that the host range of NDV is limited by the ability of its V protein to efficiently prevent innate host defenses, such as the interferon response and apoptosis (Park *et al.*, 2003a; Park *et al.*, 2003b). It was also demonstrated that for viruses infecting avian cells, the NDV V protein and the influenza NS1 protein are functionally interchangeable, even though there are no sequence similarities between the two proteins (Park *et al.*, 2003b).

2.2.3 Pathogenicity

The virulence of NDV is determined by more than one factor. The primary molecular determinant for NDV pathogenesis is the fusion protein cleavage site amino acid sequence (Seal *et al.*, 2000). The cleavability of fusion protein alone does not determine the virulence of a strain. The HN gene also plays a crucial role. The tissue tropism of NDV virus was shown to be dependent on the origin of the HN protein. It was determined that

virulence is a function of the amino acid differences in the HN protein (Huang *et al.*, 2004a).

2.2.4 Vaccination and vaccines

Control of ND is achieved primarily by mass application of live virus vaccines in drinking water among commercial poultry in Nigeria. A trial conducted to determine the efficacy of different vaccination routes in broilers showed that the ocular route was superior to the oral route which was superior to the spray technique (Degefa *et al.*, 2004). After considering the economic analysis, it was concluded that there was no proof of the ocular method being acceptable at the level of small-and medium-scale poultry farms (Degefa *et al.*, 2004).

Vaccination using non virulent NDV strains protects susceptible birds against disease by producing an antibody response either locally, systemically or both. The mucosal application of attenuated live virus induces both systemic and local immunity, whereas parenteral immunization with inactivated vaccine generally induces systemic immunity with little local protection (Al-Garib *et al.*, 2003b). Inactivated vaccines are not used in commercial broilers, but experimentally they have been shown to respond effectively (Maas *et al.*, 1999).

Limitations were encountered in the use of La Sota live virus vaccine and inactivated NDV vaccine, such as the secondary complications of vaccination with La Sota in the form of transient damage to the respiratory and intestinal epithelium (Mast *et al.*, 2005). Other limitations include unsatisfactory efficacy, safety, and difficulty in differentiating

vaccinated animals from their infected counter parts. These factors dictate the need to develop newer and more effective vaccines (Huang *et al.,* 2003).

A new pattern of NDV vaccine to be taken orally was developed using chitosan as capsule wall material, containing NDV as core material and glutaraldehyde as cross-linking material. The chitosan microsphere vaccine was shown to be safe and could induce both humoral and cell mediated immune responses as well as mucosal immunity strongly (Zhai *et al.,* 2007).

Recombinant vaccines against ND were developed which are genetically stable with engineered mutations that enhance immunogenicity (Mebatsion *et al.,* 2001). Also, vectored vaccines for ND control have been reported in the literature. One example is a fowl pox virus vectored vaccine carrying NDV genes (Boyle and Heine, 1993; McMillen *et al.,* 1994). Newcastle disease virus itself has been shown to be utilized as a vaccine vector, which could be used in the control of ND and other important viral diseases of poultry, such as infectious bursal disease and avian influenza (Huang *et al.,* 2003; Huang *et al.,* 2004b; Veits *et al.,* 2006).

2.2.5 Immunity

Birds usually develop immunity to NDV following recovery from infection or in response to vaccination. Protection is measured by the presence of antibodies to NDV although in vaccinated B-cell-depleted-chickens resistance to NDV was demonstrated. Consequently, immune protection involves other responses apart from the humoral immune response

(Seal *et al.*, 2000). Innate immunity could be induced by primary vaccination with NDV vaccines. The innate immunity induced by the primary NDV vaccination may significantly suppress the multiplication of *Escherichia coli* in chickens. Such non specific immunity appeared at one or two days and lasted until eight days post vaccination (Huang and Matsumoto, 2000).

The concept of humoral immunity is a key component in the protection against ND. Therefore, vaccination programs should be directed toward eliciting and maintaining high antibody levels to NDV in flocks of birds (Reynolds and Maraqa, 2000b). The rate of onset of 100% protective immunity had been shown to be seven days with commercial vaccines and 14 days with a recombinant Herpes virus of turkey vaccine expressing the fusion and HN protein of NDV (Heckert *et al.*, 1996). A high correlation was found between the presence of HI or neutralizing antibodies to NDV and protection against ND. The presence of specific antibodies to the HN and F polypeptides as determined by the HI or the viral neutralization (VN) tests, was determined to be responsible for protection from ND (Reynolds and Maraqa, 2000a). Even though there was a synergistic effect between specific antibodies to the HN and F polypeptides, it was suggested that the antibodies to the two proteins have different mechanisms of protection (Umino *et al.*, 1990).

Newcastle disease virus specific cell mediated immune (CMI) response was measured by a blastogenesis micro assay technique. The results indicated that specific CMI response to NDV by itself was not protective against virulent NDV challenge. The presence of VN or HI antibodies was necessary for protection against NDV (Reynolds and Maraqa, 2000a). In

another study using ex-vivo antigen-specific chicken interferon gamma (ChIFN-γ) production and cell proliferation of splenocytes from immune chickens, the data showed that live or killed NDV vaccines were capable of stimulating CMI responses to NDV as measured by the ChIFN-γ ELISA. However, most of the chickens vaccinated with the live vaccine produced ChINF-γ after antigen recall stimulation, from two to four weeks after vaccination, when only some chickens vaccinated with the inactivated vaccine showed a specific response four weeks after vaccination (Lambrecht *et al.*, 2004).

2.2.6 Antibodies kinetics

Hemagglutination inhibition antibody titer was first detected for live NDV vaccines at day seven post vaccination and reached plateau level at day 14 post vaccination. Immunization with inactivated NDV vaccines produced detectable HI response by day 14 post vaccination (Al-Garib *et al.*, 2003a). In an experimental trial with inactivated NDV vaccine in broilers a peak was obtained for HI antibodies at three to six weeks after vaccination, and there after, the antibodies remained constant for up to 18 weeks (Maas *et al.*, 1999).

A study by Al-Garib and colleagues (2003) showed that 55 % of antibody forming cells produced Ig M, 37 % produced Ig G and the remaining 8 % produced Ig A. Immunoglobulin M in serum was detected at day four post vaccination using live NDV vaccine in one fourth of the birds which reached plateau level at day seven post vaccination. It persisted at a low level to the end of the experiment (28 days). With inactivated NDV vaccine Ig M was first detected at 14 days post vaccination and remained

high until day 28. Generally at 14, 21 and 28 days post vaccination, the serum Ig M was higher in inactivated vaccine. For Ig G in serum, it was detected for live vaccine at seven days post vaccination and reached plateau at 14 days. While inactivated vaccine gave detectable titer at 14 days. For Ig A, maximum response was detected in serum between seven and 14 days after intraocular and intranasal inoculation. For the inactivated vaccine, a low Ig A response developed at 14 days post vaccination followed by a higher level at 21 days post vaccination (Al-Garib *et al*., 2003a).

There was a divergence in the rates of decay of serum neutralization (SN) or HI antibody titers after eight weeks following revaccination. While the SN titers were maintained at the same level up to 16 weeks, the HI antibody titers showed a steady decline. This indicates the possible duality of antibodies in HI and SN (Kaliannan *et al.*, 1975).

Maternal antibodies in chicks play a significant role in immune response to NDV vaccination where it had been shown that for birds with high levels of maternal antibodies there was a sharp drop in antibodies levels following vaccination with NDV vaccines (Rahman *et al.*, 2002). The rate of decay of maternally derived antibodies was about half by every five days (Rahman *et al.*, 2002). It has been found that maternal antibodies did not interfere with aerosol or intraocular immunization (Malkinson and Small, 1977).

2.2.7 Immunostimulatory and antineoplastic properties

Newcastle disease virus is considered as a potential oncolytic agent in the treatment of cancer because it could selectively kill tumor cells. A central mechanism, in the anti-tumor activity of immune cells is induction of apoptosis in the target cells (Washburn *et al.*, 2003). Newcastle disease virus was found to stimulate human monocytes to kill various human tumor cell lines. The tumoricidal activity was mediated by TRAIL a member of tumor necrotic factor family which induces apoptosis (Washburn *et al.*, 2003).

2.3 Levamisole

Levamisole is the laevo isomer of di-tetramisole, which is a racemic mixture. The parent compound tetramisole was first marketed as an anthelmintic in 1965, but it was soon noted that its anthelmintic activity resided almost entirely in the L-isomer, levamisole. Thus, it was determined that using the L-isomer alone the dosage could be reduced by half (Barragry, 1994).

Following either oral or iv administration of 40 mg/kg levamisole in chickens, levamisole was detected 30 hours after both oral and iv administration in pre lay and laying birds. The levamisole plasma concentration curve followed a three compartment open model (El-kholy *et al.*, 2006). The minimum toxic dose in chickens is 640 mg/kg (Charles and Roberson, 2001). Withdrawal time for levamisole at 40 mg/kg is nine days in eggs and eighteen days for the slaughter of birds for human consumption (El-kholy and Kemppainen, 2005).

Pharmacological action of levamisole includes its use as adjuvant, immunomodulator, anti rheumatic agent and antinematodal agent. Levamisole was used as a chemical adjuvant together with chicken interferon (molecular adjuvant) for DNA vaccine encoding the HN and F genes of NDV to potentiate immune response. The two adjuvants yielded 80 % protection in chickens against challenge with a lethal dose of the virulent NDV strain. They induced significantly higher levels of HI titer and T cell proliferation. In addition, they increased the expression of INF-γ, 1L 2, 1L 4, 1L 12 and 1L 13 which was a balanced cytokine production (Yin *et al.*, 2007).

In normal chickens, levamisole was able to enhance humoral and cellular immune responses at the dose of 0.25 mg/kg. The effect was probably mediated by the activation of the T cell function and affects only antibody response to thymus dependent antigen (Soppi *et al*., 1979). Different concentrations of levamisole affected different populations of lymphocytes. For example, 0.25 mg/kg stimulated both phyto hemagglutinin (PHA) and Con A responses of chicken lymphocytes while 2.5 mg/kg increased only PHA responses (Soppi *et al.*, 1979). In the same experiment, levamisole had no effect on the body weight and on the relative and absolute weights of bursa and thymus. They concluded that it will be best if levamisole is administered intermittently.

Prolonged intermittent treatment with a higher dose of levamisole 20 mg/bird from day old up to two weeks, and subsequently, 33 mg/bird daily for five weeks was administered to chickens infected with IBD virus in an experiment to test the effect of levamisole on response to NDV vaccinations (Panda and Rao, 1994). The results indicated that

levamisole treatment significantly boosted HI antibodies titer in NDV vaccinated chickens that were not exposed to IBD virus from fifth week onward, but HI antibodies titer of birds that were infected with IBD virus and vaccinated with NDV vaccine was much lower, suggesting that levamisole had not increased the immunizing capability in IBD virus infected birds. This was perhaps due to administration of levamisole continuously which caused further immunosuppression (Panda and Rao, 1994).

Another trial with levamisole to stimulate immunity against ND in Leghorn breed that were two months old was conducted. Levamisole at 3 mg/kg body weight given once, parenterally, 24 hours prior to or after immunization of the birds with a live La Sota vaccine or given three times, parenterally or orally, did not stimulate the humoral immune response (Chenchev *et al.*, 1981). No differences were found in the level of the total protein, protein fractions, and the titer of the HI antibodies between the test and the control birds. There was, however, prolongation of the time of effective immunity in the levamisole treated and vaccinated birds as against the birds that were vaccinated against ND only (Chenchev *et al.*, 1981).

2.4 Packed Cell Volume

Determination of PCV is routinely done to define health, anemia and polycythemia in all animals, but for diagnosis of anemia or polycythemia to be accurate, it is necessary to have an established reference intervals for the specie affected (Goodwin *et al.*, 1991; Goodwin *et al.*, 1992b). The definition of anemia in chicks varies with age. The incidence of anemia was shown to be high in young broiler chicks of various ages while the incidence of

polycythemia was high only in relatively old broilers, an indication that neonatal physiologic anemia occurs in chicks (Goodwin *et al.*, 1992a). Increase in PCV was observed with IBD infection (Chineme and Cho, 1984; Oladele *et al.*, 2005). A decrease in PCV was observed in birds challenged with NDV (Oladele *et al*., 2005).

2.5 Total Protein

Total protein changes and their significance in establishing the etiology and pathogenesis of disease are well recognized. Serum protein had been shown to decrease in chickens inoculated at one day old with IBD virus. The change occurred due to reduced levels of serum gamma-globulins at four weeks post inoculation (Naqi *et al.,* 1978).

Experimental infection of chickens with NDV Kudu 113 strain caused a decrease in daily mean total protein concentrations, beginning from day three post infection, and the lowest concentration was obtained by day seven and eleven post inoculation. The decrease in total protein was attributed to a possible decrease in albumin content (Oladele *et al.,* 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Chickens

A total of 500 day-old Ross broilers, hatched on 9 November, 2006, obtained from a commercial hatchery (Best Line Chicks) Ibadan were used for the experiment.

3.2 Antigens

Positive control IBD virus antigen used in AGID test was prepared by inoculating three weeks old cockerels devoid of maternal antibodies against IBD with a clarified 10 % (w/v) bursal homogenate. Bursae were obtained from chickens infected with a virulent strain field IBDV that had previously caused a natural outbreak of IBD. Each bird received one drop (approximately 0.02 ml) of the bursal homogenate intraocularly in each open eye. The birds were euthanized three days post-inoculation and their bursae were harvested aseptically. The bursae were pooled, weighed, with the addition of equivalent volume of phosphate buffered saline pH 7.4 and homoginised with mortar and pestle. The homogenate was centrifuged at 2000 g for 30 min and the supernatant was harvested, dispensed into aliquots and stored at –25^{o}C.

La Sota vaccine strain obtained from NVRI, Vom was used as NDV antigen for hemagglutination (HA) and HI tests.

3.3 Antibodies

Positive control antiserum for use in AGID was obtained by bleeding eight weeks old broilers that recovered from an outbreak of IBD when they were four weeks old. The sera that tested positive with the positive control antigen were pooled and stored at –25^{o}C until when required.

3.4 Red Blood Cells (RBCs)

One percent RBCs suspension was obtained after washing blood that was collected from apparently healthy local chickens using Alsever's solution as anticoagulant. The washing was performed three times with PBS. After each respective washing, intact RBCs were separated as sediments following centrifugation at 447.2 g for five minutes. Finally, 0.1 ml of the washed RBCs was suspended in 9.9 ml of PBS.

3.5 Feed and Feeding

The birds were fed *ad libitum* with a commercial broiler starter pelletized feed (Vital feeds) for five weeks. Subsequently, broiler finisher from the same source was given up to the end of the experiment at ten weeks of age. Water was also provided *ad libitum.*

3.6 Management and Housing

The broilers were reared on the deep litter. The birds were brood for two weeks and each bird was identified using a wing tag. The birds were treated prophylactically with chloramphenicol at 250 mg/L in drinking water for ten days, starting from two days after

their arrival. Also anticoccidia Embazine®, was administered in drinking water at the dose of 0.6 g/L for three days and amprolium in feed at the rate of 1 g/kg at three weeks old.

The birds were housed in the animal rooms of the Faculty of Veterinary Medicine and Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. Six different animal rooms were used based on experimental group and stage of the experiment.

3.7 Experimental Design

At two weeks of age, the broilers were randomly divided into six groups using the table of random numbers. Group A to E comprised of 60 chicks per group and each group had three sub groups of 20 chicks for 2.5 mg/kg levamisole, 5 mg/kg levamisole, and without levamisole treatments. Group G comprised of 80 chicks and was sub divided into five. Two sub groups from group G had 15 chicks each and received 2.5 mg/kg and 5 mg/kg doses of levamisole, respectively when they were 16 days of age. Two other sub groups from group G had 15 chicks each and received 2.5 mg/kg and 5 mg/kg doses of levamisole, respectively at 30 days of age. The last sub group of group G had 20 chicks and was not administered levamisole. Groups E and G were not vaccinated with IBD and La Sota vaccines. Group E was administered levamisole after NDV-challenge (Table 3.1). Groups A and B were vaccinated initially with IBD vaccine at 16 and 30 days of age, respectively and then La Sota vaccine after 14 days in each case at 30 or 44 days of age. Groups C and D were vaccinated with La Sota vaccine only at 30 and 44 days, respectively.

Table 3.1: Broiler age at respective vaccination, challenge and administration of levamisole in different groups

Group	Group Size	Age (days)			
		16	30	44	62
A (0)	20	IBDV	La Sota		ND challenge
A (2.5)	20	IBDV+L	La Sota		ND challenge
A (5)	20	IBDV+L	La Sota		ND challenge
B (0)	20		IBDV	La Sota	
B (2.5)	20		IBDV+L	La Sota	
B (5)	20		IBDV+L	La Sota	
C (0)	20		La Sota		ND challenge
C (2.5)	20	L	La Sota		ND challenge
C (5)	20	L	La Sota		ND challenge
D (0)	20			La Sota	
D (2.5)	20		L	La Sota	
D (5)	20		L	La Sota	
E (0)	20				ND challenge
E (2.5)	20				ND challenge +L
E (5)	20				ND challenge +L
G (0)	20				ND challenge
G1 (2.5)	15	L			ND challenge
G1 (5)	15	L			ND challenge
G2 (2.5)	15		L		
G2 (5)	15		L		

L = Levamisole administered at 2.5 mg/kg and 5 mg/kg
IBDV = Infectious bursal disease vaccine
La Sota = Newcastle disease vaccine La Sota
ND challenge = Newcastle disease virus challenge with Kudu 113 NDV strain

Three sub groups in group A, C, E and G were challenged with NDV Kudu 113 strain, when they were 62 days old. The experiment was terminated when the broilers were 70 days old.

3.8 Vaccines and Vaccinations

La Sota Newcastle disease vaccine was obtained from NVRI Vom. Infectious bursal disease vaccine (Bursine®-2) manufactured by Fort Dodge Animal Health USA was purchased from a commercial distributor at Kaduna. The infectious bursal disease vaccine (Forte Dodge) 1,000 doses vial was reconstituted in 200 ml of PBS. Each bird was administered 0.2 ml equivalent to one dose of the vaccine orally. The birds have been vaccinated against NDV infection when they were day-old, at the hatchery. A 200 doses vial of the La Sota vaccine was reconstituted in 40 ml of PBS. Each bird was administered 0.2 ml of the reconstituted vaccine equivalent to one dose of the vaccine orally. The ND vaccine virus titer was $10^{6.0}$ median embryo infective doses (EID_{50})/dose of live vaccine.

3.9 Administration of Levamisole

(-) – Tetramisole hydrochloride (L9756 SIGMA) was purchased from the UK.

One gramme of the pure levamisole compound was dissolved in 200 ml of sterile distilled water to make a 5 mg/ml levamisole solution. Levamisole 5 mg/ml solution was injected directly into the crop of respective birds, every other day for one week at the doses of 2.5 mg/kg or 5 mg/kg in respective sub groups at 16 and 30 days of age. Injection of levamisole into the crop was chosen for convenience and accuracy and was expected to give the same effect as oral administration.

3.10 Newcastle Disease Virus Challenge

An ampoule containing 0.5 ml of lyophilized NDV Kudu 113 strain obtained from NVRI Vom was reconstituted in 4.5 ml of PBS. Each bird received 0.02 ml containing $10^{6.5}$ EID_{50} of the virus through the oculo-nasal route.

3.11 Bleeding

All birds were bled weekly from two weeks of age to the termination of the experiment at 10 weeks. They were bled from the wing vein using 1 ml insulin syringe or 2 ml disposable syringe, depending on the age of birds during bleeding. About one to two ml of blood was collected from each bird during each bleeding. Serum samples were collected after overnight coagulation at room temperature, stored in serum vials at –25^{o}C until when required. Birds in the ND challenged groups were bled on the day of challenge (before challenge) and one week after challenge when 2 ml of blood was collected using ethylene diamine tetra acetic acid (EDTA) as anticoagulant for determination of PCV and TP.

3.12 Serology

3.12.1 Hemagglutination test

The method described in OIE manual (2004a) was used to determine the hemagglutination units (HAU) of the La Sota antigen for use in running the HI test as follows:

i) 25 µL of PBS was dispensed into each well of a plastic V-bottomed microtiter plate.

ii) 25 µL of La Sota vaccine (one 200 doses vial reconstituted with 2 ml of PBS) was placed in the first well.

iii) Twofold dilutions of 25 µL volumes of the La Sota vaccine were made across the plate up to well 11.

iv) A further 25 µL of PBS was dispensed to each well.

v) 25 µL of 1 % chicken RBCs was dispensed to each well.

vi) The solution was mixed by tapping the plate gently. The RBCs were allowed to settle for about 40 min at room temperature, when control RBCs settled to a distinct button.

vii) HA was determined by tilting the plate and the presence or absence of tear streaming of the RBCs was observed. The highest dilution that showed complete HA represented 1 HAU and counting backward two dilutions was the 4 HAU.

