# COMPARATIVE EVALUATION OF ANTIBODY PRODUCTION IN BROILERS VACCINATED WITH NEWCASTLE DISEASE VACCINES IN KANO METROPOLIS, NIGERIA

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**AUGUST, 2016.**

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**DEPARTMENT OF MICROBIOLOGY,**

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## DECLARATION

I declare that the work in this dissertation, entitled "**Comparative evaluation of antibody production in broilers vaccinated with Newcastle disease vaccines in Kano metropolis, Nigeria**" has been carried out by me in the Department of Microbiology, Faculty of Science, Ahmadu Bello University, Zaria, under the supervision of Professor J.B. Ameh and Professor E.D. Jata'u. The information derived from the literature has been duly acknowledged in the list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

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| --- | --- | --- |
| Name of student | Signature | Date |

## CERTIFICATION

This dissertation entitled "**Comparative evaluation of antibody production inbroilers vaccinated with Newcastle disease vaccines in Kano metropolis, Nigeria**" by Olubukola Olukemi SERIKI meets the regulation governing the award of the degree of Master of Science of the Ahmadu Bello University, Zaria and is approved for its contributions to knowledge and literary presentation.

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# DEDICATION

I dedicate this work to my Family; my Husband, Children, Parents and siblings who relentlessly give their support to me always.

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ***et al***: | And others |
| **OIE**: | Office internationalé  des epizootics |
| **CoEC**: | Commission of European Communities |
| **EU**: | European Union |
| **RNA**: | Ribonucleic Acid |
| **APMV**: | Avian Paramyxovirus |
| **MDT:** | Mean death time |
| **i.e**: | That is |
| **NDV**: | Newcastle Disease Virus |
| **ND**: | Newcastle Disease |
| **HI**: | Haemagglutination Inhibition |
| **HA**: | Haemagglutination |
| **Kb**: | Kilobase |
| **SPF**: | Specific pathogen Free |
| **DIVA**: | Differentiating Vaccinated Animals |
| **rNDV**: | Recombinant  Newcastle Disease Virus |

**cDNA**: Cloned Deoxyribonucleic acid

**≥**: Greater than or equal to

*****: Asterisk

**$^{o}$C**: Degrees Celsius

**±**: Plus or Minus

**μ**: micro

**>**: Greater than

**<**: Less than

## ABSTRACT

The incidence of Newcastle disease (ND) in Nigeria is high and is a persistent cause of mortality and (or) morbidity among vaccinated chickens causing huge economic losses. This study was carried out to evaluate the persistence of maternally derived antibody (MDA) against Newcastle Disease Virus (NDV), thermo-stability of the test vaccines, comparative analysis of humoral immune response against four La Sota NDV vaccines as well as the effect of time with respect to vaccination schedule. In study group I; four experimental groups A, B, C and D each which consisted of 10 birds were vaccinated with corresponding vaccines on day 14 (primer) and 28 (booster) and group E of 10 birds was maintained as unvaccinated control. Serum samples collected from five randomly selected birds among the vaccinated groups on day 17, 21, 24, 28 and 35 and from the unvaccinated group E on day 1, 3, 10, 14, 24, 28 and 35 were subjected to haemagglutination inhibition (HI) titre test. Study group II; two experimental groups i and ii consisted of 10 birds each primed on day 14 and boosted on day 35 with vaccines A and B. Serum samples were obtained on day 17, 21, 28 35 and 42 and HI titre measured. The test vaccines were subjected to varying storage temperature and Haemagglutination (HA) titre measured. It was found that MDA against NDV persisted till day 24. The results showed a significant decrease ($p<0.05$) in HI titre of chickens which were vaccinated at day 14 and 28. Chickens vaccinated at day 14 and boosted at day 35 produced better immune response. Haemaggltuination (HA) titre measured after vaccines were subjected to high temperature was as low as 0. This study has shown that MDA can serve as protection against infective form NDV in chickens for first two weeks of life and none of the test La Sota vaccine was thermo-stable. In conclusion, primary vaccination at day 14 followed by booster dose at day 35 may be followed for better immune response and protection against ND in broilers.

# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Background

Newcastle Disease (ND) is a viral disease of poultry. Mayo (2002) described the causative agent; Newcastle Disease Virus (NDV) as a member of the genus *Avulavirus*. It can present as a super acute, acute and sometimes sub-clinical contagious disease of poultry (Health *et al.,* 1991). Newcastle Disease infection takes place through direct contact with secretions of infected birds; principally via ingestion (feacal/oral route) and inhalation; fomites (feed, water, implements, sacks among others); hatching chicks may be infected through eggs for some ND virus strain but there is no clear evidence of the role of flies in mechanical transmission of the infective ND virus (Anon, 2013).

Three major panzootics of ND have been recorded and it is present in all continents except Antarctica (Alexander, 2003). Spradbrow (1999) described Epizootic and Enzootic ND in village chickens as non-self-limiting event that can smolder for several months and even years in a typical village housing one or two thousand birds. Epizootics occur when virus is introduced into a susceptible population with spectacular outbreaks and high mortalities which may cover whole villages or area within a short time and this form comes most readily to notice. Enzootic ND occurs when the virus transmits slowly in partially immune population such that there are few susceptible birds to maintain an outbreak and the occasional birds that die do not come to veterinary and public attention. These occur in commercial chickens in Nigeria (Ezeokoli *et al*., 1984; Echeonwu *et al.*, 1993). The role of exotic and wild birds in the epizootics of ND is not well understood; however, it is assumed that they play a critical role in the dissemination of virus

from the affected poultry farms to the non-affected farms within the country (Farooq *et al*., 2014).

Control measures involving slaughter, sanitary measures and vaccination are the best options in the fight against ND (Alexander, 2000). However, vaccination has been reported as the only safeguard against endemic ND (Orajaka *et al.,* 1999) but in developing countries like Nigeria, these policies are not well implemented (Okwor *et al*., 2009). The efficacy of vaccine is determined mainly by assessing the level of antibody produced in the target bird and the ability of the vaccinated birds to resist exposure to the virulent agent when compared with unvaccinated control (Spradbrow, 1993). The suggested and reported protective anti-ND virus antibody titre is HI $\geq Log_2 4$; 1:16 (OIE, 2004). By implication, antibody titre $< Log_2 4$ may not be protective. Nakamura *et al*. (2014) stated that it is curious that chickens vaccinated against ND were not protected against clinical disease caused by virulent ND virus infection and the reasons for vaccine failures against ND are still unknown. Factors which interfere with the immunization of commercial poultry may be divided into three main groups; factors associated with the vaccine itself, those associated with vaccine administration and those which are endogenous to the birds.

### 1.2 Statement of Research Problem

Newcastle disease is considered as one of the most important diseases of poultry and outbreaks with mortality up to 100% are common (Alders and Spradbrow, 2001; Sa'idu and Abdu, 2008), it causes large economic losses worldwide (Alexander, 2000). In Nigeria, until recently the occurrence of Avian Influenza, Newcastle disease was regarded as the deadliest and economically important disease of poultry (Okwor *et al.,* 2009). Newcastle disease has become

endemic in Nigeria in both local and commercial poultry with high epidemics recorded in highly susceptible flocks (Halle *et al.,* 1999; Sa'idu and Abdu, 2008) with pockets of outbreak occurring in between the annual epidemic periods. Okwor and Eze (2010) stated that commercial chickens in Nigeria are exclusively exotic which are reared intensively or semi-intensively. The intensive system combines both deep litter and battery cage system and in most part of the country, the disease is seen and diagnosed throughout the year in these commercial flocks and the incidence vary with season.

Newcastle disease was reported in most parts of Northern Nigeria with prevalence rates as; Bauchi State 56% (Nwankiti *et al*., 2010); Borno State 52.2% (El-Yuguda *et al*., 2009; Sadiq *et al*., 2011); Jigawa State (Wakawa *et al*., 2009); Nassarawa State (Salihu *et al*., 2012); Kaduna State 73.3% (Nwanta *et al*., 2006); Plateau State 51.9% (Musa *et al*., 2009) and Gombe State 55.5% (Lawal *et al*., 2015).

Okwor *et al.* (2009) conducted a research on the effect of storage conditions on the potency of ND vaccine La Sota, and concluded that in Nigeria, electricity or power supply is poor and vaccines are also handled by untrained personnel who do not know the need for cold chain system in vaccine storage; outbreaks of ND in vaccinated birds are thus quite common and one may therefore conclude that improper vaccine storage under our local power supply may be contributing to this. The preventive efficiency of a vaccine depends on its strict post and pre-manufacture quality control (Shil *et al*., 2011).