3.12.2 Hemagglutination inhibition test

The method described in OIE manual (2004a) was used to determine the titer of antibodies to NDV in sera as follows:

i) 25 µL of PBS was dispensed into each well of a plastic V-bottomed microtiter plate.

ii) 25 µL of the serum was placed into the first well of the plate.

iii) Twofold dilution of 25 µL volume of the serum was made across the plate up to well 11.

iv) 4 HAU antigen in 25 µL PBS was added to each well except the control and the plate was left for 30 min at room temperature.

v) 25 µL of 1 % chicken RBCs was added to each well and the solution was mixed by tapping the plate gently. The RBCs were allowed to settle for about 40 min at room temperature, when control RBCs settled to a distinct button.

vi) The HI titer was the highest dilution of serum that caused complete inhibition of 4 HAU of antigen. The agglutination was assessed by tilting the plate. Only those wells in which the RBCs streamed at the same rate as the control well were considered to show inhibition.

vii) The validity of results was assessed against a negative control serum (pooled sera that were HI negative) and a positive control serum (pooled sera that were HI positive with titers of $\geq \log_2 8$).

3.12.3 Agar gel immuno diffussion test

Qualitative AGID was performed as described in the OIE manual (2004b):

i) Plates of agar were prepared in plastic Petri dishes and three vertical rows of wells (6 mm in diameter and 3 mm apart) were cut using a template and tubular cutter.

ii) Pieces of agar were removed from the wells using tip of 22 G needle without damaging the walls of the wells.

iii) 50 µL of sera were dispensed into wells in the first and third rows leaving an empty well in between two sera for the positive control antibody.

iv) 50 µL of the positive control antibody were dispensed in wells in between two sera and 50 µL of the antigen were dispensed into wells in the middle row.

v) The plates were incubated at room temperature for up to 48 h in a humid chamber.

vi) The plates were examined using a viewer with a bright light source against a dark background.

3.13 Determination of Packed Cell Volume

Microhematocrit method was used to determine the PCV as described by Campbell and Coles (1986). Plain capillary hematocrit tubes were filled with the blood samples containing EDTA by capillary action the outsides of the tubes were carefully dried with a piece of gauze and then the opposite ends of the tubes were sealed using special clay. The sealed tubes were placed in a micro-hematocrit centrifuge (Hettich Hematokrit Tuv® Bayern) and were centrifuged at 5,000 g for five min. After centrifugation, the tubes were carefully placed on a special reader Hawksley Micro-haematocrit reader (Hawksley England) and the percentages of red cells were determined by noting their levels on the reader.

3.14 Determination of Total Protein

Refractometric method was used to determine the total plasma proteins as described by Campbell and Coles (1986). A hand refractometer made by American optical corporation USA (Catalog 10400A) was used. The concentration of protein in g/100 ml was determined by noting the specific gravity of plasma sample and referring it to a standard chart.

3.15 Determination of Organ Body Weight Ratios

One week post challenged, five birds from each sub group were removed and euthanized. Their body, thymus, bursae and spleens weights were determined. The values obtained were used for computation of immune organ body weight ratios. The organ weight in grammes is divided by the body weight in grammes then multiplied by 1000.

3.16 Histopathology

Samples of the bursa, spleen and thymus were collected from birds in each of the challenged groups, one week post challenge for histopathology. Samples collected were preserved in 10 % formalin, processed and embedded in paraffin wax. Five microns thick sections were made and stained with haematoxylene and eosin (H & E) and examined under a light microscope.

3.17 Statistical Analysis

Data obtained were subjected to analysis using SPSS 11.5 version. One way analysis of variance (ANOVA) was performed with Tukey post hoc multiple comparisons, which determined statistical significant difference between sub groups at 95 % confidence interval with $P \leq 0.05$ considered as significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Humoral Immune Response

4.1.1 Antibody response to infectious bursal disease vaccine

All the sera tested for precipitin antibodies following vaccination against IBD were negative.

4.1.2 Antibody response to Newcastle disease vaccine

Hemagglutination inhibition antibodies due to day-old vaccination against NDV were detected in all the sera tested at two weeks of age. The NDV mean HI antibody titers at two weeks of age for all groups were above $3\log_2$. Peak, mean ± SE $\log_2$ NDV antibody titer of 6.00 ± 0.83 was observed in subgroup 0 (C) at four weeks, following La Sota vaccine administration without IBD vaccine or levamisole.

4.1.3 Effect of infectious bursal disease vaccine on antibody response to Newcastle disease vaccine

The effect of a variant IBD virus vaccine strain which was expected to cause immunosuppression in vaccinated birds was evaluated. At 16 days of age, broilers were administrated IBD vaccine without levamisole and vaccinated with ND La Sota vaccine at 30 days of age in subgroup 0 (A). La Sota vaccine alone was administered to broilers in subgroup 0 (C) at 30 days of age. Subgroup 0 (G) were not vaccinated against IBD or ND and not administered levamisole. The mean ± SE $\log_2$ NDV HI antibody titers of

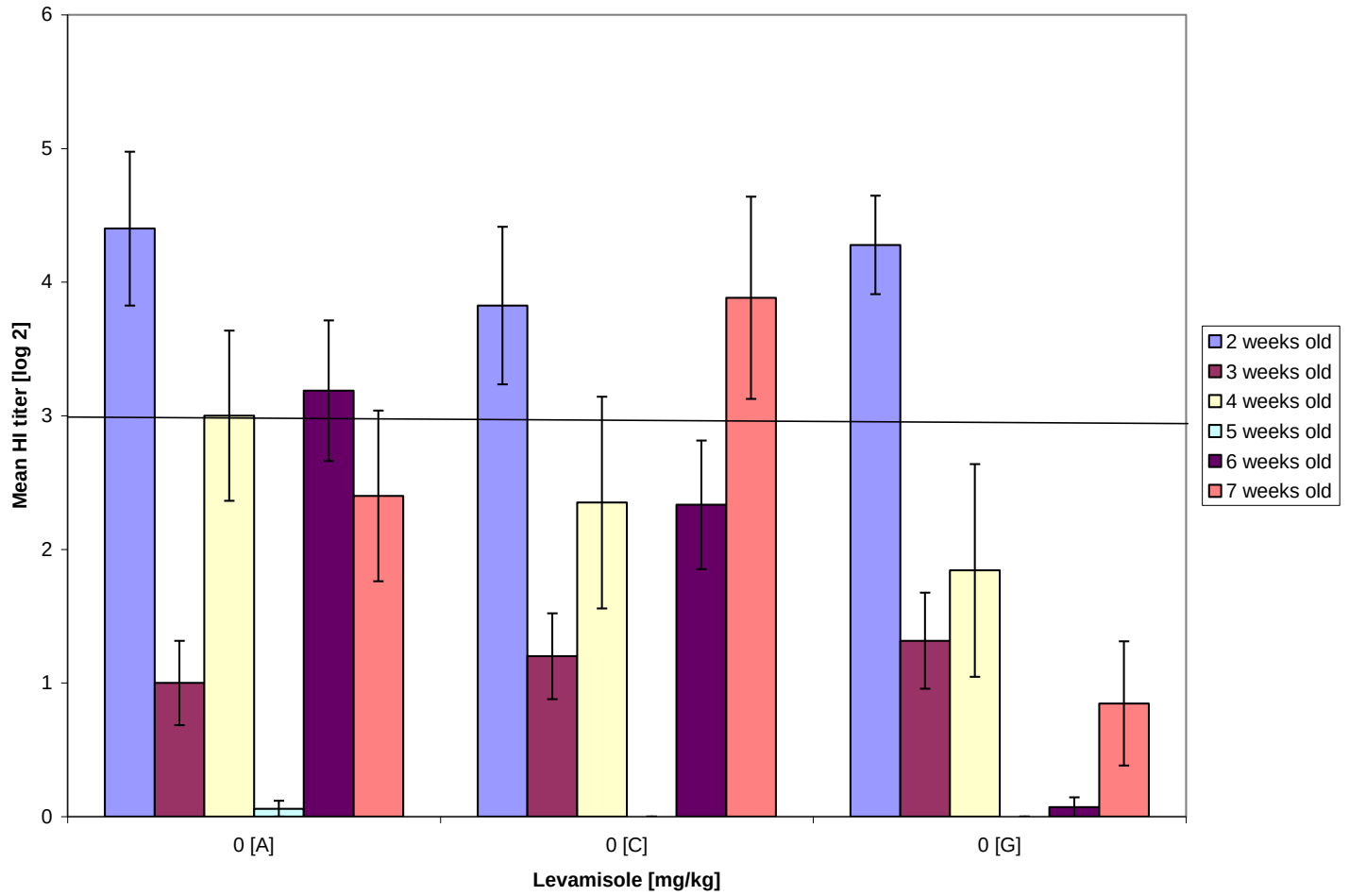


Figure 4.18: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 16 days of age. Groups: A, vaccinated with Gumboro (16 days of age) and La Sota (30 days of age) vaccines; C, vaccinated with La Sota vaccine (30 days of age); G, not vaccinated with Gumboro or La Sota vaccines. Line indicates cut off point for protective antibody titer.

subgroups 0 (A), 0 (C) and 0 (G) are shown in Figure 4.1. A highly significant difference was found at six weeks of age between subgroup 0 (A), 0 (C) and 0 (G) with NDV HI mean antibody titers of 3.19 ± 0.53, 2.33 ± 0.48 and 0.07 ± 0.07 $\log_2$ respectively ($P < 0.001$). From four to six weeks of age, the highest mean antibody titer was found in subgroup 0 (A). At seven weeks of age, subgroup 0 (C) had the highest mean antibody titer of 3.88 ± 0.76 $\log_2$. This differ significantly from 2.40 ± 0.64 and 0.85 ± 0.47 $\log_2$ of subgroup 0 (A) and 0 (G) respectively ($P < 0.01$). Subgroup 0 (A) NDV HI mean antibody titer reached 3 $\log_2$ and above at four weeks of age (before vaccination with La Sota) and above 3 $\log_2$ at six weeks of age (after vaccination with La Sota). Subgroup 0 (C) NDV HI mean antibody titer was above 3 $\log_2$ once, at seven weeks of age.

The effect of IBD vaccine at 30 days of age is shown in Figure 4.2. Subgroup 0 (B) was administered IBD vaccine at 30 days of age (without levamisole) and ND La Sota vaccine at 44 days of age. Subgroup 0 (D) was administered La Sota vaccine at 44 days of age. Highly significant difference was found at six weeks of age between subgroup 0 (B), 0 (D) and 0 (G) with NDV HI mean antibody titers of 2.63 ± 0.53, 0 ± 0 and 0.07 ± 0.07 $\log_2$ respectively ($P< 0.001$). At seven weeks of age the different mean antibody titers of 3.13 ± 0.76, 0.21 ± 0.21 and 0.85 ± 0.07 $\log_2$, for subgroup 0 (B), 0 (D) and 0 (G) respectively, were statistically significant ($P< 0.01$). At ten weeks of age, subgroup 0 (D) had the highest mean antibody titer of 5.58 ± 0.93 than 5.22 ± 0.60 and 4.80 ± 0.82 $\log_2$ of subgroup 0 (G) and 0 (B) respectively, although the differences were not statistically significant ($P> 0.05$).

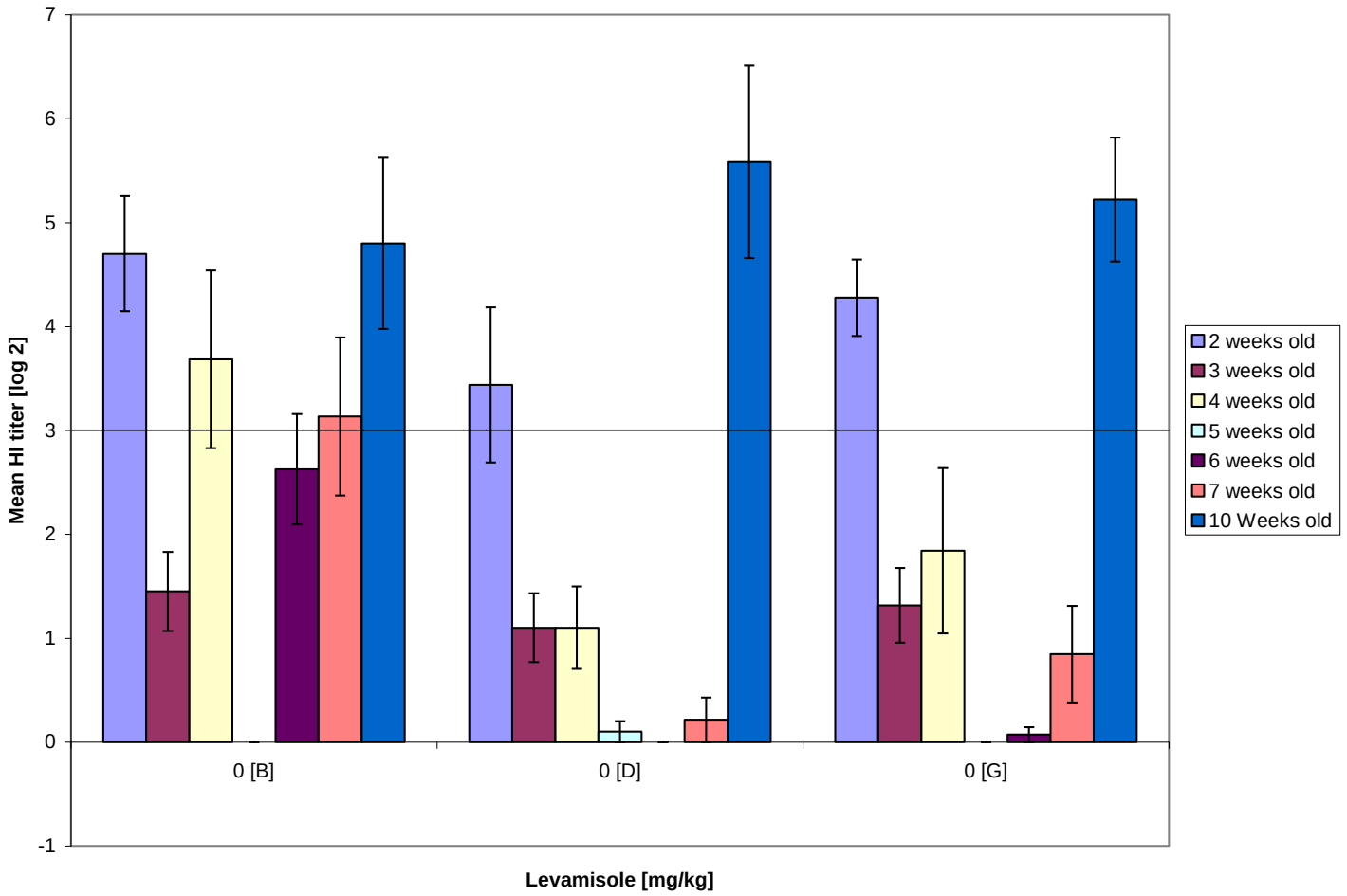


Figure 4.19: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 30 days of age. Groups: B, vaccinated with Gumboro (30 days of age) and La Sota (44 days of age) vaccines; D, vaccinated with La Sota vaccine (44 days of age); G, not vaccinated with Gumboro or La Sota vaccines. Line indicates cut off point for protective antibody titer.

In order to determine the effect of IBD vaccine administered at 16 and 30 days of age, the results of subgroup 0 (A) and 0 (B) were compared (Figure 4.3). There was no statistical significant difference between subgroups 0 (A) and 0 (B) with respect to NDV mean HI antibody titer. The NDV mean HI antibody titer of subgroup 0 (A) was higher than that of 0 (B) at five, six and nine weeks of age (0.06 ± 0.06, 3.19 ± 0.53 and 4.79 ± 0.75 $\log_2$) and (0.00 ± 0.00, 2.63 ± 0.53 and 3.47 ± 0.65 $\log_2$) respectively. The mean antibody titer of subgroup 0 (B) was higher than that of 0 (A) at three, four and seven weeks of age (1.45 ± 0.38, 3.68 ± 0.86 and 3.13 ± 0.76 $\log_2$) and (1.00 ± 0.32, 3.00 ± 0.64 and 2.40 ± 0.64 $\log_2$) respectively. The highest mean antibody titer (4.79 ± 0.75 $\log_2$) was found in subgroup 0 (A) at nine weeks of age.

4.1.4 Effect of levamisole on antibody response to Newcastle disease vaccine

Levamisole was administered to broilers every other day for one week starting from 16 days of age. The dose of 2.5 mg/kg was administered to subgroup 2.5 (C) and 2.5 (G1) while 5 mg/kg was administered to 5 (C) and 5 (G1) subgroups. Levamisole was not administered to subgroup 0 (C) and 0 (G). Broilers in group C were then vaccinated with ND La Sota vaccine at 30 days of age (Figure 4.4). There was a relative increase in the mean antibody titer in subgroup 2.5 (C) (2.94 ± 0.84 $\log_2$) and 5 (C) (2.79 ± 0.88 $\log_2$) at four weeks of age before vaccination with La Sota. After vaccination with La Sota, the highest mean antibody titer was observed in subgroup 0 (C) (3.88 ± 0.76 $\log_2$) than in 2.5 (C) (2.60 ± 0.82 $\log_2$) and 5 (C) (1.87 ± 0.70 $\log_2$) respectively at seven weeks of age.

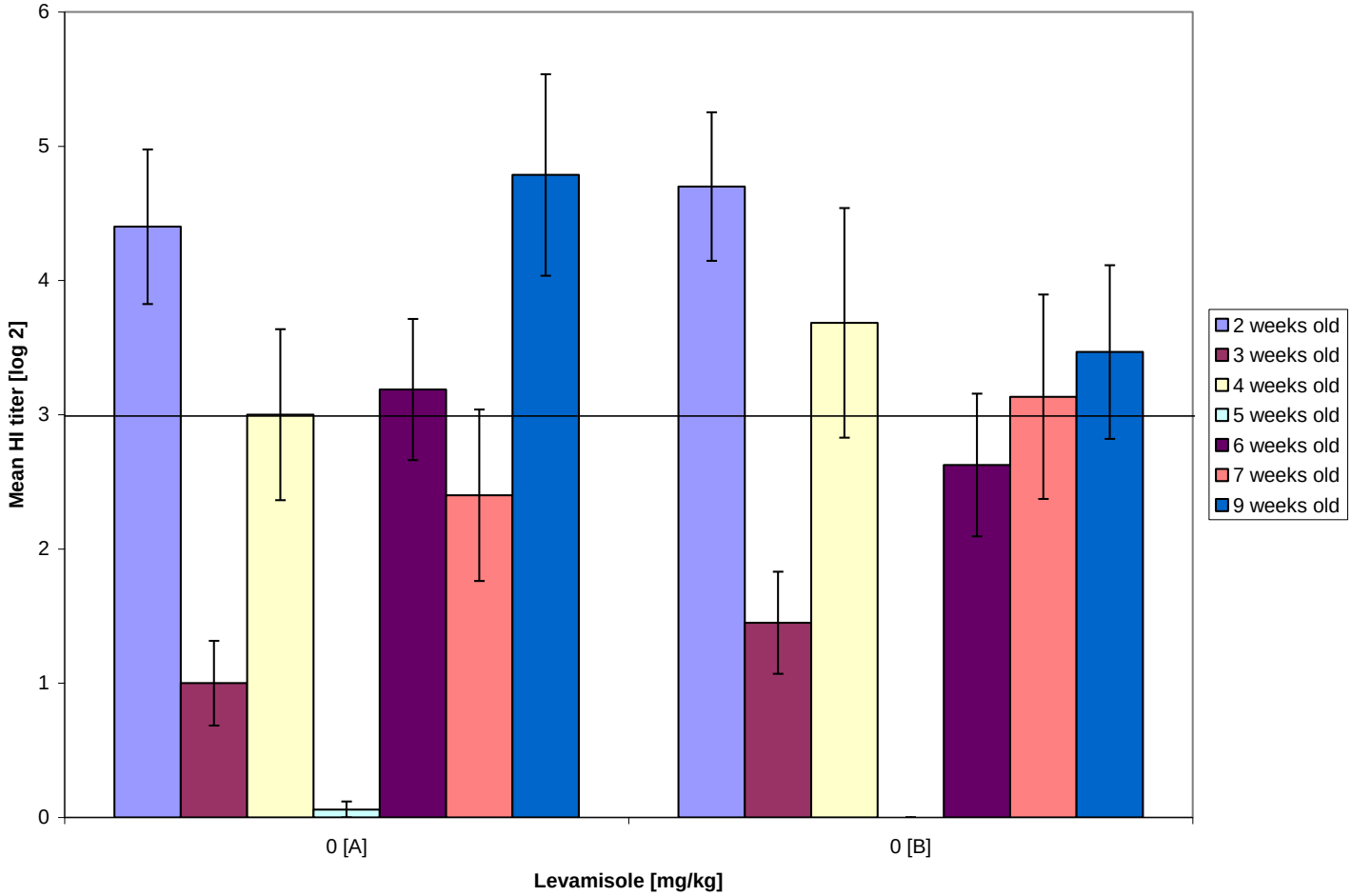


Figure 4.20: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 16 (A) and 30 (B) days of age. Groups: A, vaccinated with Gumboro (16 days of age) and La Sota (30 days of age) vaccines; B, vaccinated with Gumboro (30 days of age) and La Sota (44 days of age) vaccines. Line indicates cut off point for protective antibody titer.