Furthermore, Allan *et al*. (1978) had earlier reported that if the time interval between primary and secondary (booster) vaccination is less than twenty-one days, the antibodies produced by the first vaccination are likely to interfere with the second dose of the vaccine virus (for live vaccines such as the La Sota). The common practice among farmers in Kano (study area) is an interval of two weeks between primer and booster La Sota vaccine administration.

Ibu *et al*. (2008) conducted a research to assay the antigenic relatedness among Newcastle disease virus isolates from Nigerian Feral birds and the La Sota strain. They concluded the effects of their findings on the epidemiology and control of Newcastle Disease in Nigeria as; "it is evident that there is high level of antigenic variability among the thirteen (13) field isolate virus strains and the La Sota vaccine strain which could be of practical significance in relation to vaccine failure". Furthermore, Miller *et* al. (2013) concluded that vaccines formulated with NDV of the same genotype as the as the challenge virus have the potential to provide superior protection against transmission by reducing the magnitude of viral shedding and the La Sota vaccine virus is more distantly related to circulating viruses.

## 1.3 Justification

Vaccines are used to prevent or reduce problems that may arise when a poultry flock is exposed to field disease organism (Butcher and Miles, 1994). This notwithstanding, Newcastle Disease is enzootic in Africa, Asia and South America where it continues to cause serious losses despite the vaccination of industrialized poultry (FAO, 2006) and newly isolated strains of ND virus are continuously been reported from all over the world (Ganar *et al*., 2014). In 2013, ninety-six (96) ND virus outbreaks were reported in poultry from Cameroon, Central Africa Republic, Cote

d'Ivoire and Nigeria (Snoeck *et al*., 2013). In addition to routine outbreaks, vaccination incapacity has also been also been reported causing emergence of new ND virus strains (Chen *et al*., 2012).

The availability of data on the epidemiology of Newcastle Disease and the impact of vaccination against this disease in backyard production systems is limited (Otim *et al.,* 2007; Harrison and Alders, 2010). Shafqat *et al*. (2015) presented a field data suggesting that despite high levels of anti-NDV antibody titre $>Log_2 3$ HI in 99% of the tested birds in different farms and localities, there was a very high incidence of the disease.

Yegani *et al*. (2002) stated that due to ever increasing farm size and the proximity of one farm to another, the disease status in any given location is constantly evolving and it has become glaringly evident that no single vaccination program will be suitable for all farms in all areas and vaccine recommendation may change as new information regarding bird immunity and disease comes to light.

### 1.4 Aim

The aim of this study was to carry out a comparative evaluation of antibody production in broilers vaccinated with Newcastle disease La Sota vaccines in Kano metropolis, Nigeria.

### 1.5 Objectives

The objectives of this study were to:

1. Measure the level of maternally derived antibody (MDA).

2. Evaluate the immunogenicity of some ND La Sota vaccines in use in Kano for control of ND in broiler chickens.
3. To determine the protective indices of the vaccines.
4. Determine the effect of time interval between repeated doses of vaccine on anti-ND virus antibody production.
5. Record cases of mortality or morbidity among the study populations.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Newcastle Disease

The definition currently in use in all member states of the European Union (EU) is defined in the Directive 92/66/EEC (CoEC, 1992) as; Newcastle disease (ND) is a disease of major importance for poultry and other birds caused by specified viruses of the Avian Paramyxovirus Type 1 (APMV-1) of the family *Paramyxoviridae* characterised by respiratory and or nervous signs, partial or complete cessation of egg production or mis-shapened eggs, greenish watery diarrhea and oedema of the tissues around the eye and neck. Newcastle disease is an Office Internationalé des Epizooties (OIE) notifiable Avian disease and infection and notification of any outbreak to the OIE is mandatory (OIE, 2016). The OIE (2012a) definition for reporting an outbreak of ND is;

Newcastle disease is defined as an infection of bird caused by a virus of avian Paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a) The virus has an Intracerebral pathogenicity index (ICPI) in day-old chick *gallus-gallus* of 0.7 or greater, or,
b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term ‘multiple basic amino acids’ refers to at least three arginine or lysine residues between residue 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterization of the isolated virus by an ICPI test. In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the

nucleotide sequence of the F0 gene, 113-116 corresponds to residues -4 to -1 of the cleavage site.

## 2.2 Classification of Newcastle Disease Virus

Newcastle Disease Virus is of the order *Mononegavirales,* family *Paramyxoviridae* and sub-family *Paramyxovirinae*, genus *Avulavirus* (Mayo, 2002). It is a single stranded negative sense ribonucleic acid (RNA) virus. Ribonucleic acid viruses can be placed into seven classes according to the nature of the viral RNA and its relation to the messenger. The Paramyxoviruses belong to the Class III with genome of negative polarity to the messenger. The paramyxoviruses isolated from avian species have been classified by serological testing and phylogenetic analysis into ten sub-types designated as APMV-1 to APMV-10 (Miller *et al*., 2010); ND virus has been designated APMV-1 (Alexander and Senne, 2008b).

One of the most characteristic properties of ND Virus has been their great variation in pathogenicity for chickens (OIE, 2012b). Beard and Hanson (1984) stated that ND Virus strains can be classified as highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic). This classification is based on the results of the mean death time (MDT) of chicken embryo at < 60 hours, 60-90 hours and > 90 hours respectively. The clinical signs of a highly virulent ND Virus infection in chickens can be extremely different depending on the strain of the infecting virus. Other tests designed to differentiate strains directly assess clinical signs or death in infected birds, calculating a pathogenicity index and APMV-1 virus that does not meet the OIE definition for causing ND is referred to as a low virulence APMV-1 or NDV (Orsi *et al*., 2009).

Pathotype groupings are rarely clear-cut (Alexander and Allan, 1974) and even in infection of specific pathogen free (SPF) birds, considerable over-lapping may be seen. In addition, exacerbation of clinical signs induced by the milder strains may occur when infections by other organisms are super-imposed or when adverse environmental conditions are present (OIE, 2012b). Strains of Newcastle Disease Virus have been distinguished on the basis of the clinical signs seen in infected chickens (Alexander and Senne, 2008b). These are:

i. Viscerotropic velogenic: These are viruses responsible for diseases characterized by acute lethal infections usually with hemorrhagic lesions in the intestines of dead birds.
ii. Neurotropic velogenic: These are viruses causing diseases characterized by high mortality which follows respiratory and neurological diseases but in which gut lesions are usually absent.
iii. Mesogenic: These are viruses causing clinical signs consisting of respiratory and neurological signs with low mortality.
iv. Lentogenic: These viruses cause mild infections of the respiratory tract.
v. Asymptomatic enteric: These viruses cause avirulent infections and replication occurs primarily in the gut.

Elam (1993) stated that four forms of the disease have been identified and they are caused by different strain of the virus. They are; Doyle's form, Beach's form, Beaudette's form and the Hitchner's form. In the Doyle's form, all ages are susceptible with high mortalities reaching ninety percent**.** In Beach's form on the other hand, 10% mortality is common in adults and it could be higher hence morbidity is high and mortality variable. In immature chicks however,

mortality is as high as 90%. In the Beaudette's form, morbidity in adult is rare and mortality is variable. In the Hitchner's form of the disease, there is negligible mortality although in young birds, complications with other secondary infections may result in mortality rates as high as 30%.

## 2.3 Genomic Structure of Newcastle Disease Virus

The genome of ND virus is a non-segmented, single-stranded, negative-sense Ribonucleic acid (RNA). The ND virus isolated from different parts of the world fall into three genome size groups (Ganar *et al*., 2014); 15,186 nucleotides (nt) long in the isolates before 1960; 15,192nt in the isolates discovered in China; 15,198 in the avirulent isolates from Germany (Czegledi *et al*., 2006; de Leeuw and Peeters, 1999; Huang *et al*., 2004b; Krishnamurthy and Samal, 1998; Römer-Oberdörfer *et al*., 1999).

The genome is approximately 15 Kilobase (Kb) in length and follows the "rule of six" which is a pre-requisite for effective genome replication (Calain and Roux, 1993). The genomic RNA contains a 3′ leader sequence of 55 nucleotides and a 5′ trailer sequence of 114 nucleotides. The leader and trailer sequences are essential for virus transcription and replication, and they flank six structural genes in the order of 3′-NP-P-M-F-HN-L-5′, which encode at least seven proteins. The genome (3′ to 5′) encodes for six different proteins, i.e. Nucleocapsid (N), Phosphoprotein (P), Fusion protein (F), Matrix protein (M), Haemagglutinin-neuraminidase (HN) and RNA large polymerase (L) protein. The N, P and M proteins encompass the viral inner surface whereas the L protein constitutes the viral nucleocapsid together with NP and P proteins (Lamb and Park, 2007). Two viral glycoproteins HN and F are present in the viral envelope and mediates viral entry (Sánchez-Felipe *et al*., 2014) they act as virus neutralizing antigens and are

responsible for virus attachment and its fusion to the host cell membrane (Ganar *et al*., 2014). The mechanism is as such; the former is responsible for the attachment of the virus to host cell receptor, whereas the latter mediates fusion of the viral envelope with the host cell membrane, enabling viral entry into the cell (Peeters *et al*., 1999).

The nucleocapsid protein (N) binds to the genomic RNA forming the nucleocapsid core; and the phosphoprotein (P) and the large polymerase protein (L) are associated with the nucleocapsid core forming a tight functional herringbone-like ribonucleoprotein (RNP) complex, both in virions and infected cells (Lamb and Park, 2007). The matrix protein (M) is non-glyscosylated is present beneath the envelop and is shown to be responsible for virus shedding and budding during viral assembly (Pantua *et al*., 2006). Two additional proteins, V and W, are produced by RNA editing (Curran *et al*., 1991) during P gene transcription. V and W are accessory and are present only in the virus infected cells. The V protein is Interferon (IFN) antagonist and plays an important role in ND virus virulence (Alamares *et al*., 2010; Dortmans *et al*., 2011; Huang *et al*., 2003).

At the beginning and end of each gene are conserved transcriptional control sequences known as gene-start (3′-UGCCCAUCU/CU-5′) and gene-end (3′-AAU/CUUUUUU-5′) signals respectively. Between the gene boundaries are intergenic regions, which vary in length from 1 to 47 nucleotides (Krishnamurthy and Samal, 1998; Lamb and Park, 2007).

Newcastle disease virus follows the same general mode of transcription and replication as other non-segmented negative-sense RNA viruses (Lamb and Kolakofsky, 2001). Like other non-

segmented negative-sense viruses (NSV), there is a polar attenuation of transcriptions such that each downstream gene is transcribed less than its upstream neighbour (Nagai, 1999).The replication of RNA viral genomes is dictated by the absence of multiple translation units within the same messenger. To overcome this difficulty, three (3) main strategies have been developed;

1. The viral messenger RNA (mRNA) acts directly as the messenger and is transcribed monocistronically followed by cleavage to form different proteins;
2. The virion RNA is transcribed to yield various monocistronic mRNAs by initiating transcription at various places;
3. The genome itself is a collection of separate RNA fragments that are transcribed into monocistronic mRNAs.

## 2.4 Molecular Basis for Pathogenicity

During replication, NDV particles are produced with a precursor glycoprotein, F0, which has to be cleaved to F1 and F2 for the virus particles to be infectious. The amino sequence of the F protein cleavage site is thus postulated as the primary determinant of infection (Panda *et al*., 2004; Peeters *et al*., 1999; Samal *et al*., 2011). This post-translation cleavage is mediated by host-cell proteases found in a wide range of cells and tissues, and thus spread throughout the host damaging vital organs, but F0 molecules in viruses of low virulence are restricted in their cleavability to certain host proteases resulting in restriction of these viruses to growth only in certain host-cell types. Trypsin is capable of cleaving F0 for all NDV strains (OIE, 2004).

Most ND viruses that are pathogenic for chickens have the sequence 112R/K-R-Q-K/R-R116 at the C terminus of the F2 protein and F (phenylalanine) at residue 117, the N-terminus of the F1

protein, whereas the viruses of low virulence have sequences in the same region of 112G/E-K/R Q-G/E-R116 and L (leucine) at residue 117. Some of the pigeon variant viruses (PPMV-1) examined have the sequence 112G-R-Q-K-RF117, but give high ICPI values. Thus there appears to be the requirement of at least one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at 113 if the virus is to show virulence for chickens. Several studies have been done using molecular techniques to determine the F0 cleavage site sequence by reverse-transcription polymerase chain reaction (RT-PCR), either on the isolated virus or on tissues and feces from infected birds, followed by analysis of the product by restriction enzyme analysis, probe hybridization or nucleotide sequencing with a view to establishing a routine *in vitro* test for virulence. Determination of the F0 cleavage sequence may give a clear indication of the virulence of the virus, and this has been incorporated into the definition of Newcastle Disease (OIE, 2004).

In the diagnosis of ND, it is important to understand that the demonstration of the presence of virus with multiple basic amino acids at the F0 cleavage site confirms the presence of virulent or potentially virulent virus, but failure to detect virus or detection of ND virus without multiple basic amino acids at the F0 cleavage site using molecular techniques does not confirm the absence of virulent virus. Primer mis-match, or the possibility of a mixed population of virulent and avirulent viruses means that virus isolation and an invivo assessment of virulence will still be required (OIE, 2004). Analyses of viruses isolated in Ireland in 1990 and during the outbreaks of ND in Australia since 1998 have given strong evidence that virulent viruses may arise from progenitor viruses of low virulence (Alexander, 2001; Westbury, 2001). Virulent ND virus has

also been generated experimentally from low virulence virus by passage in chickens (Shengqing *et al*., 2002).

## 2.5 Isolation of Newcastle Disease Virus

Newcastle Disease Virus had been isolated from dead chickens and guinea fowls as well as from apparently healthy chickens, ducks and pigeons (Echeonwu *et al*., 1993). Occasionally, ND virus has been isolated from non-avian species such as pigs (Chen *et al*., 2013) and goats (Sharma *et al*., 2012). Forrester *et al*. (2013) reported ND virus isolation from a mosquito pool in Jakarta, Indonesia.

## 2.6 Occurrence of Newcastle disease

The disease was first reported in 1927 from an outbreak which occurred in Newcastle Upon Tyne, England (Alexander, 1992). Velogenic ND virus is endemic in areas of Mexico, Central and South America, widely spread in Asia, Middle East and Africa. Cases have been reported in Europe, Canada, United States of America and Australia. Lentogenic strains of ND virus are worldwide in their distribution while wide spread mesogenic pathotypes with special adaptation to pigeons (Pigeon Paramyxovirus; PPMV) do not appear to infect other poultry readily (Anon, 2013).

The first documented outbreak of ND in Nigeria occurred between December, 1952 and February, 1953 in and around Ibadan (Hills *et al*., 1953) the disease has since then remained a notable problem in the country. Scavenging local chickens may play significant roles in the maintenance and spread of Avian Inflenza (AI) and Newcastle disease viruses to the susceptible commercial poultry (Abdu *et al*., 2002). The disease has shown marked variation in seasonality

in various parts of the world (Chabauf, 1990; Alders and Spradbrow, 2001). In Nigeria however, the disease is noted to have a higher prevalence during the cold harmattan periods; October – March (Abdu *et al*., 1992; Sa'idu *et al*., 1994).

## 2.7 Newcastle Disease in Humans

According to OIE (2009), ND virus is a human pathogen. Newcastle disease viruses of both virulent and with low virulence for chickens have been reported to have infected humans. Reported infections have been non-life threatening and usually not debilitating for more than a day or two (OIE, 2012b). Live ND virus vaccines may represent a hazard to humans usually causing acute conjunctivitis following direct introduction to the eye. Infection is transient and the cornea is not involved. Mineral oil emulsion represent serious hazard to the vaccinator. Accidental injection of humans should be treated promptly by washing of the needle-prick site with removal of the material including incision of tissues as for a 'grease-gun' injury (OIE, 2012b).

## 2.8 Newcastle Disease in Poultry

Avian Paramyxovirus-1 viruses are known to infect more than 250 species of birds in 27 Orders; other avian species are likely to be susceptible (Anon, 2016). This could be as a result of natural or experimental infections (Alexander, 1997) because a vast majority of birds are susceptible to ND viruses of both high and low virulence for chickens (Leslie, 2000; OIE, 2009). In Nigeria, ND show age and specie differences (Halle *et al*., 1999; Abdu *et al*., 2005) as such, susceptibility and mortality in younger chickens is more severe (Abdu *et al*., 1992, 2005).