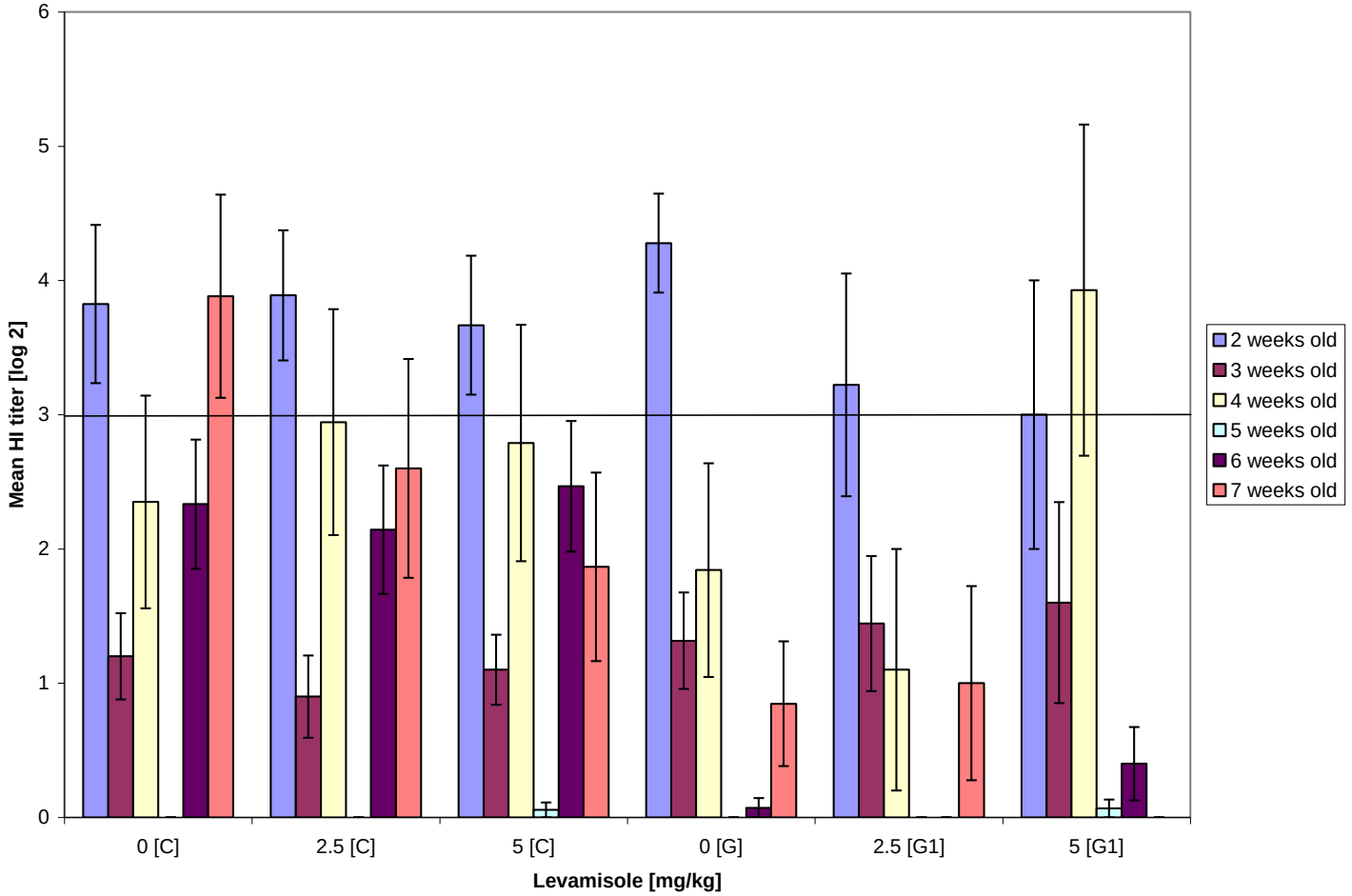


Figure 4.21: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 16 days of age. Groups: C, vaccinated with La Sota vaccine at 30 days of age; G/G1, not vaccinated with La Sota vaccine. Line indicates cut off point for protective antibody titer.

The differences in mean antibody titers for the subgroups were statistically significant at six and seven weeks of age ($P< 0.001$ and $P< 0.01$) respectively.

At 30 days of age, broilers in subgroup 2.5 (D) and 2.5 (G2) were administered levamisole at 2.5 mg/kg doses. Subgroup 5 (D) and 5 (G2) were administered 5 mg/kg levamisole. Subgroup 0 (D) and 0 (G) were not administered levamisole. Group D broilers were then vaccinated with ND La Sota vaccine at 44 days of age (Figure 4.5). Subgroup 5 (D) had the highest mean antibody titer at seven ($0.42 \pm 0.26 \log_2$) and nine ($5.11 \pm 0.69 \log_2$) weeks of age. Subgroup 0 (D) produced the highest mean antibody titer ($5.58 \pm 0.93 \log_2$) at ten weeks of age. Group G subgroups produced higher mean antibody titers than broilers administered levamisole in group D at ten weeks of age.

Newcastle disease vaccine HI antibody response was compared between group C and group D as presented in figure 4.6. Highly significant difference was observed at six, seven and eight weeks of age ($P< 0.001$) with higher mean antibody titer observed in group C. Subgroup 0 (C) produced antibody titers above 3 $\log_2$ at seven (3.88 ± 0.76), eight (6.00 ± 0.83) and nine (3.67 ± 0.72) weeks of age. The subgroups administered 5 mg/kg levamisole showed higher mean antibody titer than 2.5 mg/kg subgroups in group C and D at eight and nine weeks of age.

Unvaccinated subgroups, 5 (G1) and 2.5 (G1) were the only subgroups with NDV HI mean antibodies up to or above 3 $\log_2$ following levamisole administration (Figure 4.7).

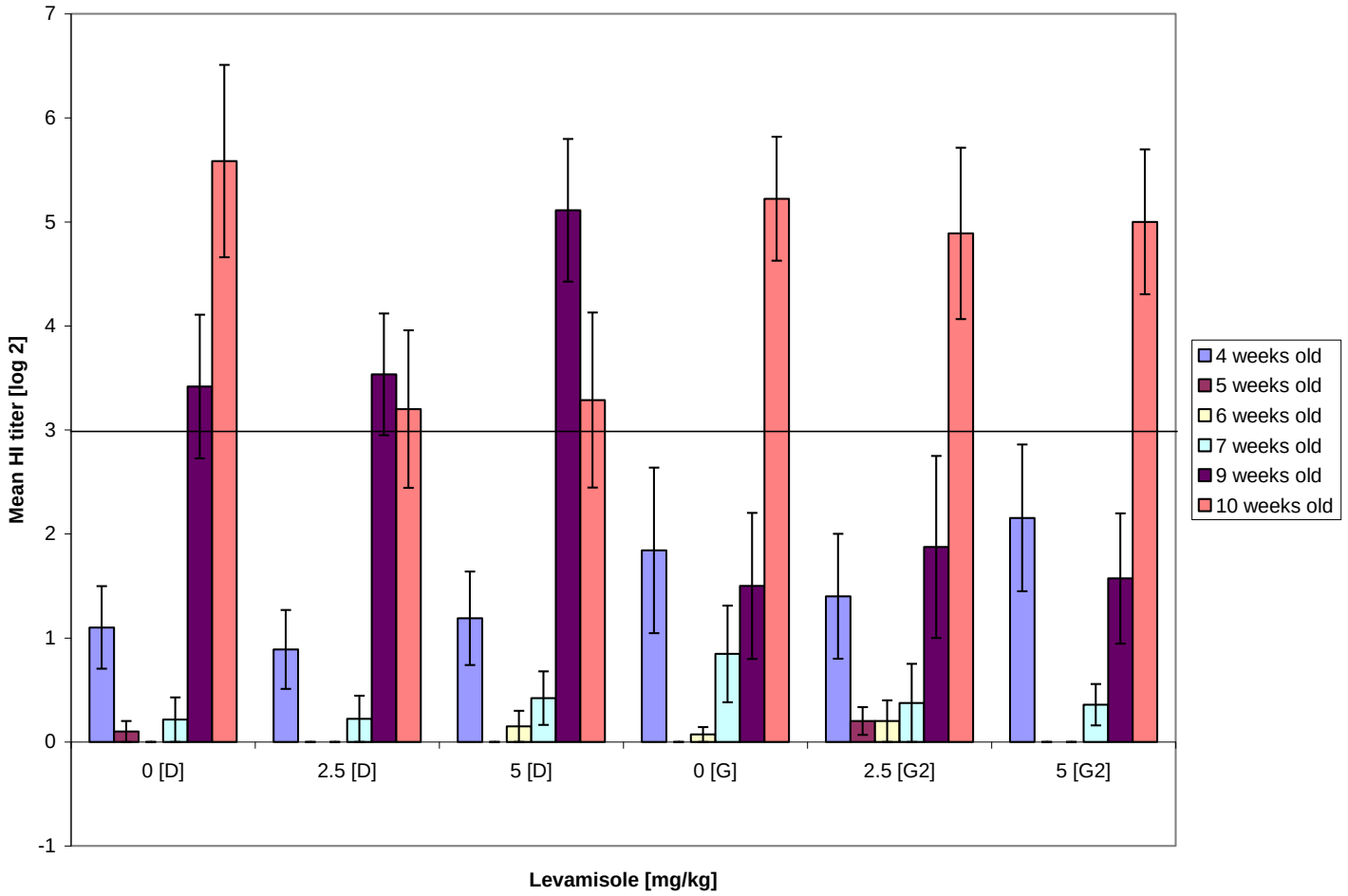


Figure 4.22: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 30 days of age. Groups: D, vaccinated with La Sota vaccine at 44 days of age; G/G2, not vaccinated with La Sota vaccine. Line indicates cut off point for protective antibody titer.

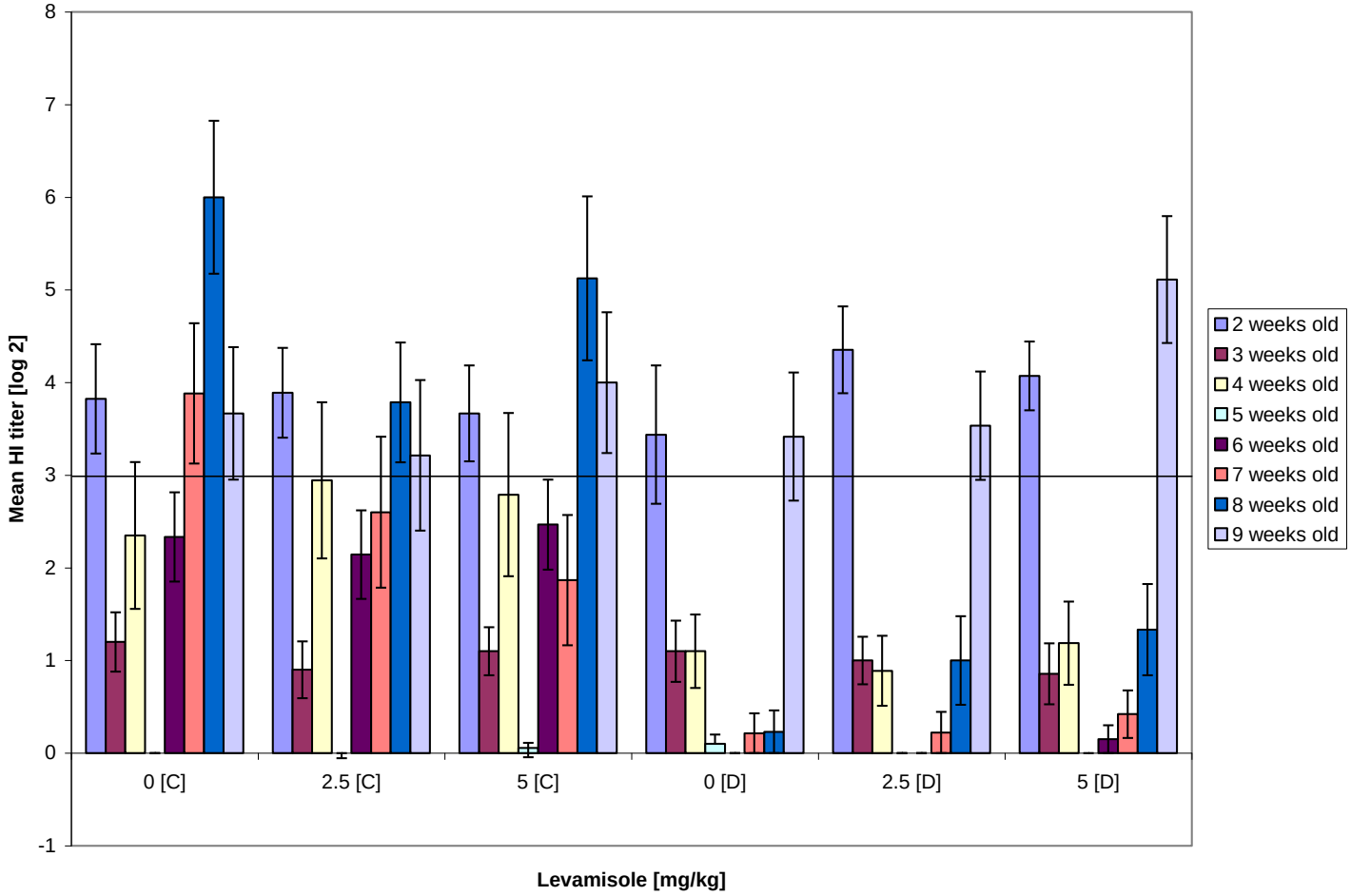


Figure 4.23: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 16 (C) and 30 (D) days of age. Groups: C, vaccinated with La Sota vaccine at 30 days of age; D, vaccinated with La Sota vaccine at 44 days of age. Line indicates cut off point for protective antibody titer.

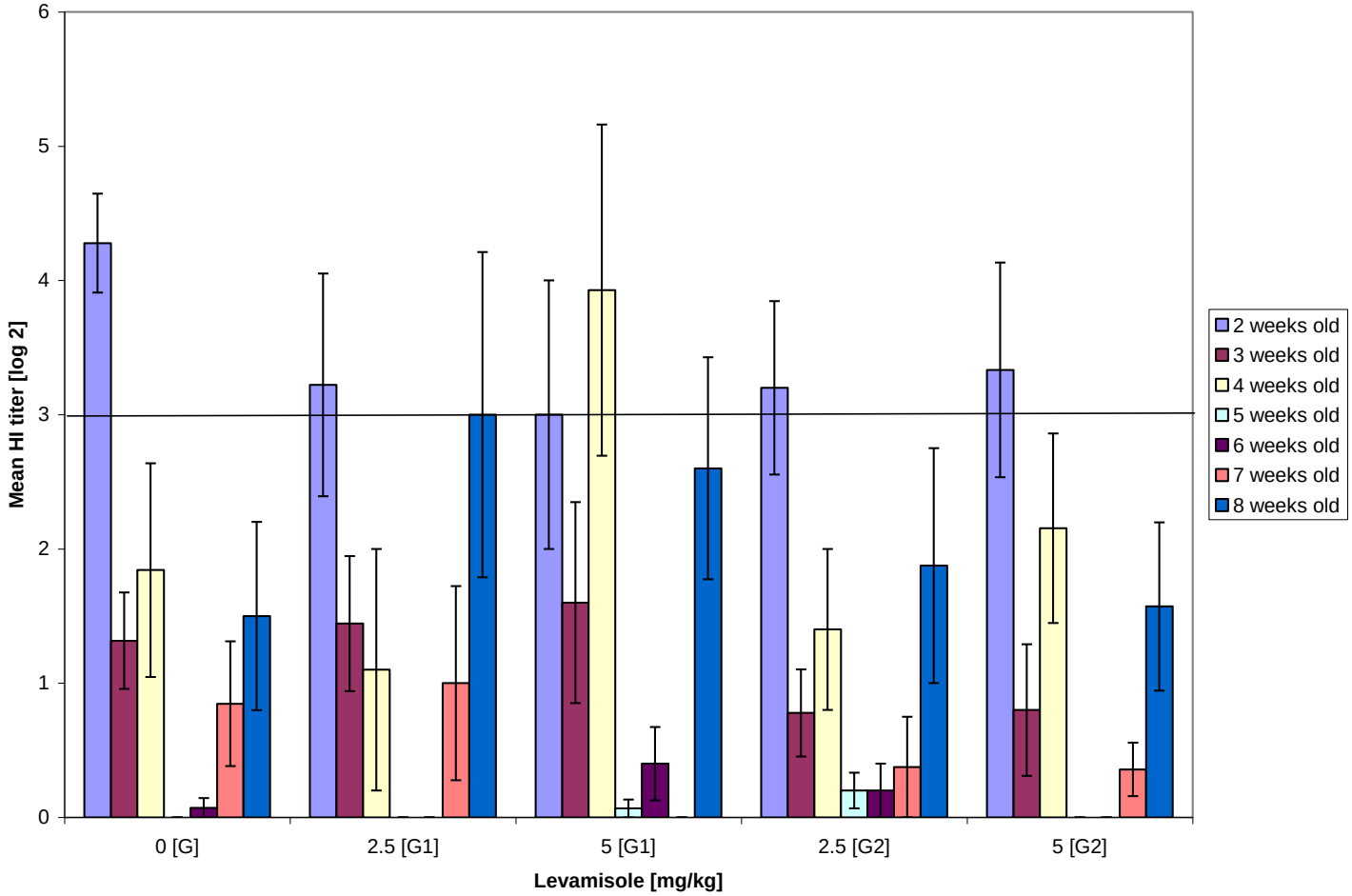


Figure 4.24: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 16 (G1) and 30 (G2) days of age. Subgroup G, G1 and G2 were not vaccinated with Gumboro or La Sota vaccines. Line indicates cut off point for protective antibody titer.

Subgroup 5 (G1) mean antibody titers were higher than the other subgroups at three (1.60 ± 0.75 $\log_2$), four (3.93 ± 1.23 $\log_2$) and six (0.40 ± 0.27 $\log_2$) weeks of age. Subgroup 2.5 (G1) mean antibody titers were higher than the other subgroups at seven (1.00 ± 0.72 $\log_2$) and eight (3.00 ± 1.21 $\log_2$) weeks of age (Figure 4.7).

4.1.5 Effect of administering levamisole concurrently with infectious bursal disease vaccine on antibody response to Newcastle disease vaccine

Levamisole was administered concurrently with IBD vaccine at 16 days of age to subgroup 2.5 (A) and 5 (A) administered 2.5 mg/kg and 5 mg/kg levamisole respectively. These subgroups were then vaccinated with ND La Sota vaccine at 30 days of age. A comparison was made between 2.5 (A), 5 (A), 2.5 (C), 5 (C), 2.5 (G1) and 5 (G1) subgroups as shown in Figure 4.8. In all the three groups, the subgroups with higher mean antibody titers at two weeks of age became lower at three weeks of age. Higher mean antibody titers were observed in 5 (A) than 2.5 (A) subgroup at three, five and seven weeks of age. Subgroup 5 (A) produced the highest mean antibody titer than all other subgroups at three, five and seven weeks of age. Subgroup 5 (A) was the only subgroup with NDV HI mean antibody titer above 3 $\log_2$ at seven weeks of age. Antibody response to ND vaccine was higher in group A than C and G1 respectively with highly significant difference at six and seven weeks of age ($P < 0.001$).

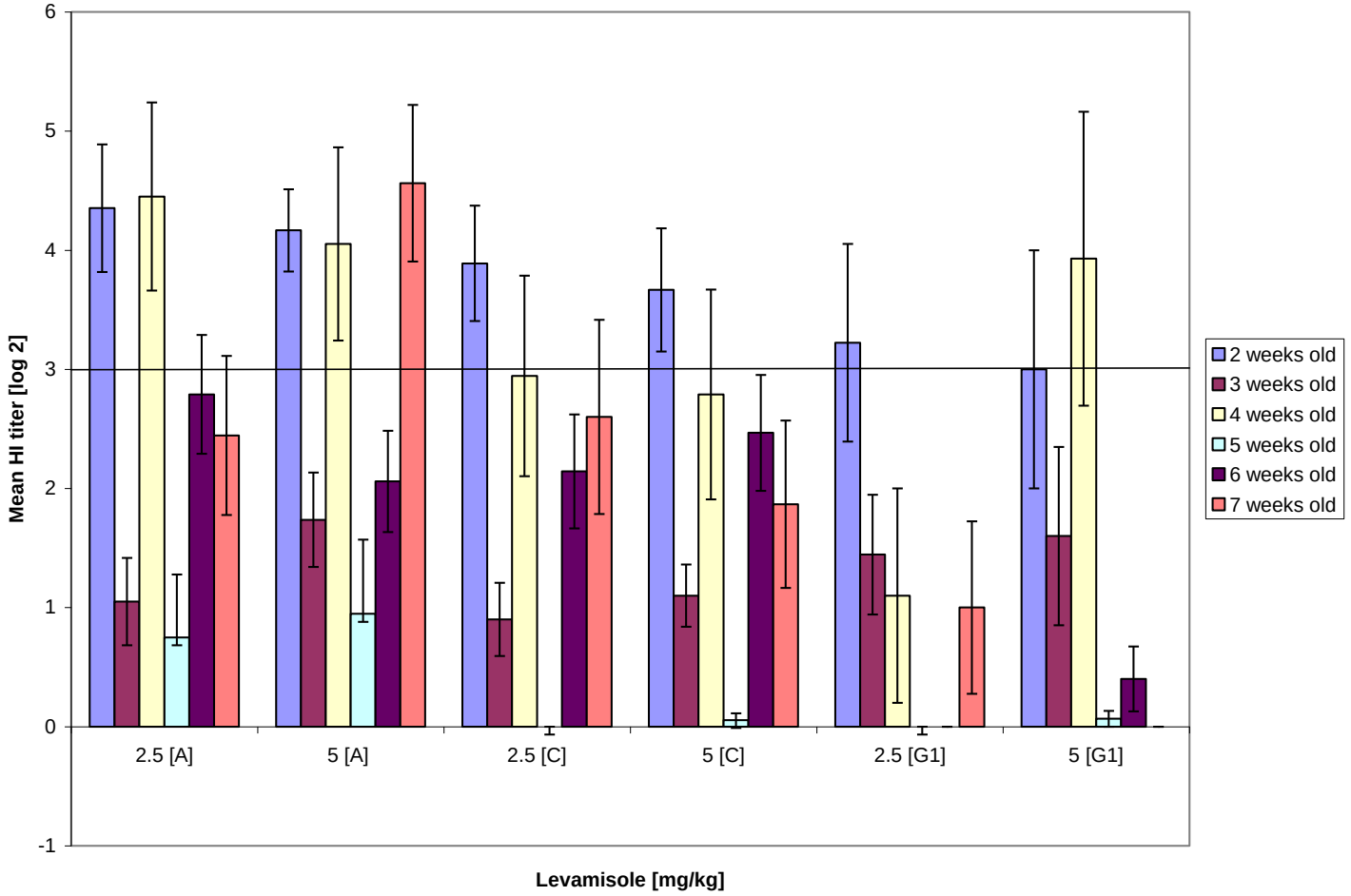


Figure 4.25: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 16 days of age. Groups: A, vaccinated with Gumboro (16 days of age) and La Sota (30 days of age) vaccines; C, vaccinated with La Sota vaccine (30 days of age); G1, not vaccinated with Gumboro or La Sota vaccines. Line indicates cut off point for protective antibody titer.

Levamisole was administered concurrently with IBD vaccine at 30 days of age to subgroup 2.5 (B) and 5 (B) at the dose rate of 2.5 mg\kg and 5 mg\kg respectively. This was followed by La Sota vaccination at 44 days of age. Results were compared between 2.5 (B), 5 (B), 2.5 (D), 5 (D), 2.5 (G2) and 5 (G2) subgroups as shown in Figure 4.9. Subgroup 5 (B) produced the highest NDV HI mean antibody titer of 2.13 ± 0.48 and 1.75 ± 0.70 $\log_2$, at six and seven weeks of age, respectively. These antibody titers differed significantly when compared with those of group D and G at six ($P< 0.001$) and seven ($P< 0.05$) weeks of age. At ten weeks of age group G produced higher NDV HI mean antibody titers than group B and D, respectively.

Antibody response of broilers to vaccination against ND was compared between subgroups administered IBD vaccine concurrently with levamisole at 16 days of age and subgroup administered IBD vaccine without levamisole (Figure 4.10). In subgroup 2.5 (A) and 5 (A) mean antibody titers were higher than subgroup 0 (A) at three, four, five and seven weeks of age. Generally subgroup 5 (A) had better antibody response than 2.5 (A) and 0 (A) respectively. The difference in the mean antibody titer between group A and G subgroups was highly significant at six and seven weeks of age ($P< 0.001$) (Figure 4.10).

The group vaccinated with IBD vaccine at 30 days was evaluated for differences in antibody response between subgroups administered levamisole and those not administered levamisole groups B and G (Figure 4.11). Subgroup 0 (B) mean antibody titers were higher than 5 (B) and 2.5 (B) respectively, at six and seven weeks of age. The highest mean

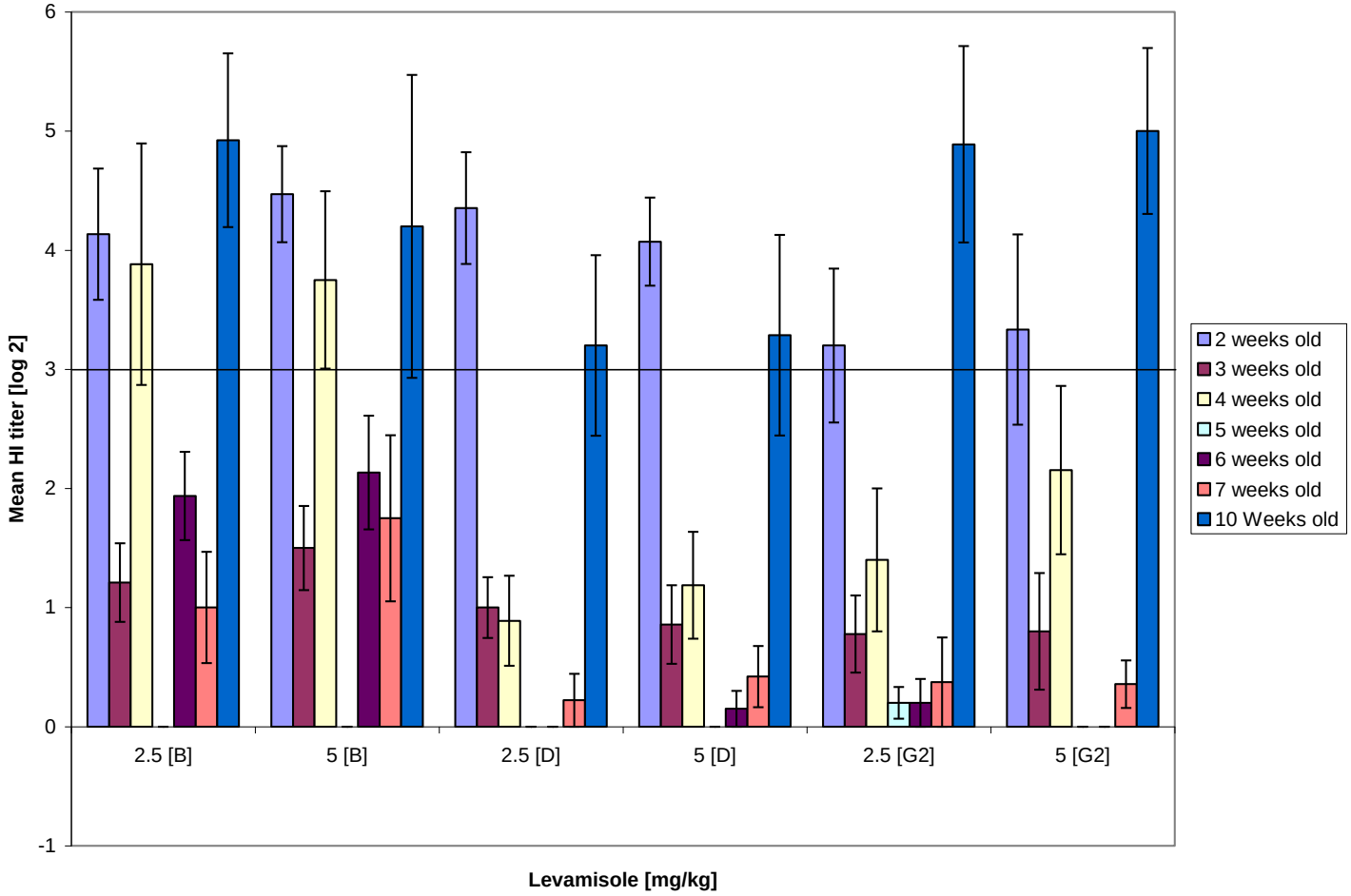


Figure 4.26: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 30 days of age. Groups: B, vaccinated with Gumboro (30 days of age) and La Sota (44 days of age) vaccines; D, vaccinated with La Sota vaccine (44 days of age); G2, not vaccinated with Gumboro or La Sota vaccines. Line indicates cut off point for protective antibody titer.

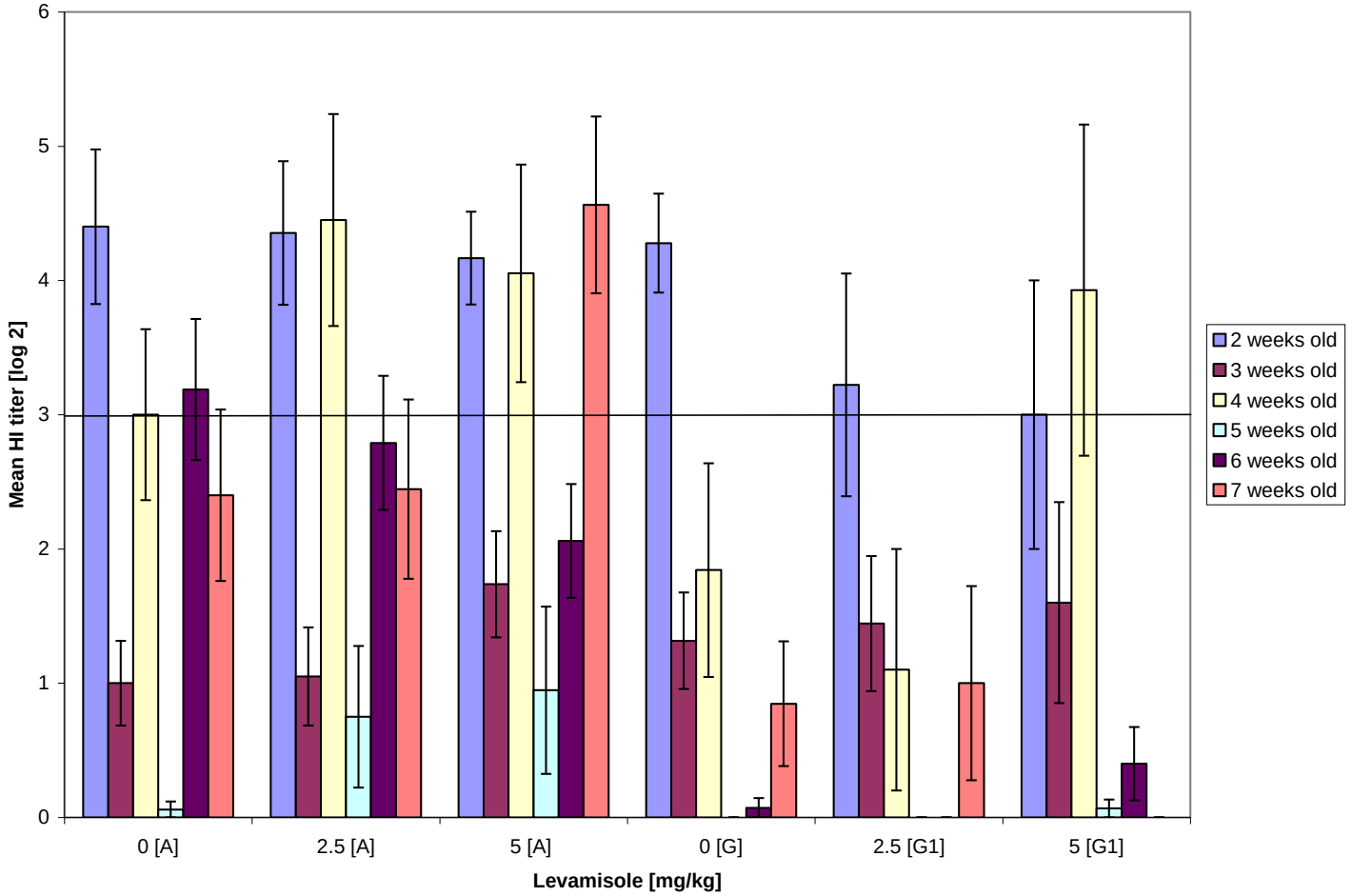


Figure 4.27: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 16 days of age. Groups: A, vaccinated with Gumboro (16 days of age) and La Sota (30 days of age) vaccines; G/G1, not vaccinated with Gumboro or La Sota vaccines. Line indicates cut off point for protective antibody titer.

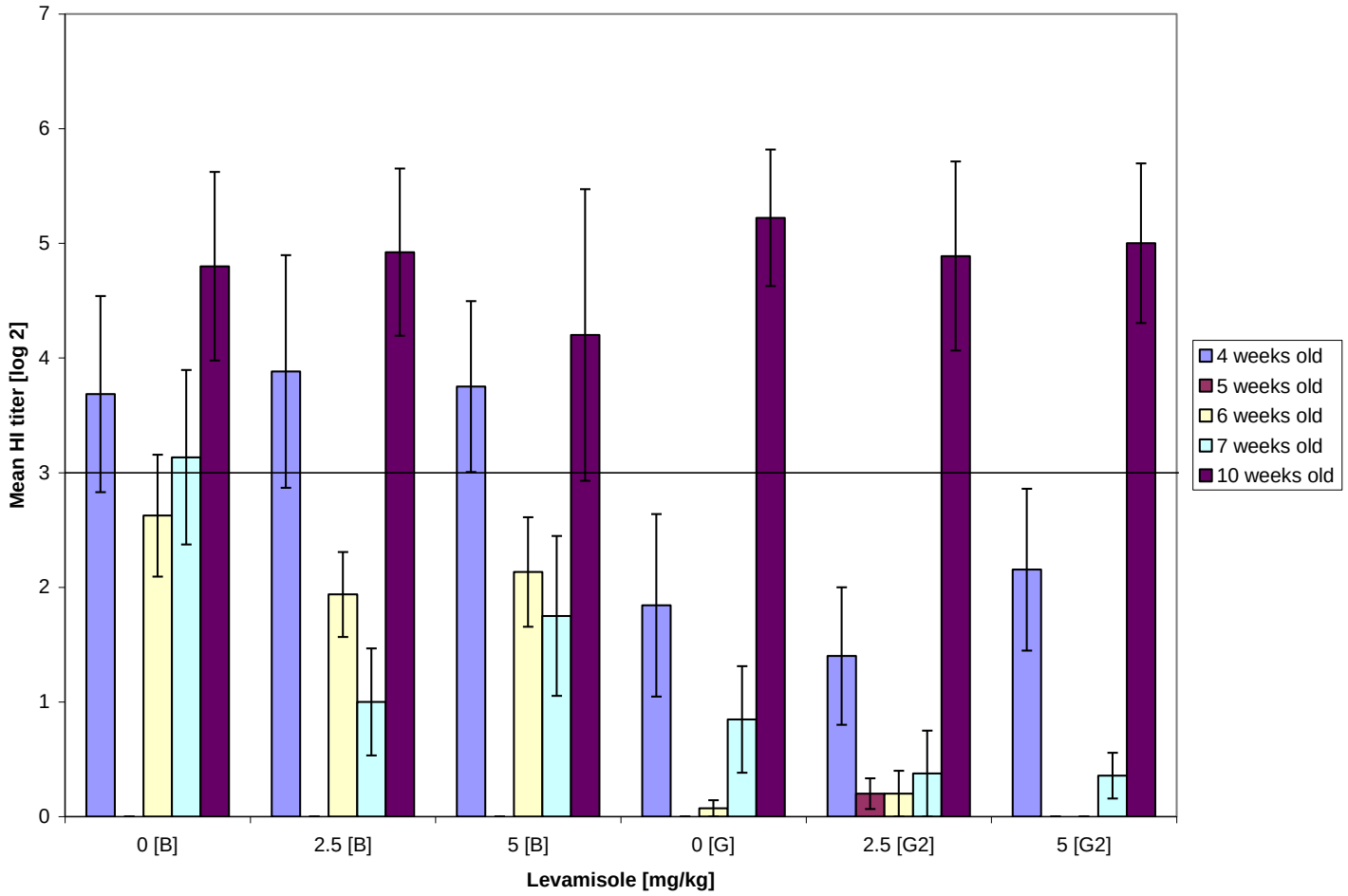


Figure 4.28: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 30 days of age. Groups: B, vaccinated with Gumboro (30 days of age) and La Sota (44 days of age) vaccines; G/G2, not vaccinated with Gumboro or La Sota vaccines. Line indicates cut off point for protective antibody titer.

antibody titer of 5.22 ± 0.60 $\log_2$ was found in subgroup 0 (G) at 10 weeks of age. The differences in mean antibody titers between groups were statistically significant at five ($P< 0.01$), six ($P< 0.001$) and seven ($P< 0.01$) weeks of age.

The mean antibody titers in group A and B were compared (Figure 4.12). The antibody titers in levamisole administered subgroups of group A were higher than similar subgroups in group B. There was a statistical significant difference with the highest antibody titer of 4.56 ± 0.67 $\log_2$ in subgroup 0 (A) ($P<0.01$) at seven weeks of age. The highest mean antibody titer of 4.79 ± 0.75 $\log_2$ observed in subgroup 0 (A) at nine weeks of age, although the difference was not statistically significant ($P> 0.05$).

4.1.6 Percentage of broilers positive for hemagglutination inhibition antibodies to Newcastle disease vaccine

The total number of birds positive to NDV vaccine with HI antibody titers ≥ 1 $\log_2$, out of the total number of birds tested from each group was determined. In group A, the per cent positive increased and decreased every other week. However, in subgroup 5 (A) the percentage positive persistently increased from five to seven weeks of age. Subgroup 5 (A) obtained the highest per cent positive value at three, four and seven weeks of age (Table 4.1). The per cent antibody positive values for subgroup 0 (C) consistently decreased from 50 % at three weeks of age to 0 % at five weeks of age. The values then increased to 93 % at eight weeks of age following vaccination with La Sota vaccine at 30 days of age. Subgroup 0 (C) obtained the per cent positive values at seven, eight and nine weeks of age which were higher than those in subgroup 2.5 and 5 (C) (Table 4.2).

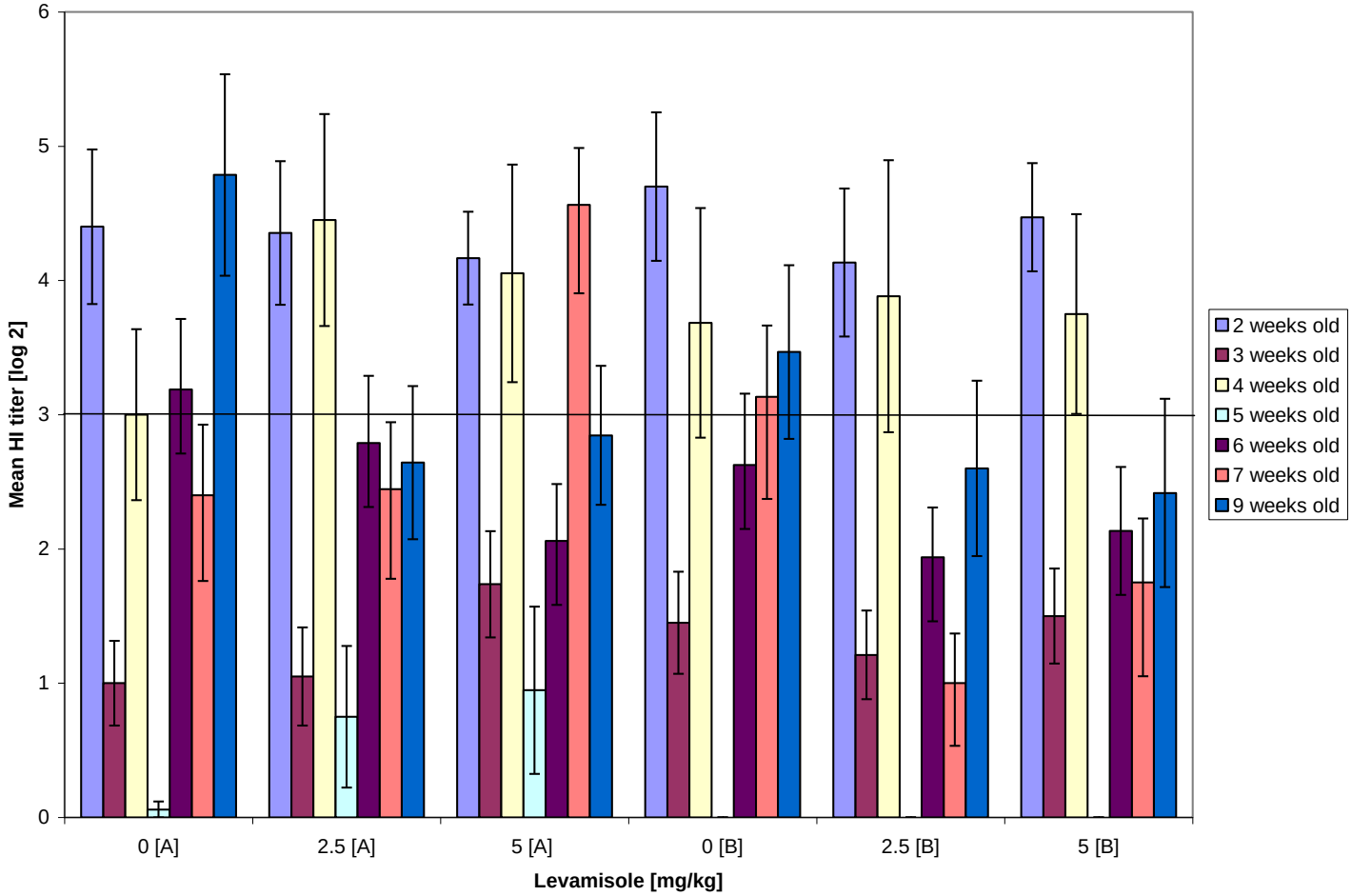


Figure 4.29: Hemagglutination inhibition antibodies to Newcastle disease vaccine in broilers administered levamisole every other day for one week, at 16 (A) and 30 (B) days of age. Groups: A, vaccinated with Gumboro (16 days of age) and La Sota (30 days of age) vaccines; B, vaccinated with Gumboro (30 days of age) and La Sota (44 days of age) vaccines. Line indicates cut off point for protective antibody titer.