Poultry species virulent ND virus strains are commonly found in Pigeons and double crested Cormorants (Diel *et* al., 2012; Kim *et al*., 2008; Pchelkina *et al*., 2013), it is also found occasionally in some other wild birds (Kaleta and Kummerfeld, 2012). A high percentage of pigeons in Nigeria have been shown to be positive for the anti-ND virus antibodies although the titre was low (Sai'du *et al*., 2004). Velogenic ND virus (vNDV) isolated from pigeons in India was used to induce ND in chickens without prior adaptation (Roy *et al*., 2000). Newcastle disease outbreaks have been reported in guinea fowl in some African countries; Nigeria and Niger (Echeonwu *et al*., 1993; Idi *et al*., 2001). Wambura (2010) showed that guinea fowl exhibited a higher prevalence of ND antibodies when compared with pigeons.

### 2.8.1 Age susceptibility

Abdu *et al*. (2005) reported that ND affects both young and old birds. Chicks up to two weeks old with high maternal antibodies may be less susceptible (Ezeokoli *et al*., 1984). This is because maternal antibodies play a role in protecting chicks against ND virus infection. However, maternal antibodies gradually decline in chicks and at two weeks of age, it is not able to protect the bird against the disease (Sa'idu *et al*., 2006). Chicks that are 3-4 weeks old are highly susceptible to the disease while layer flocks within 9-10 weeks old are more resistant due to high antibody titre levels as a result of vaccination at 6 weeks of age (Halle *et al*., 1999).

### 2.8.2 Species Susceptibility

Newcastle Disease Virus was reported in both local and exotic chickens (Echeonwu *et al*., 1993). An accurate assessment of the distribution of NDV throughout the world is difficult to achieve due to the wide spread use of live vaccines. However, Anon (2013) summarised as; chickens are

highly susceptible to ND but turkeys do not tend to develop severe signs; Game birds (Pheasants, partridges, quails and guinea fowls) and parrots (order Psittaciformes) vary in susceptibility; cockatiels are susceptible; wild birds and water fowl (order Anseriformes) may harbour the virus subclinically; young cormorants have demonstrated disease associated with APMV-1; ostriches and pigeons are susceptible; raptors are usually resistant but acute ND has been reported in bearded vulture, white-tailed sea eagle, wild osprey and some species of falcon; gulls, owls, pelicans have been affected by ND virus; passerine birds (order Passeriformes) are variable in their susceptibility; some species show no signs of disease but excrete ND virus while others may develop severe disease; there has been reports of ND related deaths in crows and ravens; acute ND has been recorded in penguins.

Other birds that have shown serological evidence of ND include laughing dove, mourning dove, Muscovy duck, khaki Campbell ducks, geese, mallard ducks, peacock, village weavers, gray-headed sparrow, red bishop, gray canary, scaly fronted weaver and bearded barbet (Sa'idu *et al*., 2004; Bisalla *et al*., 2005; Ibrahim *et al*., 2005).

### 2.8.3 Breed susceptibility

Ezeokoli *et al*. (1984) reported that local chickens are less likely to develop clinical ND compared to the improved intensively reared breed. This is true because local chickens scavenge for food and may be exposed to and infected by milder forms of the ND virus and they gradually develop a substantial amount of immunity which protects them during more severe outbreaks. Some rural dwellers believe that the different breeds of local chickens show difference in susceptibility patterns to the disease (Ibrahim and Abdu, 1992). However, all breeds of local

chickens are equally susceptible to ND virus (Sa'idu *et al*., 2005). Breed specific morbidity rate were reported to be higher for improved breeds (40-80%) than for local breeds (10.6%). This implies that exotic breeds are twice as likely as local breeds to be infected with ND (Halle *et al*., 1999).

## 2.9 Clinical Signs and Symptoms

According to OIE (2009), ND virus is so virulent that many birds die without showing any clinical signs also, depending on the environment and degree of resistance of the birds, not all signs may be shown or some in mild or sub-clinical forms. Newcastle Disease Virus affects the respiratory, nervous, and digestive systems with variable symptoms depending on factors such as: virus, host species, age of host, co-infection with other organisms, environmental stress and immune status. Clinical signs as seen in chickens include: gasping, stretching of neck, sneezing, coughing, tracheal rales, dyspnoea and opisthotonus (Okoye *et al.,* 2000). Other signs may include drop in feed and water consumption, drooped wings, convulsion, trembling of head and neck, backward movement and weakness or paralysis of the leg. Abdu *et al.* (2002) described the following as signs of ND; ruffled feathers, oedema of the head, cloudy eyes, conjunctivitis, severe depression, yellow and whitish diarrhea, dehydration, emaciation and sudden death. There could be a rise in rectal temperature following NDV infection (Oladele *et al.,* 2005).

In turkeys, signs of ND include; tracheal rales, circling, non-coordination, weakness or paralysis of legs and wings, profuse yellowish or greenish diarrhea, ruffled feathers, depression and dehydration (Abdu *et al*., 2005). In Guinea fowls, the following signs were noted; circling, torticolis, inability to fly or falling while in flight, conjunctivitis, anorexia, diarrhea, paralysis of

legs and wings, coughing, sneezing, paddling movement and death (Abdu *et al*., 2005; Haruna *et al.,*1993).

As signs of clinical disease vary widely and diagnosis may be complicated further by the different responses to infection by different hosts, clinical signs alone do not present a reliable basis for diagnosis of ND. However, the characteristic signs and lesions associated with the virulent pathological types will give rise to strong suspicion of the disease (OIE, 2009). In addition, ND virus has been classified into five pathotypes based on the disease induced in chickens under laboratory conditions (Beard and Hanson, 1984; Saif *et al*., 2008).

These are;

### 2.9.1 Viscerotropic velogenic Newcastle Disease

The disease spreads rapidly with marked depression and loss of appetite, sharp drop in egg production, increased respiratory rate, profuse bright green diarrhea, edematous swelling of the head, cyanosis of the combs and conjunctivitis, prostration with many birds dying within a few days; mortality can be as high as > 90% in susceptible flock.

### 2.9.2 Neurotropic velogenic Newcastle Disease

This is characterised predominantly by acute respiratory and nervous signs, sudden depression, loss of appetite, drop in egg production, respiratory distress (severe coughing, gasping), nervous signs (head tremors, wings and leg paralysis, torticolis). Mortality rate in adult is 50 – 70% which maybe higher in young chicks.

### 2.9.3 Mesogenic Newcastle Disease

There is gross weight loss, drop in egg production and quality (with shelf-life of 1-3weeks). Acute respiratory disease with coughing and gasping nervous signs usually develop late and mortality rate is about 50%.

### 2.9.4 Lentogenic Newcastle Disease

Manifestation is usually subclinical with mild respiratory signs, temporary loss of appetite, drop in egg production, no nervous signs and negligible mortality except concurrent disease is present.

### 2.9.5 Asymptomatic enteric Newcastle Disease

This include all forms of the disease presenting as sub-clinical enteric infections.

## 2.10 Incubation Period

The incubation period of ND varies with strain of virus, route and dose of infection and can range from 2-15 days with an average of 4-6 days for velogenic ND but some species may be over 20 days. Alexander and Senne (2008a) reported that incubation period and clinical diseases observed with ND virus infection depends on multiple factors although the typical range is 3-6 days depending on the species and immunity of host infected with a virulent ND Virus and the amount and strain of infecting virus the host is exposed to. For the purposes of OIE Terrestrial Animal Health Code, the incubation period for ND is 21 days (Anon, 2013).

## 2.11 Pathology of the Disease

### 2.11.1 Common lesions and post-mortem findings

Okwor and Eze (2010) reported that gross lesions are variable and young chickens and those dying acutely may have no lesions. Most commonly observed post-mortem lesions were pin-point hemorrhages at the tip of proventricular glands, hemorrhagic ulcers in intestinal walls and caecal tonsils, petechial hemorrhage in colon, hemorrhagic lungs, tracheitis with congestion and catarrhal exudates (Hasan *et al*., 2010).

#### 2.11.2 Microscopic Lesions

The most consistent microscopic lesions in the central nervous system (CNS) of chickens infected with ND are those of a non-suppurative disseminated encephalomyelitis characterised by neuronal degeneration, gliosis, hypertrophy and proliferation of the endothelial cells and perivascular lymphocytic infiltration (Alexander, 1990). Necrosis of the cerebral parenchyma and markedly thickened arteries were also observed (Okoye *et al*., 2000; Oladele *et al*., 2005).