Table 4.1: Percentage of broilers with positive hemagglutination inhibition antibody titers of $\geq$ 1 $\log_2$ to Newcastle disease vaccine, Group A, administered Gumboro vaccine with levamisole (at 16 days of age), La Sota vaccine (at 30 days of age) and challenged with Newcastle disease virus (at 62 days of age).

Group A	Age in days							
	14	21	28	35	42	49	63	70
Levamisole [in mg/kg]	Per cent positive							
0	86.7	42	65	5.9	81.3	60	92.9	84.6
2.5	94.1	35	75	10	78.9	50	71.4	88.2
5	100	63.2	78.9	15.8	76.5	87.5	84.6	90.9

Table 4.2: Percentage of broilers with positive hemagglutination inhibition antibody titers of $\geq$ 1 $\log_2$ to Newcastle disease vaccine, Group C, administered levamisole (at 16 days of age), La Sota vaccine (at 30 days of age) and challenged with Newcastle disease virus (at 62 days of age).

Group C	Age in days								
	14	21	28	35	42	49	56	63	70
Levamisole [in mg/kg]	Per cent positive								
0	82.4	50	45	0	75	82.4	92.9	86.7	100
2.5	83.3	35	55.6	0	71.4	60	85.7	71.4	81.8
5	77.8	55	47.4	5.6	80	43.8	81.3	76.9	100

Control (broilers not vaccinated against IBD and ND) subgroup 5 (G1) had relatively higher per cent antibody positive values up to six weeks of age when compared to subgroup 0 and 2.5 (G1) (Table 4.3).

In group B, the weekly changes in per cent positive antibody values were relatively minimal in all subgroups. After administration of levamisole, subgroup 2.5 (B) had the highest per cent antibody positive values at six and ten weeks of age. After vaccination with La Sota, subgroup 0 (B) had the highest per cent antibody positive values at nine week of age (Table 4.4).

In group D there was a consistent decrease in per cent antibody positive values in all subgroups. The values in subgroup 2.5 (D) and 5 (D) decreased from two to five weeks of age. The values in Subgroup 0 (D) decreased from two to six weeks of age (Table 4.5).

Control (broilers not vaccinated against IBD and ND) subgroup 2.5 (G2) and 5 (G2) . The lowest value of 10 % in subgroup 2.5 (G2) was at six weeks of age. Decrease to 0 % was found in subgroup 5 (G2) at five and six weeks of age (Table 4.6).

4.1.7 Percentage of broilers with HI titers for Newcastle disease virus of $\geq 3 \log_2$

Percentage of broilers that had ND HI antibody titers of $\geq 3 \log_2$ are presented in table 4.7. At two weeks of age, subgroups 0 (G), 0 (B), 5 (B), 2.5 (D) and 5 (D) had $\geq$ 85 % antibody titers of $\geq 3 \log_2$. Subgroup 5 (A) had the highest, 81 % $\geq 3 \log_2$ antibodies at seven weeks of age.

Table 4.3: Percentage of broilers with positive hemagglutination inhibition antibody titers of $\geq$ 1 $\log_2$ to Newcastle disease vaccine, Group G1, administered levamisole (at 16 days of age) and challenged with Newcastle disease virus (at 62 days of age).

Group G1	Age in days							
	14	21	28	35	42	49	56	70
Levamisole [in mg/kg]	Per cent positive							
0	100	52.6	31.6	0	7.1	23.1	33.3	100
2.5	66.7	55.6	20	0	0	28.6	66.7	100
5	62.5	60	64.3	6.7	13.3	0	46.7	71.4

Table 4.4: Percentage of broilers with positive hemagglutination inhibition antibody titers of $\geq$ 1 $\log_2$ to Newcastle disease vaccine, Group B, administered Gumboro vaccine with levamisole (at 30 days of age) and La Sota vaccine (at 44 days of age).

Group B	Age in days							
	14	21	28	35	42	49	63	70
Levamisole [in mg/kg]	Per cent positive							
0	90	55	57.9	0	68.8	66.7	86.7	80
2.5	86.7	52.6	58.8	0	75	38.5	66.7	92
5	94.1	68.8	75	0	66.7	50	66.7	60

Table 4.5: Percentage of broilers with positive hemagglutination inhibition antibody titers of $\geq$ 1 $\log_2$ to Newcastle disease vaccine, Group D, administered levamisole and La Sota vaccine at 30 and 44 days of age respectively.

Group D	Age in days								
Levamisole [in mg/kg]	14	21	28	35	42	49	56	63	70
	Per cent positive								
0	62.5	45	35	5	0	7	7.7	100	83.3
2.5	88.2	55.6	33.3	0	0	5.6	23.5	93.3	66.7
5	100	42.9	37.5	0	5	15.8	33.3	94.4	57.1

Table 4.6: Percentage of broilers with positive hemagglutination inhibition antibody titers of $\geq$ 1 $\log_2$ to Newcastle disease vaccine, Group G2, administered levamisole at 30 days of age.

Group G2	Age in days							
	14	21	28	35	42	49	56	70
Levamisole [in mg/kg]	Per cent positive							
0	100	52.6	31.6	0	7.1	23.1	33.3	100
2.5	90	44.4	40	20	10	11.1	50	88.9
5	77.8	40	53.8	0	0	23.1	35.7	91.7

Table 4.7: Percentage of broilers with Newcastle disease vaccine hemagglutination inhibition antibody titers of $\geq 3 \log_2$

Group	Levamisole [in mg/kg]	Age in days								
		14	21	28	35	42	49	56	63	70
		Per cent positive $\geq 3 \log_2$								
A[a]	0	80	5	60	0	56	47	NS	71	85[g]
	2.5[e]	82	15	74	10	63	44	NS	57	88[g]
	5[e]	83	21	63	11	41	81	NS	69	82[g]
C[b]	0	71	20	35	0	58	65	93	60	100[g]
	2.5[e]	83	20	44	0	50	40	71	50	82[g]
	5[e]	78	15	42	6	47	25	75	69	100[g]
G	0	89	21	21	0	0	23	25	NS	89[g]
G1	2.5[e]	67	22	10	0	0	14	50	NS	100[g]
	5[e]	63	20	43	0	13	0	40	NS	64[g]
B[c]	0	85	25	53	0	56	53	NS	60	73
	2.5[f]	80	21	53	0	31	15	NS	47	85
	5[f]	94	25	75	0	47	17	NS	42	60
D[d]	0	63	25	15	0	0	7	8	66	83
	2.5[f]	88	11	11	0	0	6	18	60	60
	5[f]	86	7	19	0	5	11	28	78	57
G2	2.5	60	0	30	0	0	11	38	NS	89
	5	56	0	38	0	0	0	29	NS	83

NS= Not sampled e= Administered levamisole at 16 days of age
g= Post challenge antibody titer f= Administered levamisole at 30 days of age
a\c= Administered Gumboro and La Sota vaccines at 16\30 and 30\44 days of age respectively;
b\d= Administered La Sota vaccine at 30\44 days of age respectively

Subgroup 2.5 (G2) had 89 % while 2.5 (B) had 85 % $\geq$ 3 $\log_2$ antibody titers at ten weeks of age. Subgroup 0 (C) had 93 % of birds having $\geq$ 3 $\log_2$ antibody titers at eight weeks of age. Subgroup 0 (D) had 83 % of birds having $\geq$ 3 $\log_2$ antibody titers at ten weeks of age.

4.2 Effect of Levamisole on Total Protein

Administration of levamisole at 16 days of age in group A, C and G broilers, had no significant effect on their total protein values at 62 days of age (P> 0.05). The values ranged from 3.1 ± 0.17 g\dl in subgroup 2.5 (A) to 4.0 ± 0.31 g\dl in subgroup 5 (G1) (Figure 4.13).

4.3 Effect of Levamisole on Packed Cell Volume

The effect of administration of levamisole at 16 days of age on mean PCV was evaluated at 62 days of age (Figure 4.14). There was statistically significant difference on mean PCV of broilers at 62 days of age (P< 0.001).The mean PCV values of broilers in vaccinated group A and C were lower than those in non vaccinated group G and E. The highest mean PCV value of 35.2 ± 1.93 (mean ± SE) was observed in subgroup 2.5 (G1). The lowest mean PCV value of 27.6 ± 1.03 was observed in subgroup 2.5 (A). Levamisole administered at 16 days of age caused further decrease in mean PCV value of vaccinated subgroup 2.5 (A) and 5 (C) (29.2 ± 1.43). On the other hand, levamisole caused slightly higher mean PCV value of non vaccinated subgroup 2.5 (G1) and 5 (G1) [34.2 ± 1.69].

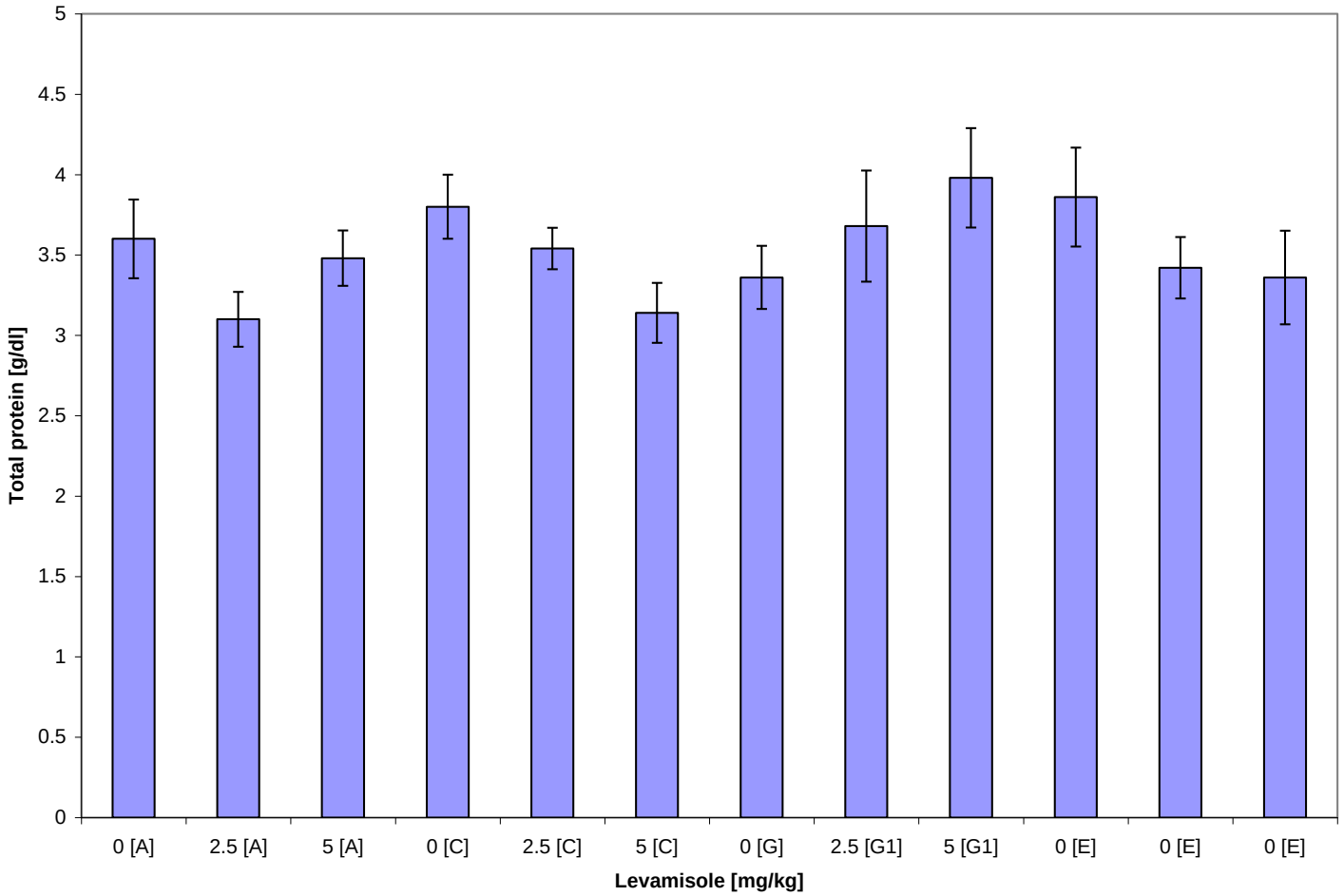


Figure 4.30: Total protein levels of broilers at 62 days of age. Groups: A, administered Gumboro vaccine with levamisole (16 days of age) and La Sota vaccine (30 days of age); C, administered levamisole (16 days of age) and La Sota vaccine (30 days of age); G/G1 and E, not vaccinated with Gumboro or La Sota vaccines; G1, administered levamisole at 16 days of age.

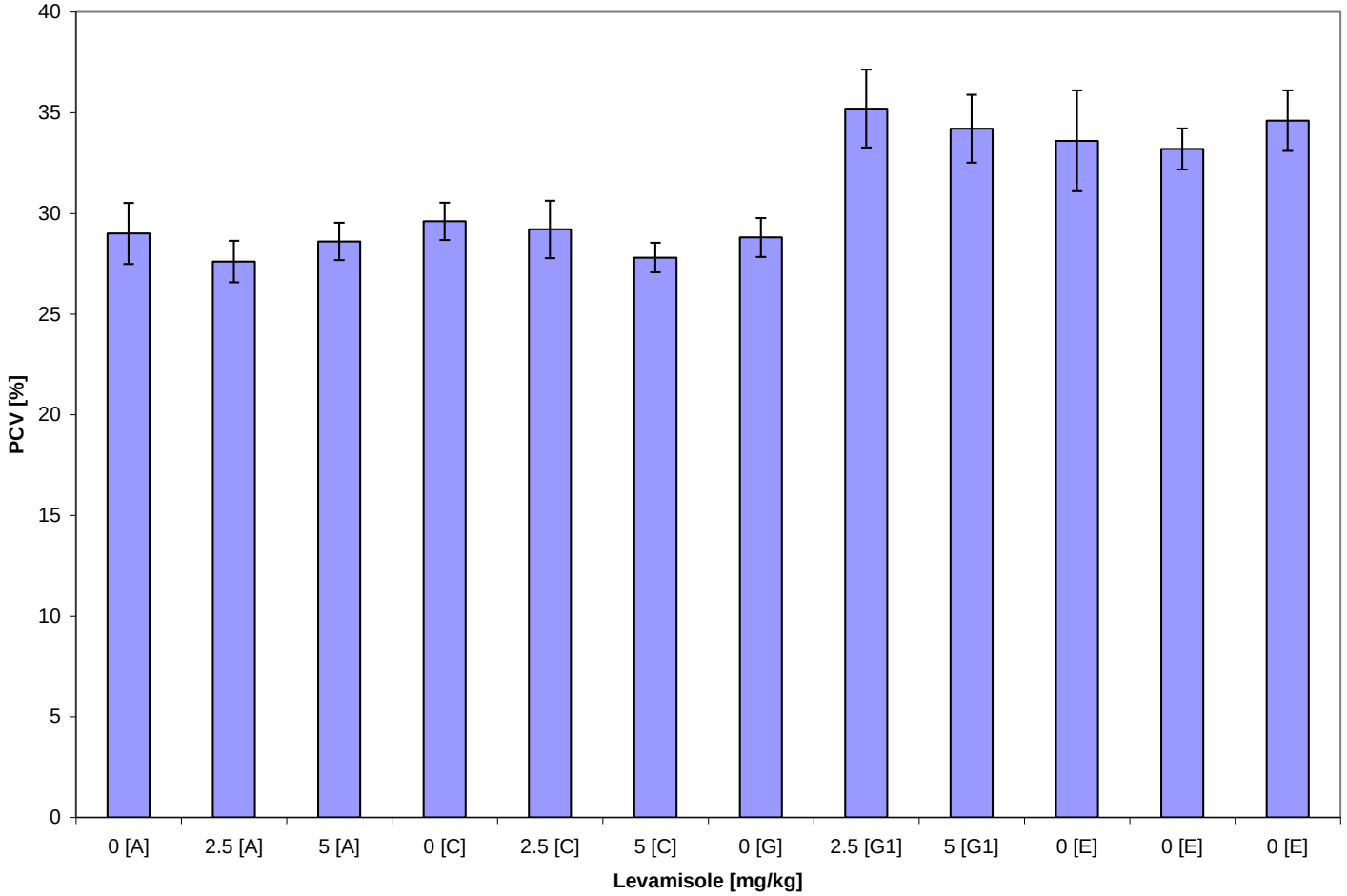


Figure 4.31: Packed cell volume of broilers at 62 days of age. Groups: A, administered Gumboro vaccine with levamisole (16 days of age) and La Sota vaccine (30 days of age); C, administered levamisole (16 days of age) and La Sota vaccine (30 days of age); G/G1 and E, not vaccinated with Gumboro or La Sota vaccines; G1, administered levamisole at 16 days of age.

4.4 Effect of Challenge with Newcastle Disease Virus Kudu 113 Strain

Broiler groups A, C, G1 and E challenged with NDV Kudu 113 strain did not show clinical signs and none died. No gross lesions were observed in the birds euthanized, one week post challenge.

4.5 Effect of Levamisole on Organs to Body Weight Ratios

There was a statistical significant difference between the lowest and the highest bursal body weight (BBW) ratio ($P< 0.05$) at 69 days of age. Subgroup 2.5 (A) showed the lowest BBW ratio while subgroup 2.5 (E) showed the highest BBW ratio. Other differences observed were not statistically significant ($P> 0.05$). Each subgroup administered 5 mg/kg levamisole showed better BBW ratio than subgroup administered 2.5 mg/kg levamisole, except in group E. Broilers administered levamisole in group C had higher BBW ratio than those administered levamisole in group A. Similarly, broilers administered levamisole in group E had higher BBW ratio than those administered levamisole in group G. Among subgroups without levamisole, 0 (A) had the highest BBW ratio. Broilers not administered levamisole had higher BBW ratio than those administered levamisole in group A and G (Figure 4.15).

Subgroup 2.5 (E) had the lowest spleen body weight SBW ratio while subgroup 2.5 (G1) had the highest SBW ratio. Each subgroup administered 5 mg/kg levamisole had higher SBW ratio than subgroup administered 2.5 mg/kg levamisole, except in group G1. Broilers administered levamisole in group A had higher SBW ratio than those administered levamisole in group C. Similarly, broilers administered levamisole in group G had higher

SBW ratio than those administered levamisole in group E. Broilers administered levamisole had higher SBW ratio than those not administered levamisole in group A and G. The differences in the SBW ratios were not statistically significant (P> 0.05) (Figure 4.16).

Each subgroup administered 5 mg/kg levamisole had higher thymus body weight (TBW) ratio than subgroup administered 2.5 mg/kg levamisole. Broilers not administered levamisole in group A had higher TBW ratio than those administered levamisole. In subgroups that were not administered levamisole, 0 (A) had the highest TBW ratio. Broilers administered levamisole in group C had higher TBW ratio than those administered levamisole in group A. Similarly, broilers administered 5 mg/kg levamisole in group E had higher TBW ratio than those administered 5 mg/kg levamisole in group G1. The differences in the TBW ratios were not statistically significant (P> 0.05) (Figure 4.17).

4.6 Histopathology

Histological appearance of the bursa at 69 days of age was normal in most groups. In subgroup 2.5 (G1) and 0 (C), some bursae showed atrophic changes. These changes included depletion of follicular lymphoid cells and infolding of epithelium of some follicles. The bursa in subgroup 0 (A) had active follicles with less conspicuous cortex and medulla (Plate I). The bursa in subgroup 2.5 (A) showed atretic follicles with marked interstitial or inter follicular edema and proliferation of connective tissue (Plate II). The bursa in subgroup 5 (A) had fully developed follicles with high cellularity of (Plate III).

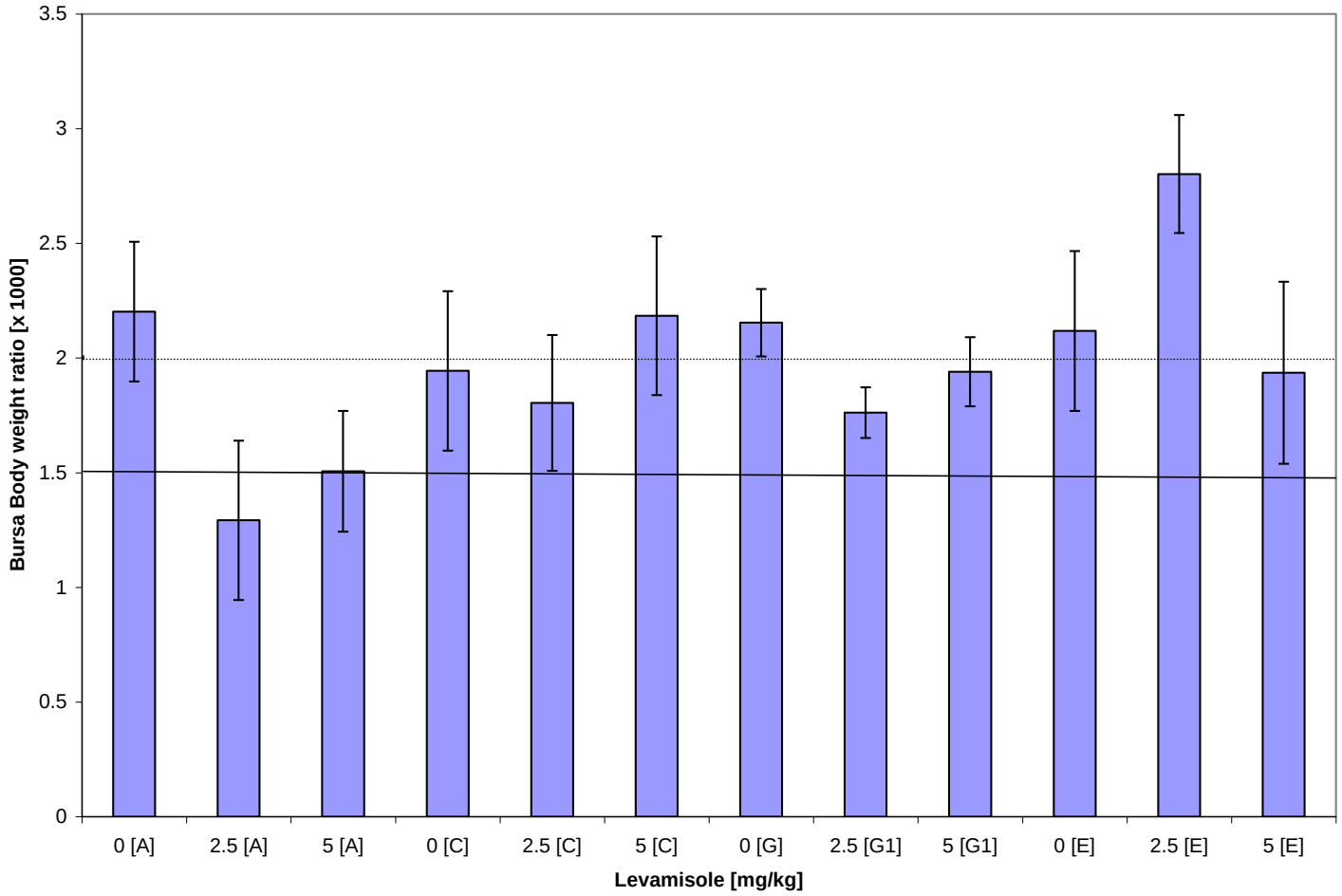


Figure 4.32: Bursa body weight ratio at 69 days of age. Groups: A, administered Gumboro vaccine with levamisole (16 days of age) and La Sota vaccine (30 days of age); C, administered levamisole (16 days of age) and La Sota vaccine (30 days of age); G/G1 and E, not vaccinated with Gumboro or La Sota vaccines; administered levamisole at 16 and 62 days of age respectively. Intact line indicate cut off point for good while dash line indicate cut off point for excellent bursal ratio.

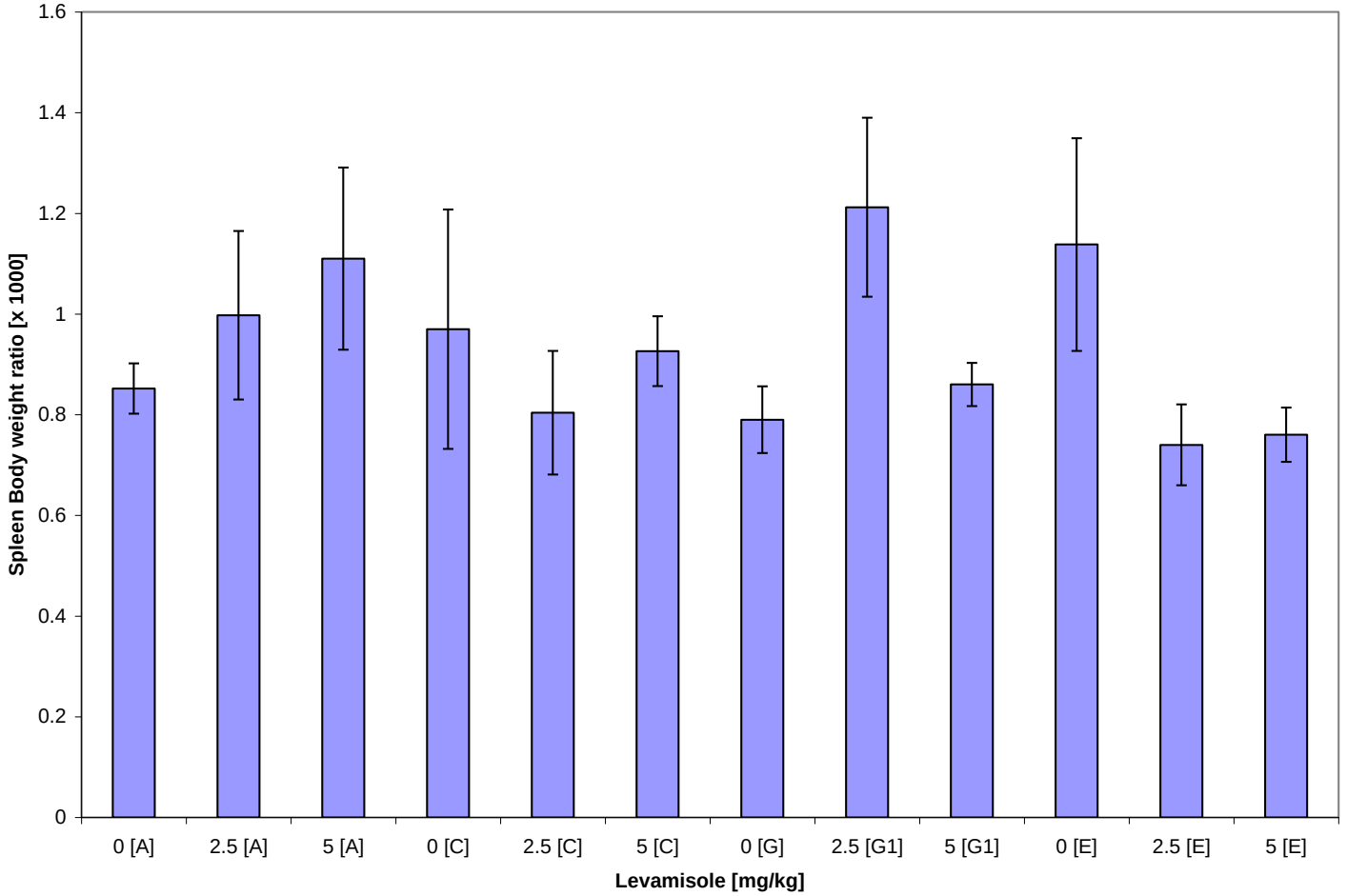


Figure 4.33: Spleen body weight ratio of broilers at 69 days of age. Groups: A, administered Gumboro vaccine with levamisole (16 days of age) and La Sota vaccine (30 days of age); C, administered levamisole (16 days of age) and La Sota vaccine (30 days of age); G/G1 and E, not vaccinated with Gumboro or La Sota vaccines; administered levamisole at 16 and 62 days of age respectively.

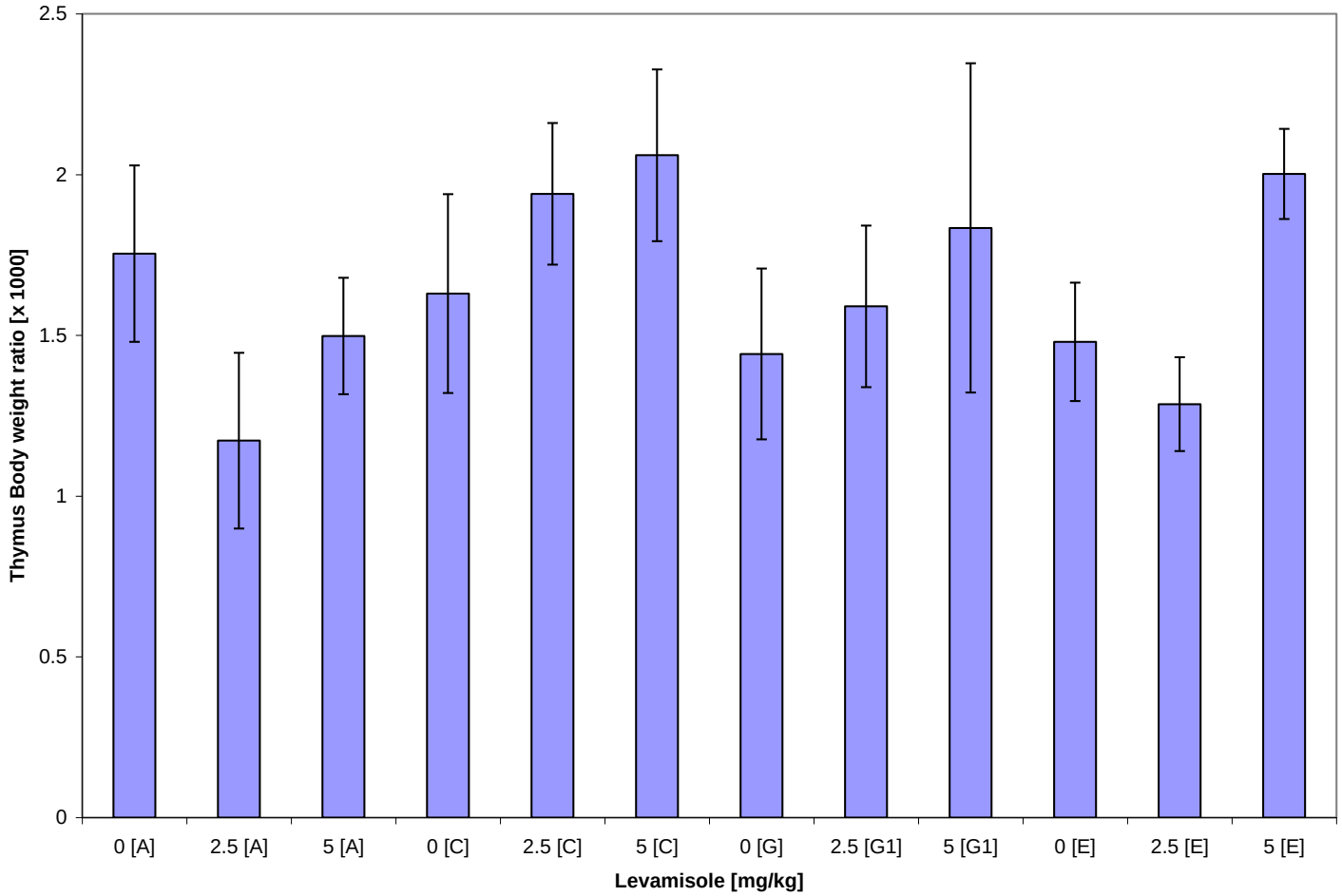


Figure 4.34: Thymus body weight ratio at 69 days of age. Groups: A, administered Gumboro vaccine with levamisole (16 days of age) and La Sota vaccine (30 days of age); C, administered levamisole (16 days of age) and La Sota vaccine (30 days of age); G/G1 and E, not vaccinated with Gumboro or La Sota vaccines; administered levamisole at 16 and 62 days of age respectively.



Plate I: Bursa of broilers administered Gumboro (16 days of age) and La Sota (30 days of age) vaccine without levamisole, one week post Newcastle disease challenge (62 days of age). Active follicles (F1) and no histologic lesions observed, H & E x 40.

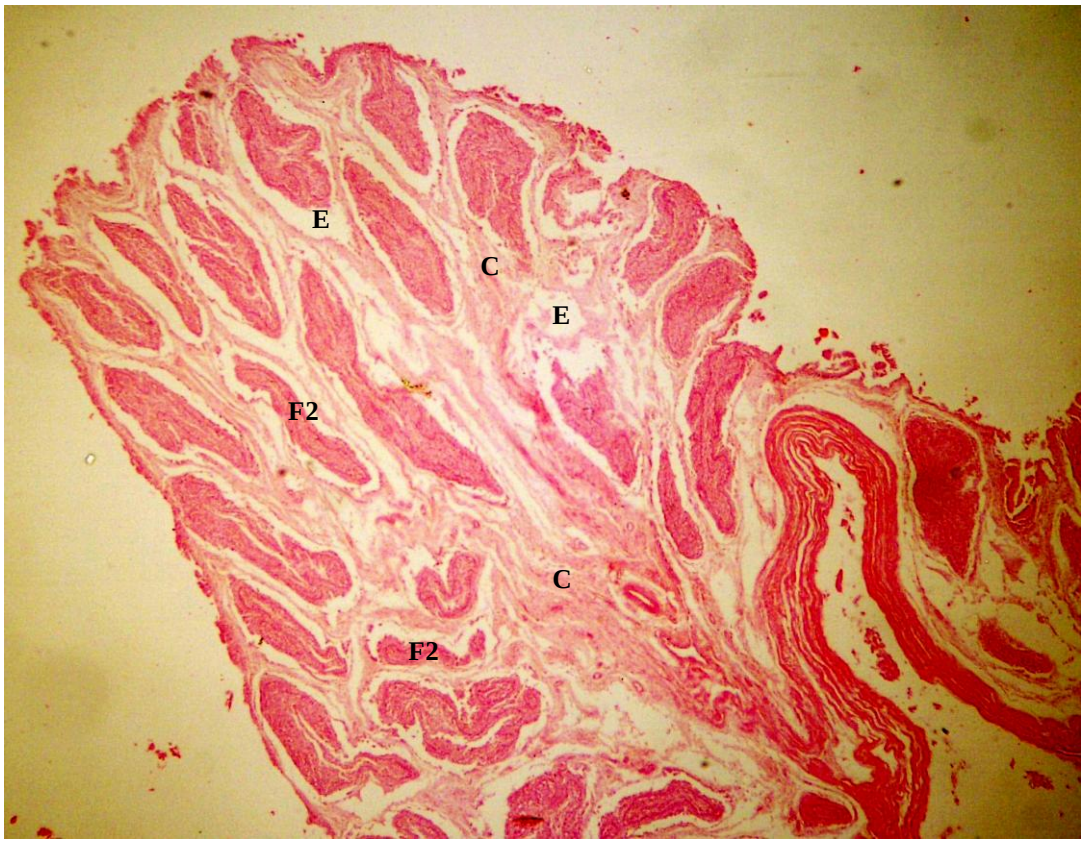


Plate II: Bursa of broilers administered 2.5 mg/kg levamisole with Gumboro (16 days of age) and La Sota vaccine (30 days of age), one week post Newcastle disease challenge (62 days of age). Note atretic follicles (F2); inter follicular edema (E) and proliferation of connective tissue (C), H & E x 40.

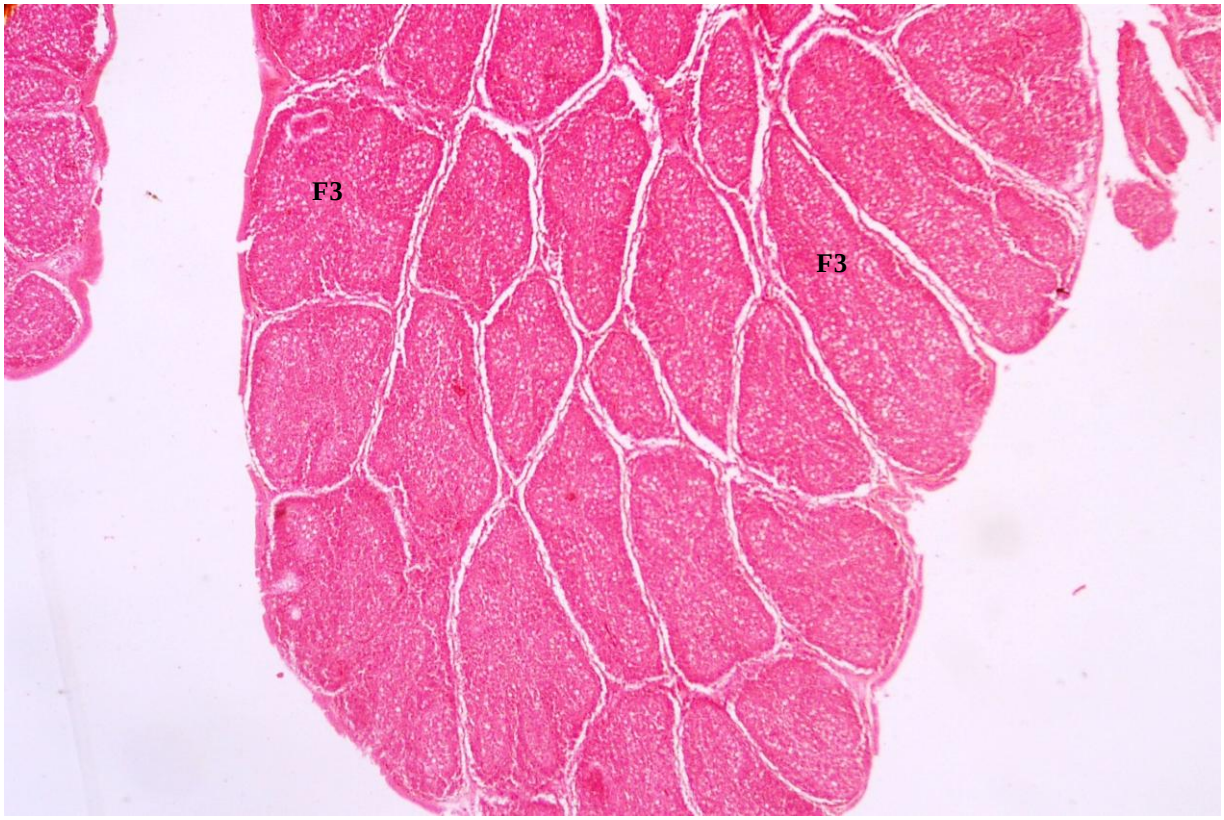


Plate III: Bursa of broilers administered 5 mg/kg levamisole with Gumboro (16 days of age) and La Sota vaccine (30 days of age), one week post Newcastle disease challenge (62 days of age). Note full development and cellularity in follicles (F3), H & E x 40.

There was no histopathologic lesion (NHL) in the thymus of broilers that were not administered levamisole (Plate IV). There was a decrease in cellularity with diffuse edema in the parenchyma of the thymus of broilers in subgroup 2.5 (A) (Plate V). The thymus of subgroup 5 (A) had increased cellularity with less conspicuous cortex and medulla (Plate VI).

There was NHL in the spleen of subgroup 0 (E) although there was a decrease in cellularity in the white pulp (Plate VII). In subgroup 2.5 (E) there was diffused edema and hypercellular (lymphoid cells) parenchyma in the spleen (Plate VIII). Spleen of subgroup 5 (E) broilers had diffused increase in cellularity as well as germinal centers in the white pulp (Plate IX).