### 2.12 Newcastle Disease Diagnosis

Different methods have been developed for diagnosis of ND virus infection. These can be broadly classified as;

i. Presumptive: these are based on clinical signs and or post-mortem findings or lesions.
ii. Unequivocal: these involve virus isolation (from samples), identification and characterization.
iii. Sero-diagnosis: these involve monitoring specific NDV antibody levels in convalescent or acute blood samples. Newcastle disease virus can be employed as an antigen in a wide

range of serological tests enabling neutralization or Enzyme-linked Immunosorbent Assay (ELISA) and Haemagglutination-inhibition (HI) to be used for assessing anti-NDV antibodies in birds (OIE, 2013).

Laboratory diagnostic tests of ND virus can be classified as;

1. Those involving identification of the virus, and,
2. Those involving serological tests

### 2.12.1 Identification of the virus

This process involves all the following:

1. Virus isolation: According to OIE (2012a), when investigations of ND are the result of severe disease and high mortality in poultry flocks, it is usual to attempt virus isolation from recently dead birds or moribund birds that have been killed humanely. Samples from dead birds should consist of oronasal swabs, as well as samples collected from lung, kidneys, intestine (including contents), spleen, brain and liver and heart tissues. These may be collected separately or as a pool. Intestinal samples are usually processed separately from others.

Samples from live birds should include both tracheal and cloacae swabs; the latter should be visibly coated with fecal material. Small delicate birds may be harmed by swabbing, but the collection of fresh feces may serve as an adequate alternative. Where opportunities for obtaining samples are limited, it is important that cloacae swabs (or feces) and tracheal swabs (or tracheal tissue) be examined as well as organs or tissues that are grossly affected or associated with the clinical disease. Samples should be taken in the early stages of the disease (OIE, 2012a).

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0–7.4, containing antibiotics. Protein-based media, for example, brain–heart infusion (BHI) or tris-buffered tryptose broth (TBTB), have also been used and may give added stability to the virus, especially during transportation. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml); streptomycin (2 mg/ml); gentamycin (50 µg/ml); and mycostatin (1000 units/ml) for tissues and tracheal swabs, but at fivefold higher concentrations for feces and cloacae swabs. It is important to readjust the solution to pH 7.0–7.4 following the addition of the antibiotics. If control of *Chlamydophila* is desired, 0.05–0.1 mg/ml oxytetracycline should be included. Feaces and finely minced tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to four (4) days (OIE, 2012b).

2. Virus culture: The supernatant fluids of feaces or tissue suspensions obtained through clarification by centrifugation at 1000*g* for about 10 minutes at a temperature not exceeding 25°C are inoculated in 0.2 ml volumes into the allantoic cavity of each of at least five embryonated specific pathogen free (SPF) fowl eggs of 9–11 days' incubation. After inoculation, these are incubated at 35–37°C at interval of 4–7 days (Alexander and Senne, 2008a). Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C and the allantoic fluids tested for HA activity. Fluids that give a negative reaction should be passaged into at least one further batch of eggs (OIE, 2009).

3. Virus identification: Haemagglutination detected in bacteriologically sterile fluids harvested from inoculated eggs may be due to the presence of any of the sixteen haemagglutinin subtypes of Influenza-A viruses or of the eight other Paramyxovirus serotypes (non-sterile fluid could contain bacterial HA). Newcastle Disease Virus can be confirmed by the use of specific antiserum in a haemagglutination inhibition (HI) test. Usually chicken antiserum that has been prepared against one of the strains of NDV is used (OIE, 2009).

4. Pathogenicity index: The extreme variation in virulence of different NDV isolates and the widespread use of live vaccines means that the identification of an isolate as NDV from birds showing clinical signs does not confirm a diagnosis of ND, so that an assessment of the virulence of the isolate is also required. In the past such tests as the mean death time in eggs, the intravenous pathogenicity test and variations of these tests have been used (Alexander and Senne, 2008b), but by international agreement, a definitive assessment of virus virulence is based on the intracerebral pathogenicity index (ICPI) test.

The current OIE (2012b) definition also recognizes the advances made in understanding the molecular basis of pathogenicity and allows confirmation of virus virulence, but not lack of virulence, by *in-vitro* tests that determine the amino acid sequence at the F0 protein cleavage site.

The intracerebral pathogenicity index test is briefly described as follows;

i) Fresh infective allantoic fluid with a HA titre $>2^4$ (>1/16) is diluted 1/10 in sterile isotonic saline with no additives, such as antibiotics.

ii) Approximately, 0.05 ml of the diluted virus is injected intracerebrally into each of ten chicks hatched from eggs from an SPF flock. These chicks must be over 24-hours and under 40-hours old at the time of inoculation.

iii) The birds are examined every 24 hours for 8 days.

iv) At the end of each observation, the birds are scored: 0 if normal, 1 if sick, and 2 if dead. (Birds that are alive but unable to eat or drink should be killed humanely and scored as dead at the next observation. Dead birds must be scored as 2 at each of the remaining daily observations after death).

v) Intracerebral pathogenicity index is the mean score per bird per observation over the 8-day period.

The most virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic and asymptomatic enteric strains will give values close to zero.

5. Phylogenetic studies: This utilizes improved techniques for nucleotide sequencing, the availability of sequence data of more ND viruses in computer databases and the demonstration that even relatively short sequence lengths could give meaningful results and phylogenetic analyses have led to a considerable increase in such studies in recent years. Considerable genetic diversity has been detected, but viruses sharing temporal, geographical, antigenic or epidemiological parameters tend to fall into specific lineages and this has proven valuable in assessing both the global epidemiology and local spread of ND (Mase *et al.,* 2002; Aldous *et al.,* 2003; Wehmann *et al.,* 2003; Weingart *et al.,* 2003).

Although in the past, phylogenetic studies have been impracticable as a routine tool, the greater availability and increased speed of production of results obtained using sophisticated, commercially available kits for Reverse transcriptase-Polymerase chain reaction (RT-PCR) and automatic sequencers now means such studies are within the capabilities of many more diagnostic laboratories and can give meaningful results that are contemporaneous rather that retrospective (Aldous and Alexander, 2001). Aldous *et al.* (2003) proposed that genotyping of ND virus isolates should become part of diagnostic virus characterisation for reference laboratories by producing a 375-nucleotide sequence of the F gene, which includes the F0 cleavage site, routinely for all viruses and comparing the sequences obtained with other recent isolates and eighteen viruses representative of the recognised lineages and sub-lineages. Such analysis should allow rapid epidemiological assessment of the origins and spread of the viruses responsible for ND outbreaks.

### 2.13 Molecular techniques in diagnosis

In addition to the use of RT-PCR and other similar techniques for the determination of the virulence of NDV or for phylogenetic studies, there has been increasing use of molecular techniques to detect NDV in clinical specimens, the advantage being the extremely rapid demonstration of the presence of virus. Care should be taken in the selection of clinical samples as some studies have demonstrated lack of sensitivity in detecting virus in some organs and particularly in fecal samples (Gohm *et al.,* 2000; Creenlan *et al.,* 2002; Koch, 2003). Tracheal or oropharyngeal swabs are often used as the specimens of choice because they are easy to process and usually contain little extraneous organic material that can interfere with RNA recovery and amplification by Polymerase chain reaction (PCR). However, tissue and organ samples and even

feaces have been used with some success. The system used for RNA extraction will also affect the success of RT-PCR on clinical specimens and even with commercial kits, care should be taken in selecting the most appropriate or validated for the samples to be analyzed.

Usually RT-PCR systems have been used to amplify a specific portion of the genome that will give added value; for example by amplifying part of the F gene that contains the F0 cleavage site so that the product can be used for assessing virulence (Jestin and Jestin, 1991; Council of Europe, 1997; Gohm *et al.,* 2000; Barbezange and Jestin, 2002; Park *et al.,* 2002; Shengquig *et al.,* 2002). Perhaps the most serious problem with the use of RT-PCR in diagnosis is the necessity for post-amplification processing because of the high potential for contamination of the laboratory and cross contamination of samples. Extreme precautions and strict regimens for handling samples are necessary to prevent this.