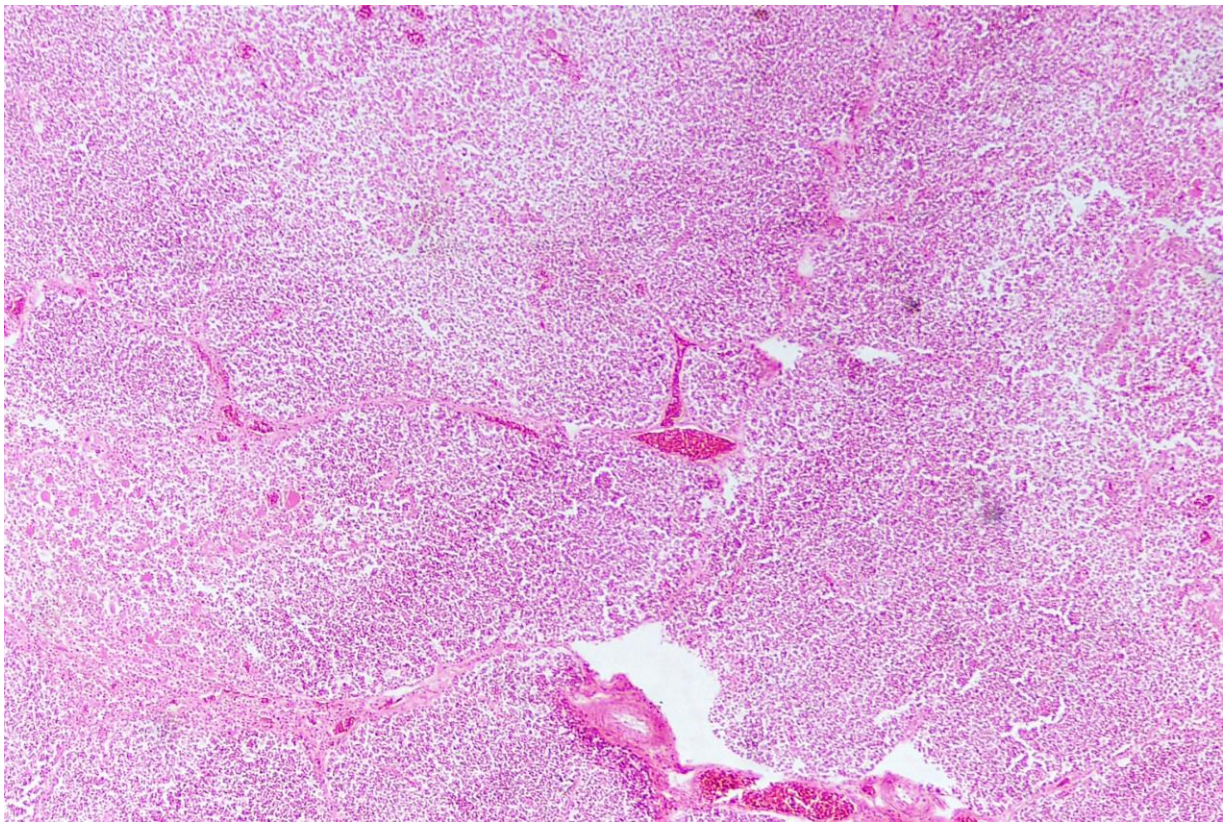


Plate IV: Thymus of broilers administered Gumboro (16 days of age) and La Sota (30 days of age) vaccine without levamisole one week post Newcastle disease virus challenge (62 days of age). No histologic lesions observed; the white space is an artifact, H & E x 100.

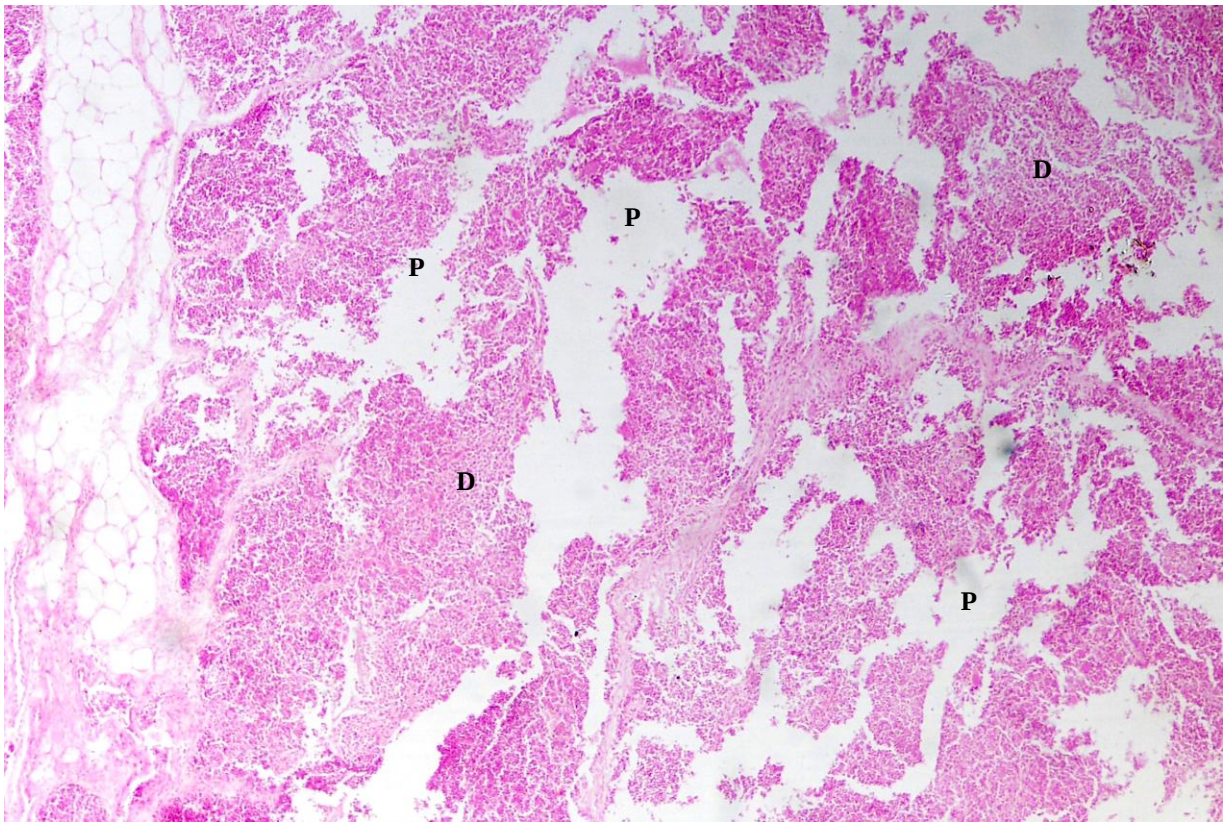


Plate V: Thymus of broilers administered 2.5 mg/kg levamisole with Gumboro (16 days of age) and La Sota vaccine (30 days of age), one week post Newcastle disease virus challenge (62 days of age). Note diffuse edema in the parenchyma (P) and decrease in cellularity (D), H & E x 100.

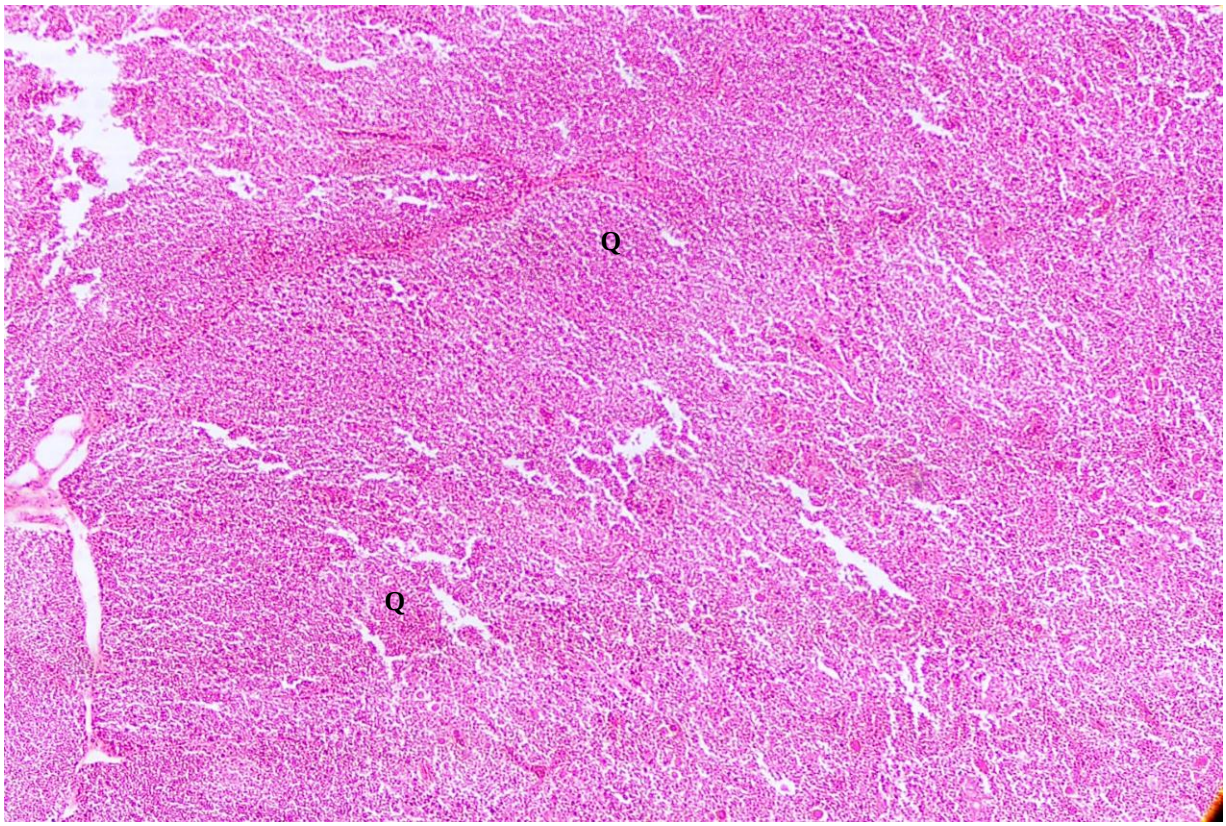


Plate VI: Thymus of broilers administered 5 mg/kg levamisole with Gumboro (16 days of age) and La Sota vaccine (30 days of age), one week post Newcastle disease virus challenge (62 days of age). Note increased cellularity in the parenchyma (Q), H & E x 100.

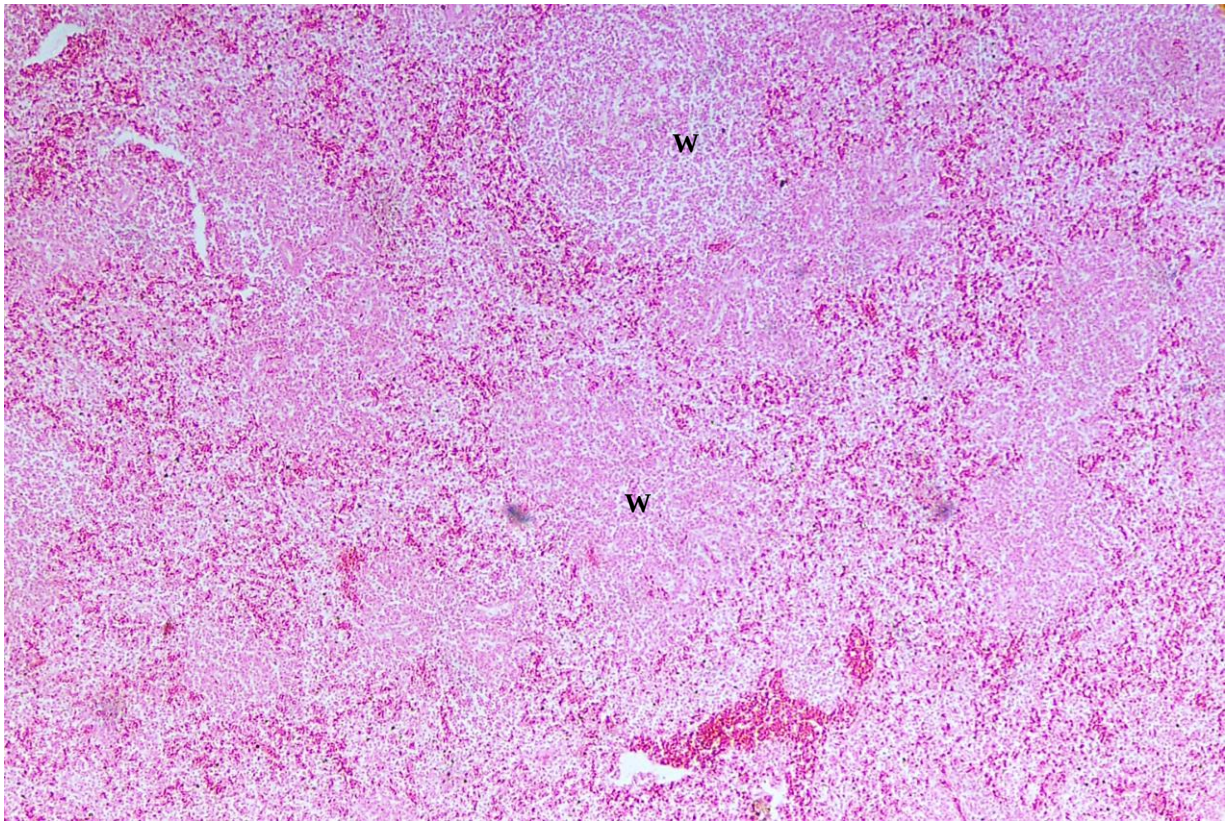


Plate VII: Spleen of broilers not administered levamisole, Gumboro and La Sota vaccines one week post Newcastle disease virus challenge, at 62 days of age. Note decrease cellularity in white pulp (W) H & E stain x 100.



Plate VIII: Spleen of broilers not administered Gumboro and La Sota vaccines, one week post Newcastle disease virus challenge and administration of 2.5 mg/kg levamisole at 62 days of age. Note diffuse edema (B) and hypercellular parenchyma (K), H & E stain x 100.

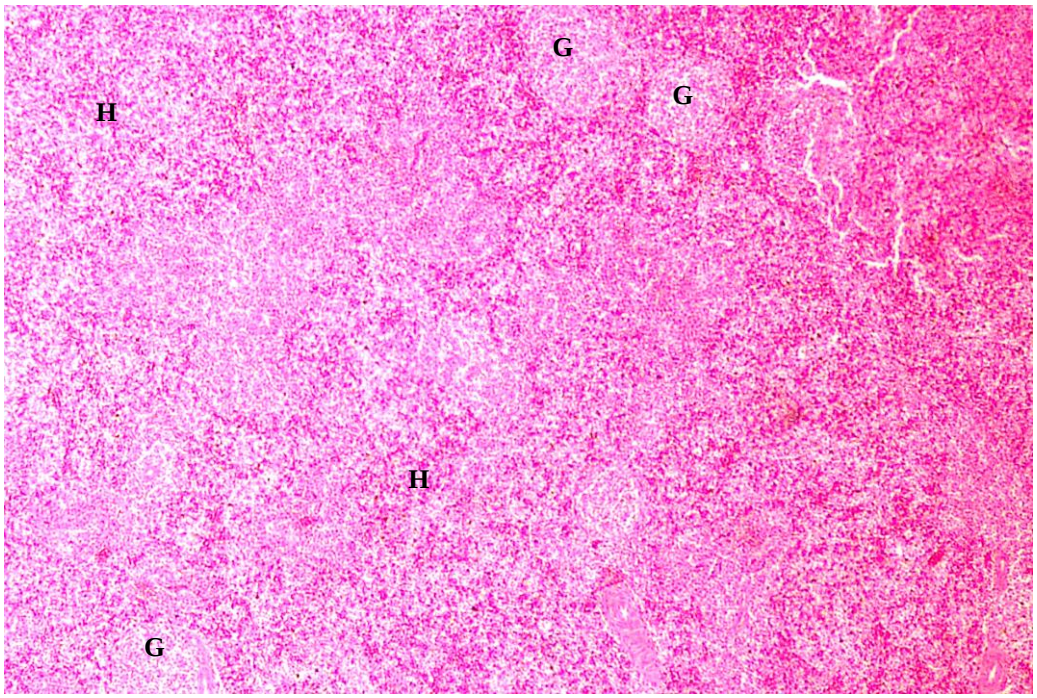


Plate IX: Spleen of broilers not administered Gumboro and La Sota vaccines, one week post Newcastle disease virus challenge and administration of 5 mg/kg levamisole at 62 days of age. Note increase in cellularity (H) and germinal centers (G) in white pulp, H & E stain x 100.

CHAPTER FIVE

5.0 DISCUSSION

The study was conducted to assess the best method of using levamisole to potentiate the humoral immune response to vaccination with ND La Sota vaccine which is often compromised by prior vaccination with immunosuppressive IBD vaccines (Rautenschlein *et al.*, 2007).

In the present study, precipitin antibodies were not detected by the AGID test following IBD vaccination. It was established that vaccination with IBD vaccines that are immunosuppressive, do not affect the production of antibodies to IBD virus itself (Kibenge *et al*., 1988). The absence of precipitin antibodies could be due to very low precipitin antibody titer in the sera tested. It had been reported previously that birds vaccinated with some IBD vaccines had been protected against challenge with IBD virus even though their sera tested negative or had low precipitin antibody titer (Okoye, 1985). Explanation consistent with negative AGID test in birds that were immune to IBD was that low antibody titers can not be detected by AGID test. Sera that were negative or with low antibody titer by AGID test were positive with higher antibody titers by ELISA (Oladele *et al*., 2004). Another possible explanation is that, precipitin antibodies may be present in the sera but did not react with the antigen used in the AGID test. This second possible explanation has to be ascertained experimentally.

The high antibody titer against NDV at two weeks of age in this experiment is similar to that obtained following day-old vaccination with B1 strains ND vaccine (Hofacre *et al*.,

1986). Chickens vaccinated at one day only with the B1 strains vaccines showed a decrease in antibody titers by 28 days of age. The antibody titers then increased by 42 days of age. On the other hand, chickens revaccinated at 14 days of age with the same ND vaccine showed a decrease in antibody titers from 14 to 42 days of age. However, chickens vaccinated with the B1 strains ND vaccines at 14 days of age only, showed sharp increase in antibody titers from 14 to 42 days of age (Hofacre *et al*., 1986). Similarly, chickens vaccinated with La Sota vaccine at five days of age had high antibody titers at two weeks of age. The titers then decreased sharply at three weeks of age (Ahmad *et al*., 2007). In these experiments, an increase or decrease in the level of antibody titer to NDV obtained following vaccinations were attributed to either low or high concentration of humoral antibody present at the time of vaccination respectively (Hofacre *et al*., 1986 and Ahmad *et al*., 2007). In the present experiment, analysis of weekly sera revealed further information on the pattern of increase or decrease in antibody titers to NDV. Sharp decreases were observed at three weeks of age then subsequent increase and decrease alternatively on weekly basis. These fluctuations in antibody titers were probably due to continued stimulation possibly by circulating ND vaccine virus. Exposure to a field NDV would have caused a steady increase or decrease in antibody titers.

Levamisole administered at 16 days of age potentiated humoral immune response following vaccination against ND at day-old. Higher antibody titers were shown in broilers administered levamisole at four weeks of age and this immunopotentiative effect was higher in broilers that had lower antibody titer at two weeks of age. The fluctuations obtained weekly during increase or decrease in the levels of antibody titers before and after

La Sota vaccine administration, as well as the sero conversion pattern in the control group indicated the possible presence of circulating NDV. This NDV was probably from birds vaccinated at day-old and the subsequent La Sota vaccine administered. It was reported that live ND vaccine virus must replicate in the host with occasional adverse reactions and possible excretion of the vaccine virus. Also, NDV transmission often occur sub clinically (Al-Garib *et al*., 2003a). Sufficient flock immunity is the only means to prevent or stop the transmission of NDV. However, flock immunity against ND could only be achieved when $\geq$ 85 % of vaccinated birds had antibody titers of $\geq 3 \log_2$ (van Boven *et al*., 2008). In the present study, birds administered La Sota vaccine attained flock immunity status four weeks post vaccination. Birds that were not administered IBD and La Sota vaccines nevertheless attained flock immunity but at a later age, 62 to 69 days, probably due to day-old vaccination against ND and the possible circulating ND vaccine virus.

The immunopotentiative effect of levamisole in this study was shown to be associated with concurrent administration of IBD vaccine. Levamisole was immunopotentiative at 5 mg/kg and 2.5 mg/kg when administered at 16 and 30 days of age respectively. This indicates that the lower dose of levamisole is more immunopotentiative in older birds. Immunopotentiation due to administration of levamisole had been shown to be dose dependent in normal chickens. It was shown by Soppi *et al*. (1979) that 0.25 mg/kg levamisole potentiated antibody response to thymus dependent antigen. They suggested that different concentrations of levamisole may affect different populations of lymphocytes. More recently, Yin *et al*. (2007) suggested that the effectiveness of levamisole and IFN-γ when used as adjuvants had to do with enhancement of balanced

cytokine production. The present study has demonstrated an inverse relationship between the dose of levamisole that produces immunopotentiation and the age in immunosuppressed birds.

An earlier report indicated that immunopotentiation by levamisole occur in birds immunosuppressed by IBD virus (Singh and Dhawedkar, 1993). In the present experiment, the immunopotentiation effect of levamisole was higher at 16 days of age. This could be related to the immunosuppressive effect of IBD virus in chickens less than three weeks of age (Lucio and Hitchner, 1980; Lukert, 1983; Kibenge *et al*., 1988).

Infectious bursal disease vaccine used in this study had immunopotentiative effect on antibody response to NDV in vaccinated birds. This is in contrast to earlier reports of immunosuppression of response to NDV vaccination (Ali *et al.*, 2004; Rautenschlein *et al*., 2007). The immunopotentiative effect of the IBD vaccine might be a result of stimulation of immune organs. It is possible that this immunopotentiation was due to a component of the IBD vaccine. A similar non-specific immunostimulation of immune organs by another IBD vaccine had been reported by Okoye (1985). He suggested that the immunopotentiation was produced by some vehicle or preservative in the vaccine. In the present experiment, administration of levamisole seems to have inhibited the action of the immunostimulatory component of the IBD vaccine. Birds vaccinated with the IBD vaccine and administered 2.5 mg/kg levamisole showed some level of immunosuppression whereas those administered 5 mg/kg levamisole were immunopotentiated. This indicates that

levamisole antagonizes the immunopotentiative effect of the IBD vaccine used in this study.

Levamisole slightly potentiated the humoral immune response to ND vaccination at day-old. This effect was limited to the period when levamisole was still in the birds system. Also the effect was limited to levamisole administered at 16 days of age. Levamisole did not potentiate the antibody response to La Sota vaccine administered one week after treatment with levamisole. Withdrawal period for levamisole at 40 mg/kg is nine days in eggs and eighteen days in meat (El-kholy and Kemppainen, 2005). This implies that the withdrawal time for levamisole at 5 mg/kg might be less than seven days. This indicates that age of chicken and concurrent therapy with levamisole is important in the potentiation of immune response to vaccination against NDV by levamisole. Chenchev *et al.* (1981) were unable to stimulate immunity against ND in birds administered NDV La Sota vaccine using levamisole. This was probably due to the age (eight weeks) of the birds during the trial.

Apart from the dose of levamisole, the level of NDV antibodies at the time of administration played a role in determining the magnitude of the immunopotentiation by levamisole. Among birds administered IBD and La Sota vaccines, it was shown that 2.5 mg/kg or 5 mg/kg dose of levamisole that produced lower NDV antibody titer subsequently produced a higher NDV antibody titer the following week of age. Hence, it is important to note the level of NDV antibodies in addition to the age of birds to be administered levamisole in a vaccination program.

The ability of broilers to respond immunologically to stimulation by vaccination against ND or the possible circulating ND vaccine virus decreased gradually as the chickens aged. At five weeks of age, there were little or no detectible antibodies to ND, irrespective of levels of the antibodies in the broilers prior to five weeks. Comparatively, higher antibody titers in broilers administered IBD vaccine and levamisole at 16 days of age were apparent even at five weeks of age, likely as a result of immunopotentiation by levamisole.

From the results of this study the appropriate age to administer the first booster vaccination against ND in commercial broilers would be three weeks of age when the antibody titers are relatively low. High antibody titers at two weeks of age could interfere with vaccination against ND with live NDV vaccines. Another booster vaccination could be administered by six weeks of age. Vaccination at five weeks of age against ND should be avoided. This is because broilers showed poor humoral immune response to NDV at five weeks of age. Vaccination is effective when conducted in the presence of low antibody titer (Hofacre *et al.*, 1986).

Results of this study showed that all the birds challenged with a virulent ND virus were protected from morbidity and mortality. Moreover, there was an increase in the levels of their NDV HI antibodies post challenge. This shows that infection of the challenged ND virus occurred. Infection and circulation of NDV could only be prevented if challenge occurred when there is flock immunity (Hofacre *et al.*, 1986). It is evident from this study that prevention of circulation of NDV among broilers could not be achieved by administering La Sota vaccine once at four or six weeks of age. Revaccinations are

required for attainment and maintenance of flock immunity against NDV in broilers. However, revaccination with live ND vaccines four weeks post La Sota vaccine administration should be avoided. This is because flock immunity occurred in this study, four weeks post La Sota vaccine administration. High antibody titer against NDV may interfere with the replication of live ND vaccine virus if administered.

The total protein values obtained were within normal range of 3 – 6 g/dl in chickens (Campbell and Coles, 1986). This result shows that levamisole at very low doses may not affect the total protein levels of broilers. The minimum toxic dose of levamisole in chickens is 640 mg/kg (Charles and Roberson, 2001).

Decrease in PCV values were demonstrated in broilers vaccinated with IBD and La Sota vaccines. In an earlier study with chickens, NDV La Sota or NDV Komarov vaccine administered at three weeks of age caused decrease in PCV from one to eleven days post vaccination. The decrease in PCV was related to an increase in neuraminidase activity that occurred within the same period (Oladele *et al*., 2006). In the present study, the decrease in PCV was demonstrated 32 days post administration of La Sota vaccine. This further emphasizes the possible presence of circulating La Sota vaccine virus which might have produced neuraminidase. There was no fall in the PCV of broilers that were vaccinated at the hatchery against ND which were not administered La Sota vaccine even though their PCV levels were affected by levamisole administration. Levamisole was shown to cause further decrease in PCV of broilers vaccinated with La Sota vaccine. On the other hand, levamisole caused higher PCV in broilers that were not administered La Sota vaccine. This

appears to be the first indication of levamisole causing further decrease on PCV in chickens administered La Sota vaccine. The effect of levamisole on PCV was dose dependent in all the broilers treated. Also, the doses of levamisole that were immunopotentiative caused minimal decrease in PCV in birds administered La Sota vaccine.

Ratings established by Benneth and Stephens (2006) for BBW ratio in broilers was used to determined the cumulative effects of vaccination against IBD and\or ND, NDV challenge and levamisole administration. Broilers administered levamisole and IBD vaccine had the lowest BBW ratio. This low BBW ratio which is an indication of immunosuppression was not observed in the same group of birds that were not administered levamisole. This is an indication of the possible stimulation of the bursa by the IBD vaccine in the absence of levamisole. A comparison of levamisole dose showed 5 and 2.5 mg/kg levamisole to have caused better stimulation of the bursa at 16 and 62 days of age respectively. This further shows that 2.5 mg/kg levamisole has higher immunopotentiative effect than 5 mg/kg levamisole in older broilers. Also, the administration of 2.5 mg/kg levamisole during challenge with NDV at 62 days of age enhanced the response of the bursa following NDV challenge. This indicates that administration of levamisole to NDV infected chickens may be useful.

In the present study all groups showed lower spleen ratio contrary to their higher bursal ratio. This is in agreement with an earlier study by Smith and Hunt (2004) which demonstrated a negative relationship between spleen size and immunocompetence. This

means that a lower spleen ratio is an indication of a higher immunocompetence, contrary to the bursal ratio.

The effect of levamisole on the thymus was directly proportional to the different doses administered irrespective of the age of broilers during administration. The effect of levamisole was more pronounced in La Sota vaccinated broilers, especially when the result of the thymus body weight ratio was compared between all groups tested. This shows the significance of cell mediated immunity at providing protection against NDV. It also shows that levamisole enhances cell mediated immunity generally in both vaccinated and non vaccinated chickens. The immunostimulatory effect of levamisole on the thymus observed in this study is dose dependent, but age independent. It is also possible that previous observation made on the ability of levamisole to significantly increase humoral immune response to thymus dependent antigen, while no effect was observed on the antibody response to thymus independent antigen had to do with the ability of levamisole to enhance the thymus regardless of the age of the bird (Soppi *et al.*, 1979).

Histopathological examination in broilers administered IBD vaccine and levamisole at 16 days of age showed more immunologically active bursa and thymus at 5 mg/kg levamisole dose. In both immune organs, edema and depletion of cells reflected decreased immune responsiveness while increased cellularity signified increased immune responsiveness. The bursa and the thymus of broilers that were vaccinated with IBD vaccine at 16 days of age without levamisole being administered showed normal architectures. This shows lack of immunomodulation of the immune organs in the absence of levamisole.

The NDV challenge virus caused depletion of lymphocytes in the spleen of broilers that were not administered levamisole as well as IBD and La Sota vaccines. Administration of 2.5 and 5 mg/kg levamisole caused increased immunologic activity of the spleen probably through different mechanisms. Hyperplasia with edema was observed with 2.5 mg/kg levamisole while hyperplasia and germinal centers were observed in the spleen of birds treated with 5 mg/kg levamisole. Germinal centers are induced in the spleen by localization of immune complexes directly on follicular dendritic cells (Jeurissen *et al.,* 1998). Administration of levamisole during NDV challenge might have caused localization of NDV antibody complexes on follicular dendritic cells in the spleen.

Several factors are responsible for the success or failure for the control of ND through vaccination. Most of the factors are not being considered when vaccinating poultry against ND in the field. This study was able to provide more information that would assist the Farmers in preventing as well as controlling ND outbreaks in commercial poultry particularly in immunocompromized birds. The study has shown that concurrent administration of 5 mg/kg levamisole with IBD vaccine in broilers less than three weeks old would potentiate humoral immune response to ND vaccination. In older broilers, concurrent administration of 2.5 mg\kg levamisole with IBD vaccine would potentiate humoral immune response to ND vaccination. Broilers could be vulnerable to NDV infection at five weeks of age because of very low or total lack of antibodies to NDV. Further studies need to be conducted, especially field studies to ascertain the efficiency of using levamisole for enhancing immunity in the chickens under various field conditions.

SUMMARY CONCLUSIONS AND RECOMMENDATIONS

Summary

Antibodies to IBD virus were not detected in broilers by AGID test following vaccination with IBD vaccine at 16 or 30 days of age.

The IBD vaccine used (Bursine®- 2) probably had a component that was immunostimulatory.

Levamisole at the dose of 5 or 2.5 mg/kg potentiated the immune response to ND vaccination and challenge at 16 or 30 and 62 days of age respectively.

Levamisole 5 mg/kg showed higher stimulation on the thymus irrespective of age.

Administration of levamisole and La Sota vaccine caused a decrease in the PCV of broilers.

The levels of humoral antibodies to NDV were low or absent at five weeks of age.

Flock immunity against ND occur four weeks post administration of La Sota vaccine at 30 days of age.

All birds vaccinated against ND at day-old or with La Sota vaccine were protected when challenge with NDV Kudu 113 strain.

Conclusions

Levamisole at the dose rate of 5 mg/kg was effective at potentiating the immune response of broilers to La Sota vaccination at 30 days of age when administered every other day for one week, starting from the day of IBD vaccination at 16 days of age.

A lower dose of 2.5 mg/kg levamisole was effective at potentiating the immune response of broilers to La Sota vaccination at 44 days of age when administered every other day for one week, starting from the day of IBD vaccination at 30 days of age.

Levamisole caused a decrease in PCV of broilers administered La Sota vaccine.

The immunopotentiative effect of levamisole is related to increased cellular activity in the bursa, thymus and spleen.

Revaccination with La Sota vaccine at 30 days following day-old vaccination, confer flock immunity against ND four weeks post administration of La Sota vaccine.

All broilers had little or no antibodies against ND in their sera at five weeks of age irrespective of vaccination against ND and\or IBD and levamisole administration.

Recommendations

For effective management of immonosuppresive effect of IBD virus, poultry farmers should administer 5 mg/kg levamisole in drinking water (every other day) for one week, after vaccinating chickens with IBD vaccine at two weeks.

Booster vaccination with ND La Sota should be avoided at two weeks of age in broilers that have been vaccinated against ND at day-old or at four weeks interval following ND La Sota vaccine administration.

Research should be conducted to ascertain the mechanism (s) through which levamisole affects PCV in chickens.

It is recommended that the antigenic relationship between the IBD vaccine virus in Bursine®-2 and the IBD field virus used in the experiment be determined.

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APPENDICES

Appendix I: Weekly HI antibody titers ($\log_2$) for Newcastle disease vaccine in experimental broilers (mean ± SE)

Group	Age (weeks)	Samples tested	Mean ± Std. Error of Mean
A (0)	2	15	4.40 ± 0.576
	3	19	1.00 ± 0.315
	4	20	3.00 ± 0.637
	5	17	0.06 ± 0.059
	6	16	3.19 ± 0.526
	7	15	2.40 ± 0.638
	9	14	4.79 ± 0.750
	10	13	5.69 ± 0.880
A (2.5)	2	17	4.35 ± 0.535
	3	20	1.05 ± 0.366
	4	20	4.45 ± 0.790
	5	20	0.75 ± 0.528
	6	19	2.79 ± 0.499
	7	18	2.44 ± 0.668
	9	14	2.64 ± 0.570
	10	17	5.12 ± 0.618
A (5)	2	18	4.17 ± 0.345
	3	19	1.74 ± 0.396
	4	19	4.05 ± 0.811
	5	19	0.95 ± 0.623
	6	17	2.06 ± 0.424
	7	16	4.56 ± 0.658
	9	13	2.85 ± 0.517
	10	11	4.45 ± 0.743
B (0)	2	20	4.70 ± 0.553
	3	20	1.45 ± 0.380
	4	19	3.68 ± 0.855
	5	20	0.00 ± 0.000
	6	16	2.63 ± 0.531
	7	15	3.13 ± 0.761
	9	15	3.47 ± 0.646
	10	15	4.80 ± 0.823
B (2.5)	2	15	4.13 ± 0.551
	3	19	1.21 ± 0.330
	4	17	3.88 ± 1.014
	5	19	0.00 ± 0.000
	6	16	1.94 ± 0.370
	7	13	1.00 ± 0.467
	9	15	2.60 ± 0.653

	10	13	4.92 ± 0.729
B (5)	2	17	4.47 ± 0.403
	3	16	1.50 ± 0.354
	4	16	3.75 ± 0.744
	5	17	0.00 ± 0.000
	6	15	2.13 ± 0.477
	7	12	1.75 ± 0.698
	9	12	2.42 ± 0.701
	10	10	4.20 ± 1.272
C (0)	2	17	3.82 ± 0.589
	3	20	1.20 ± 0.321
	4	20	2.35 ± 0.792
	5	19	0.00 ± 0.000
	6	12	2.33 ± 0.482
	7	17	3.88 ± 0.757
	8	14	6.00 ± 0.825
	9	15	3.67 ± 0.715
	10	14	5.71 ± 0.384
C (2.5)	2	18	3.89 ± 0.484
	3	20	0.90 ± 0.307
	4	18	2.94 ± 0.842
	5	18	0.00 ± 0.000
	6	14	2.14 ± 0.479
	7	15	2.60 ± 0.815
	8	14	3.79 ± 0.648
	9	14	3.21 ± 0.813
	10	11	4.45 ± 0.767
C (5)	2	18	3.67 ± 0.518
	3	20	1.10 ± 0.261
	4	19	2.79 ± 0.881
	5	18	0.06 ± 0.056
	6	15	2.47 ± 0.487
	7	15	1.87 ± 0.703
	8	16	5.12 ± 0.884
	9	13	4.00 ± 0.760
	10	15	5.33 ± 0.398
D (0)	2	16	3.44 ± 0.747
	3	20	1.10 ± 0.332
	4	20	1.10 ± 0.397
	5	20	0.10 ± 0.100
	6	17	0.00 ± 0.000
	7	14	0.21 ± 0.214
	8	13	0.23 ± 0.231

	9	12	3.42 ± 0.690
	10	12	5.58 ± 0.925
D (2.5)	2	17	4.35 ± 0.469
	3	18	1.00 ± 0.256
	4	18	0.89 ± 0.378
	5	20	0.00 ± 0.000
	6	18	0.00 ± 0.000
	7	18	0.22 ± 0.222
	8	17	1.00 ± 0.477
	9	15	3.53 ± 0.584
	10	15	3.20 ± 0.757
D (5)	2	14	4.07 ± 0.370
	3	14	0.86 ± 0.329
	4	16	1.19 ± 0.449
	5	20	0.00 ± 0.000
	6	20	0.15 ± 0.150
	7	19	0.42 ± 0.257
	8	18	1.33 ± 0.492
	9	18	5.11 ± 0.685
	10	14	3.29 ± 0.841
E (0)	2	11	4.09 ± 0.595
	3	13	1.69 ± 0.444
	4	13	3.69 ± 1.070
	5	15	0.00 ± 0.000
	6	15	0.27 ± 0.267
	7	15	0.00 ± 0.000
	8	15	1.67 ± 0.741
	10	13	6.15 ± 0.478
E (2.5)	2	14	3.71 ± 0.450
	3	13	1.15 ± 0.436
	4	14	2.00 ± 0.646
	5	15	0.00 ± 0.000
	6	15	0.00 ± 0.000
	7	14	0.43 ± 0.202
	8	13	2.00 ± 0.784
	10	13	5.15 ± 0.492
E (5)	2	11	5.18 ± 0.377
	3	13	1.15 ± 0.436
	4	13	3.54 ± 1.042
	5	16	0.00 ± 0.000
	6	16	0.00 ± 0.000
	7	15	0.60 ± 0.363
	8	14	2.64 ± 0.599
	10	11	4.27 ± 0.875

G (0)	2	18	4.28 ± 0.369
	3	19	1.32 ± 0.359
	4	19	1.84 ± 0.796
	5	17	0.00 ± 0.000
	6	14	0.07 ± 0.071
	7	13	0.85 ± 0.465
	8	12	1.50 ± 0.702
	10	9	5.22 ± 0.596
G1 (2.5)	2	9	3.22 ± 0.830
	3	9	1.44 ± 0.503
	4	10	1.10 ± 0.900
	5	8	0.00 ± 0.000
	6	7	0.00 ± 0.000
	7	7	1.00 ± 0.724
	8	6	3.00 ± 1.211
	10	6	5.83 ± 0.654
G1 (5)	2	8	3.00 ± 1.000
	3	5	1.60 ± 0.748
	4	14	3.93 ± 1.234
	5	15	0.07 ± 0.067
	6	15	0.40 ± 0.273
	7	15	0.00 ± 0.000
	8	15	2.60 ± 0.827
	10	14	4.71 ± 1.040
G2 (2.5)	2	10	3.20 ± 0.646
	3	9	0.78 ± 0.324
	4	10	1.40 ± 0.600
	5	10	0.20 ± 0.133
	6	10	0.20 ± 0.200
	7	8	0.38 ± 0.375
	8	8	1.88 ± 0.875
	10	9	4.89 ± 0.824
G2 (5)	2	9	3.33 ± 0.799
	3	5	0.80 ± 0.490
	4	13	2.15 ± 0.706
	5	15	0.00 ± 0.000
	6	15	0.00 ± 0.000
	7	14	0.36 ± 0.199
	8	14	1.57 ± 0.626
	10	12	5.00 ± 0.696

Appendix II: Body, bursa, spleen and thymus weights of broilers challenged with Newcastle disease virus, one week post challenge (mean ± SE)

Group	Sample size	Weight (g)			
		Body	Spleen	Bursa	Thymus
A (0)	5	2220 ± 203	1.88 ± 0.185	4.96 ± 0.894	4.14 ± 0.561
A (2.5)	4	2008 ± 183	2.08 ± 0.449	2.30 ± 0.518	2.65 ± 0.596
A (5)	5	1960 ± 208	2.15 ± 0.381	3.12 ± 0.825	3.03 ± 0.566
C (0)	5	2050 ± 94	2.11 ± 0.321	4.07 ± 0.849	3.44 ± 0.778
C (2.5)	5	2280 ± 116	2.06 ± 0.290	4.74 ± 0.541	4.50 ± 0.690
C (5)	5	2190 ± 198	2.04 ± 0.270	4.91 ± 1.063	4.64 ± 0.857
G (0)	5	2020 ± 115	1.56 ± 0.033	4.39 ± 0.500	3.08 ± 0.344
G1 (2.5)	5	2180 ± 149	2.67 ± 0.477	3.88 ± 0.445	3.57 ± 0.685
G1 (5)	5	2280 ± 90	1.97 ± 0.141	4.42 ± 0.370	4.37 ± 0.904
E (0)	5	2020 ± 108	2.37 ± 0.552	4.29 ± 0.849	3.05 ± 0.494
E (2.5)	5	2200 ± 91	1.62 ± 0.177	6.18 ± 0.672	3.31 ± 0.455
E (5)	5	2060 ± 62	1.56 ± 0.100	4.47 ± 0.493	4.13 ± 0.363

Appendix III: Packed cell volume and total protein of broilers at 62 days of age (mean ± SE)

Group	Sample size	PCV (%)	Total protein (g/dl)
A (0)	5	29.0 ± 1.517	3.6 ± 0.245
A (2.5)	4	27.6 ± 1.030	3.1 ± 0.170
A (5)	5	28.6 ± 0.927	3.5 ± 0.171
C (0)	5	29.6 ± 0.927	3.8 ± 0.200
C (2.5)	5	29.2 ± 1.428	3.5 ± 0.129
C (5)	5	27.8 ± 0.735	3.1 ± 0.186
G (0)	5	28.8 ± 0.970	3.4 ± 0.196
G1 (2.5)	5	35.2 ± 1.934	3.7 ± 0.346
G1 (5)	5	34.2 ± 1.685	4.0 ± 0.309
E (0)	5	33.6 ± 2.502	3.9 ± 0.308
E (2.5)	5	33.2 ± 1.020	3.4 ± 0.191
E (5)	5	34.6 ± 1.503	3.4 ± 0.291