One of the strategies used to avoid post-amplification processing is to employ real-time Reverse transcriptase polymerase chain reaction (qRT-PCR) techniques. The advantages of such assays are that qRT-PCR assays based on the fluorogenic hydrolysis probes or fluorescent dyes eliminate the post-amplification processing step and results can be obtained in less than three hours. The most successful application of an qRT-PCR assay was in the USA during the ND outbreaks of 2002–2003, when the assay described by Wise *et al*. (2004) was employed and showed a sensitivity of 95% when compared with virus isolation for more than 1400 specimens. The assay has three sets of primers and probes that are used in separate reactions: a matrix primer/probe set that is designed to detect most strains of NDV, a fusion primer/probe set that can identify virulent strains of NDV (including many PPMV-1 viruses) and a primer/probe set

designed to detect low virulent strains of the virus. Samples are first screened with the matrix primers/probe then positive specimens are tested with the low virulent and fusion and primers/probe sets to confirm presence of low or highly virulent virus, respectively. The primers and probes in this report were validated on lentogenic, mesogenic and velogenic strains circulating in the United States of America. At the peak of the outbreak, between 1000 and 1500 samples were tested daily by qRT-PCR. A disadvantage of qRT-PCR is that, at present, the special thermocyclers required are extremely expensive and this would deter many laboratories from employing this system.

One further important challenge is that while the vast majority of ND virus isolates are genetically quite close, some have been shown to be genetically distinct. For example, one group of viruses, which were placed in Genogroup 6 by Aldous *et al*. (2003) and subsequently Class I by Czegledi *et al*. (2006), are so different from all the other NDV isolates, i.e. Class II viruses, such that different primers would be necessary for their detection in RT-PCR tests. As with virulence determination, it is important that PCR techniques alone are not used to record a negative result in investigations of suspected ND (Czegledi *et al*., 2006).

### 2.14 Serological tests

Newcastle disease virus may be employed as an antigen in a wide range of serological tests, enabling neutralisation or Enzyme linked immunosorbent assays (ELISA) and Haemagglutination inhibition (HI) to be used for assessing antibody levels in birds. At present, the HI test is most widely used for detecting antibodies to ND virus in birds, although many poultry producers are using commercial ELISA kits to assess post-vaccination antibody levels.

### 2.14.1 Haemagglutination (HA) and Haemagglutination inhibition (HI) tests

Chicken sera rarely give nonspecific positive reactions in the HI test and any pretreatment of the sera is unnecessary (OIE, 2013). There are variations in the procedures for HA and HI tests as practiced in different laboratories. The following recommended examples apply in the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic Phosphate buffered saline (PBS) (0.01 M), pH 7.0–7.2, and RBC taken from a minimum of three SPF chickens and pooled in an equal volume of Alsever's solution. (If SPF chickens are not available, blood may be taken from unvaccinated birds monitored regularly and shown to be free from antibodies to NDV). Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate (OIE, 2012b).

Haemagglutination-inhibition titre may be used to assess the immune status of a flock. In vaccinated flocks that are being monitored serologically, it may be possible to identify anamnestic responses (ability of lymphoid tissues to recognize and respond to antigens to which they have already been exposed) as the result of a challenge infection with field virus (Allan *et al.,* 1978), but great care should be exercised as variations may occur from other causes. For example, it has been demonstrated that APMV-3 virus infections of ND-virus vaccinated turkeys will result in substantially increased titre to ND virus (Alexander *et al.,* 1983).

### 2.14.2 Enzyme-linked immunosorbent assay (ELISA)

There are a variety of commercial ELISA kits available and these are based on several different strategies for the detection of anti-ND virus antibodies, including indirect, sandwich and

blocking or competitive ELISAs using Monoclonal antibodies (MAbs). At least one kit uses a subunit antigen. Usually such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use are followed carefully.

The HI test and ELISA may measure antibodies to different antigens; depending on the system used, ELISAs may detect antibodies to more than one antigen while the HI test is probably restricted to those directed against the HN protein. However, comparative studies have demonstrated that the ELISAs are reproducible and have high sensitivity and specificity; they have been found to correlate well with the HI test (Adair *et al.,* 1989).

Conventional ELISAs have the disadvantage that it is necessary to validate the test for each species of bird for which they are used. Competitive ELISAs usually employ MAbs which, because of their specificity for single epitopes, may not recognise all strains of *Avian paramyxovirus* -1 (OIE, 2012a).

### 2.14.3 Monoclonal antibodies (MAbs)

Mouse monoclonal antibodies directed against strains of ND virus have been used in HI tests for rapid identification of ND virus without cross reactions with other Avian *Paramyxovirus* serotypes that may be evident in polyclonal sera. These antibodies have been produced that give reactions in HI tests that are specific for particular strains or variant ND virus isolates (Alexander *et al.,* 1997). Panels of MAbs have been used to establish antigenic profiles of ND virus isolates based on whether or not they react with the viruses. This is quite important in grouping and

differentiating isolates of ND virus and very useful for understanding the epidemiology of outbreaks (Alexander *et al.,* 1997).

## 2.15 The Newcastle Disease Vaccine

The term vaccine was coined by Edward Jenner in 1796 from a latin word "*vacca*" translated as cow. A vaccine is a preparation of a weakened or killed pathogen such as bacterium or virus, or a portion of the pathogen's structure which upon administration stimulates antibody production or cellular immunity against the pathogen but is incapable of causing severe infection (Yegani *et al.,* 2002) and they are the most effective and inexpensive prophylactic tool in veterinary medicine (Huang *et al.,* 2003).

The ND virus vaccine can be categorized into two groups; killed and live form vaccines. Butcher and Miles (1994) described the live type poultry vaccine as one that contains a virus or bacteria that must infect the chicken and multiply in its body to produce immunity preferably with minimal reaction. Advantages of live type vaccine are ease of administration, low price, rapid onset of immunity and a broader scope of protection because chickens are exposed to all stages of the replicating virus. Disadvantages include problem with uniform vaccine application, excessive vaccine reactions, unwanted spread of the vaccine-type virus to the neighboring poultry houses and extreme handling requirements necessary to maintain viability of the vaccine organism (Butcher and Miles, 1994).

Inactivated or killed viruses can also be used for poultry vaccine production. This killed type vaccine is produced from viruses or bacteria that have been activated by chemical treatment and

are usually combined with adjuvants such as Aluminium hydroxide or an oil. These adjuvants enhance immunity response by increasing the stability of the vaccine in the body, which then stimulates the immune system for a longer period of time. The advantages of killed-type vaccines are assurance of administration of a uniform dose (birds are individually injected), safety (organism has been inactivated), development of uniform levels of immunity (each bird receives the same dose), no chance of spread of vaccine organism to neighboring poultry farms, increased product stability and a choice of wider variety of virus strains. The disadvantages however include increased costs (labour and products), slower onset of immunity, narrower spectrum of protection, and presence of localized tissue damage at site of injection due to reaction with adjuvants (Butcher and Miles, 1994; OIE, 2012a).

Major ways of ND vaccine administration include; addition of the vaccine containing virus to drinking water, instillation into the conjunctiva sac or external nares, intramuscular injection, or dissemination of virus into air as dust or spray. Recently, there have been developments in the application of oral vaccines in chickens, through the use of oiled-rice coated with 1-2ND vaccine (Wambura *et al.,* 2007). A lentogenic vaccine for use *in ovo* has been licensed for use in the United States of America (OIE, 2009). Some mesogenic strains are given by wing-web intradermal inoculation, but most importantly, vaccines have been constructed to give optimum results through application by specific routes (OIE, 2012a).

### 2.15.1 Safety in the Use of Newcastle Disease Vaccines

Vaccines produced by conventional means are imperfect in much respect with regard to safety, efficacy and cost (Huang *et al.,* 2003). Differentiating vaccinated animals (DIVA) from their

infected counterparts is a major constraint in using live attenuated vaccines and may cause problems in disease control measures (Huang *et al.,* 2003). The unsatisfactory efficacy, safety, antigenic variation and the emergence of new disease dictates the need to develop newer and more effective vaccines. This is true for development of vaccines against poultry diseases in which cost constraint and the need to vaccinate *en masse* are primary concerns (Huang *et al.,* 2003).

Through the use of recombinant DNA technology, it is now possible to generate live virus vaccines that promise significant improvements in safety, efficacy, and cost. By introducing multiple gene deletion mutations in the genome of a virus, it is possible to generate a new class of attenuated vaccines that are safe and may not result in reversion to virulence. Recombinant DNA technology has also made it possible to generate vaccines utilizing viruses as vectors for the expression of protective antigens of other viruses. This new class of vaccine is called "vectored vaccine." Several studies in recent years highlighted the potential of ND virus (NDV) to be used as a vaccine vector for avian diseases (Krishnamurthy *et al*., 2000; Huang *et al*., 2001; Nakaya *et al*., 2001).

### 2.15.2 Reverse Genetics System in Newcastle Disease Vaccine Production

The development of methods to recover non-segmented negative sense virus (NSV) from cloned DNA (cDNA) in recent times opened up the possibility of genetically manipulating the virus group and ND virus inclusive. This technique is called 'Reverse genetics', it provides a means to investigate the functions of various virus encoded genes (Palase *et al.,* 1996; Nagai, 1999) and also allow the use of these viruses to express heterologous genes (Bukreyev *et al.,* 1996;

Mebatsion *et al.,* 1996; Schnell *et al.,* 1996; Hasan *et al.,* 1997; He *et al.,* 1997; Sakai *et al.,* 1999). This serve as a new method for generating improved vaccine and vaccine vectors. Recovery systems for recombinant NSV are now based on intracytoplasmic reconstitution of the ribonucleoprotein complex, which is the template for viral polymerase and is the prerequisite for initiating an infectious cycle (Palase *et al.,* 1996; Conzelmann, 1998; Robert and Rose, 1998). The first recovery systems based on a lentogenic vaccine strain (La Sota) of ND virus, were reported simultaneously by two independent groups in 1999 (Peeters *et al.,* 1999; Römer-oberdörfer *et al.,* 1999). In the first reported system, the full-length NDV cDNA from LaSota strain (ATCC-VR699) was assembled in pOLTV5 transcription vector containing a T7 DNA-dependent-RNA polymerase promoter with two "G" residues, followed by two unique restriction sites containing the full-length clone, the autocatalytic ribozyme from hepatitis delta virus, and the transcription termination signal from bacteriophage T7 (Peeters *et al.,* 1999).

Individual clones of the ND virus transcriptase complex (NP, P and L) were cloned in a eukaryotic expression vector. To generate infectious ND virus, primary chicken embryo fibroblast cells or QM 5 cells were infected with fowl pox virus-T7 recombinant and co-transfected with the full length clone of ND virus and the support plasmids. After incubation for 3 to 6 days in medium containing 5% allantoic fluid, the recombinant virus harvests from these cells were amplified in 9-to-11-day-old embryonated specific pathogen-free eggs. The use of allantoic fluid in the medium was to supply the necessary proteases for the cleavage of F protein. Creation of genetic tags in the form of additional nucleotides after L gene or changing the F cleavage site into a consensus cleavage site of virulent strains did not affect recovery of infectious virus. The co-transfection protocol for generating recombinant ND virus (rNDV) was

claimed to be efficient in that it can generate several infective centers in infected monolayers (Peeters *et al*., 1999). The second system reported for recovery of a lentogenic ND virus from cloned cDNA essentially used the same strategy of assembling the full-length antigenomic expression plasmid and support plasmids (Römer-Oberdörfer *et al*., 1999). However, this system made use of BHK 21 cell; clone BSR T7/5, stably expressing the T7 RNA polymer.

### 2.15.3 Newcastle Disease Virus as a Vaccine Vector

Samal (2011) stated that ND virus is a good vaccine candidate for both human and animal use. It is useful for rational design of live attenuated vaccines and vaccine vectors because of its modular nature of transcription, minimum recombination frequency and an absence of DNA phase during replication. Both live attenuated and recombinant viruses are explored as vaccine and vaccine vectors with various degrees of success (Ganak *et al*., 2014). A number of ND virus based vaccines are generated for treating various human viral infections (Ganak *et al*., 2014). Recombinant ND virus confers immunogenic response against antigens like Human influenza virus haemagglutinin protein (HA) (Di Napoli *et al*., 2010; Ge *et al*., 2007); Human Immunodeficiency Virus (HIV) and Simian Immunodeficiency Virus (SIV) Gag protein (Lawrence *et al*., 2013; Nakaya *et al*., 2001, 2004); HIV glycoproteins (Khattar *et al*., 2011, 2013), F-glycoprotein of Human respiratory syncytial virus (Martinez-Sobrido *et al*., 2006); HN protein of Human parainfluenza virus 3 (Bukreyev *et al*., 2005) and spike glycoprotein of Severe acute respiratory syndrome SARS-CoV (Di Napoli et al., 2007).

The use of ND virus as vaccine for veterinary pathogens is an excellent choice (Ganak *et al*., 2014). Recombinant ND virus expressing VP2 is used as a dual vaccine against ND virus and

Infectious bursal disease infections in chickens (Huang *et al*., 2004). Recombinant ND virus expressing the HA protein of H5N1 (Ge *et al*., 2007; Nayak *et al*., 2009; Park *et al*., 2006; Römer-Oberdörfer *et al*., 2008) and H7N7 influenza virus (Park *et al*., 2006; Schroer *et al*., 2009) protects against both ND and influenza virus infection in chicken.

**2.16 The Research Newcastle Disease La Sota vaccines**

Four different brands of lyophilized ND vaccines were designated as A, B, C and D respectively for the purpose of the research.

The vaccine 'A' is a freeze dried live attenuated ND virus vaccine prepared from the reference strain La Sota. It is produced from Jordan; presentation is in vials of 200, 500, 1000, 2500 and 5000 i.u. The vaccine is indicated for the protection of poultry against ND. Each dose contains a minimum of 106.0 $EID_{50}$ of the attenuated NDV. It can be administered via drinking water, spray, intraocular or intranasal routes. It has no reported side effect, no withdrawal period. Storage is between 2- 8$^{o}$C.

The vaccine 'B' is a lyophilized attenuated live vaccine against ND. It is produced from Israel. It contains the live lentogenic strain of NDV isolated in 1946 by Dr. Baudette during a natural outbreak of ND in New Jersey and was attenuated in sixteen chicken embryo passages. The vaccine is used extensively around the world and can be applied for active immunization of chickens and turkeys against velogenic NDV.  It is applied by eye or nasal drops, coarse spray or aerosols, beak dipping or drinking water. The vaccine is stored at 2- 8$^{o}$C and protected from light.

The vaccine ‘C’ is a freeze-dried lentogenic La Sota (Georgian strain) grown in specific-pathogen free chick embryo. It is produced from India. It can be applied by eye or nasal drops, spray or drinking water. It is used extensively around the world. Optimum storage temperature is between 2 – 8$^{o}$C.

The vaccine ‘D’ is a freeze-dried La Sota strain vaccine produced from Italy. It can be admistered via drinking water, eye or nasal drops, coarse spray or aerosols, beak dipping or drinking water. It is stored optimally at 2 – 8$^{o}$C.

### 2.17 Newcastle Disease Virus Reservoir

This is supported by the following:

2.17.1 Sources of virus

These include:

a. Respiratory secretions / discharges and feaces of infected birds
b. All parts of the carcass of infected birds
c. Virus is shed during the incubation period, during clinical stages and for a limited period during convalescence (Anon, 2013).

2.17.2 Recycling of Infection within Flocks

Virulent ND virus can be maintained in a poultry flock through a cycle of waning immunity followed by “immunity boosting” symptomless infection, and the infecting virus then passed on to other birds with sufficiently lowered immunity (Hanson, 1976).

2.17.3 Spread of ND virus Newcastle Disease Virus can spread from one susceptible host to another via;

1. Domestic Birds

Domestic birds which include Ducks, Turkeys, Doves, Geese, Guinea fowls, and others, can harbor ND virus. In farms with mixed species, the afore mentioned birds can become infected with ND virus with or without overt clinical signs (depending on the infecting strain and the species of bird) and can shed the virus continuously in their feces, acting as a source of infection for chickens (Oladele *et al*., 2012). Japanese quails can carry ND virus for at least 14 days after infection (Lima *et al*., 2004). There is not enough evidence to establish whether chickens can become long-term carriers of ND virus however, in Nigeria, local chickens move about on a free range system and are always unvaccinated (Okwor *et al*., 2009) they therefore suffer severe outbreaks of ND especially during the November to March periods with heavy contamination of the environment that eventually spread to commercial chickens (Abdu *et al*., 1992). Concurrent infections of avian influenza and Newcastle disease occur in free ranging village chickens (Wakawa *et al*., 2009).

2. Wild birds

Wild birds and Water-fowls may act as reservoir hosts for lentogenic pathotypes of ND; subsequently, these viruses may become virulent following mutation upon establishment in domestic poultry. Some psittacine birds have been reported to shed ND virus intermittently for over one year and are associated with introduction into poultry (Anon, 2013).

## 2.18 Newcastle Disease Prevention and Control

Newcastle disease is listed by the world organization for animal health i.e. OIE and due to its potential for very serious and rapid spread irrespective of international borders, resulting in serious socio-economic consequences, requirements for international trade of live animals and animal products are laid down in the terrestrial code (OIE, 2009). Control programs to prevent the (re)introduction of velogenic ND virus (vNDV) into domestic poultry flock are essentially vaccination and quarantine of imported birds which must be complemented with monitoring programs (Kim *et al*., 2008).

Biosecurity is using common-sense practices to protect your poultry and birds from all types of disease agents- viruses, bacteria, funguses or parasites (USDA, 2016). There are three main elements of biosecurity FAO (2006); These are:

i. Segregation and traffic control which emphases on preventing disease agents from entering the farm by keeping potentially infected animals and contaminated objects away from the farm, formation of barriers; physical and or conceptual, use of foot dips and disinfecting car tyres when entering into the farm.
ii. Cleaning which is the most reliable step involves all steps taken to physically remove all dirts i.e., 80% of the contamination.
iii. Disinfection which is considered as the least reliable step as it is dependent on the quality of cleaning but if properly done may kill all residual contaminants on the farm

Flies may be able to transmit APMV-1 mechanically (Anon, 2016). Pets like rats and other rodents can serve as vector for poultry disease and insects; the Darkling beetle has been established as a carrier agent of Newcastle disease and other poultry infections. It is also

important to keep a neat premise free of stagnant water. Dead birds should be disposed immediately to reduce disease agent load on the farm. The suggested safe way include

a. Composting
b. Incinerating
c. Deep burial plus lime (calcium carbonate)
d. Digestion

Quarantine procedures should be strictly adhered to when introducing new stock or re-introducing birds that have recovered from sickness into the farm. It is recommended that birds in this category should be isolated in a separate shed or cage that should be far away as possible from the resident birds and thereafter observed for a minimum of two weeks for any signs of sickness (Anon, 2016).

Epidemiological data regarding changes in antigenicity / pathogenicity of field isolates in a particular geographical location should be generated and should serve as bases in formulation of vaccination strategy for effective control of the disease under field conditions (Singh *et al.,* 2005). Miller *et al*. (2013) conducted a research on the effect of ND virus vaccine antibodies on the shedding and transmission of challenge virus and concluded that it was possible to significantly decrease viral replication and shedding with high levels of antibodies and those levels could be more easily reached with vaccine formulated with ND virus of the same genotype as the challenge virus. However, when the level of heterologous antibodies were relatively high, it was possible to prevent transmission and virus shedding by allowing optimal time for the development of immune response before exposure to vND virus.

Okwor and Eze (2010) recommended that farmers should source and vaccinate their flocks with the help of veterinarians prior to the dry harmattan periods November – February and the peak rainy period of June and July. Movements in and around farms should be restricted and biosecurity measures applied. Counter stress drugs such as vitamins and minerals should be incorporated in poultry rations as supplements during these periods and birds should not be exposed to unnecessary cold. There should be regular, strategic vaccination programs against ND for local chickens, guinea fowls and pigeons in village settings (Idi *et al.,* 2001, Sai'du *et al.,* 2004, Wambura, 2010). However, prophylactic vaccination is practiced in all but few countries that produce poultry on a large scale (OIE, 2009).

### 2.19 Treatment of Newcastle Disease

There are no specific treatments effective against ND (OIE, 2012a). Farm practices in Kano State (study area) include administration of antibiotics to combat bacterial (secondary) infection in combination with virucidial solution. Vitamins and electrolytes are also given to the chickens for supportive therapy.

### 2.20 Situation of Newcastle Disease in Nigeria

In most part of Nigeria, the disease is seen and diagnosed throughout the year in commercial flocks and the incidence varies with season (Okwor and Eze, 2010). The harmattan period in Nigeria is characterised by wind; drop in ambient temperature, dryness among other harsh environmental conditions which is believed to lower the immune status of the birds making it possible for ND to manifest in commercial birds with ordinary or lowered herd immunity to ND (Okwor and Eze, 2010). In the South-East Tropical savannah region of Nigeria, high prevalence

of ND occurred during the November – February period and another relatively increased prevalence was observed during the June – July period. However, during the months March – May, the lowest prevalence of the disease was recorded (Okwor and Eze, 2010).

Ibitoye *et al*. (2013) reported 80.9% of ND during January 2007 and December 2011 in a study conducted in Sokoto. They reported incremental fluctuations in the prevalence of ND from 2007 – 2011 with monthly and bird-type distribution. The highest number of cases was recorded in March and October accounting for 88.4 and 94.4%, respectively suggesting high risk of the disease at these periods. Layers had the highest prevalence (89.0%), followed by Cockrels and Pigeons (66.7%). They also reported that the intensive system of management had the highest disease occurrence of 84.7%. Low outbreaks of the disease were recorded in the months from May to September. Lawal *et al*. (2015) reported 55.5% prevalence rate of ND diagnosed during 2004 – 2014 in Gombe State. They reported that ND cases are five times more likely to occur during the months of September – February compared with other months during the study period.

In Nigeria, the population of poultry is estimated to be 137.6 million with backyard poultry population constituting 84% (115.8 million) and 16% (21.7 million) of exotic poultry (Anon, 2006). The population of local chickens that wander and scavengers is high and ND is difficult (Okeke and Lamorde, 1998); these birds are hardly ever vaccinated and roam about acting as reservoirs and carriers of disease to commercial farms and themselves (Olabode *et al*., 1992). Chollom *et al*., 2013 reported high seroprevalence of anti ND virus-Haemagglutination Inhibition (HI) antibodies in local chickens at a live bird market in Jos with titre as high as 10

$log_2$. In a study to estimate circulating HI antibodies among unvaccinated local chickens in live bird markets and household in Zamfara State, Jibril *et al*. (2014) reported 35.8% and 26.8% seropositive rate with the value obtained from the live bird market showing a statistically significant higher prevalence rate when compared with chickens from household. They reported 35.7% prevalence rate in male chickens and 28.9% from among female chickens; however, this difference was reportedly insignificant ($p > 0.05$). Ohore *et al*. (2002) reported 73.3% prevalence of anti ND virus HI antibodies in local chickens in Ibadan. Ameji *et al*. (2011) reported 25.5% prevalence rate of ND in local chickens in live bird markets in Kogi State.

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 The Study area

The research was conducted in Kano State located in North-Western Nigeria. Kano State was created on 27th May 1967 from parts of Northern region. It borders Katsina State to the North-west, Jigawa State to the North-East, Bauchi State to the South-East and Kaduna State to the South-West. Kano State consists of forty-four (44) local Government Areas. Its coordinates are 11.7574$^o$N, 8.6601$^o$E and covers an area of 20,131km$^2$. The birds were brooded in a backyard pen house located at Sabon-gari, Kano for seven weeks by providing adequate heat, water, feed (Grand cereals Nig. Ltd), vitamins (to act as anti-stress as a result of blood collection) and antibiotics and coccidiostats (to curb bacterial and coccidial infections respectively). Strict bio-security measures were maintained.

### 3.2 The Study Birds

Seventy (70) day-old chicks of Marshall breed were purchased from Phed-Agrovet outlet located at Zungeru Road, Kano. The birds were said to have been purchased from Obasanjo Holdings Limited, Ota, Ogun State Nigeria and no history of ND vaccination was presented.

### 3.3 The Control Virus

This served as source of antigen during the HA and HI tests. It was commercially purchased. The live attenuated vaccine virus is supplied commercially in lyophilized form by the National Veterinary Research Institute (NVRI), VOM, Plateau state. It was reconstituted in Phosphate buffered saline (PBS) solution with pH 7.2 (Appendix 1.0)

## 3.4 Study Design

The birds were divided into two groups; 1 and 2 consisting of 50 and 20 chickens respectively. Group 1 was subjected to efficacy test while group 2 was subjected to time interval test. Other tests conducted include; Maternal antibody persistence, vaccine potency test and test for thermostability.

## 3.5 Collection of Blood Samples

Blood samples were collected using syringe into well labeled bottles. Using the cephalic (wing) vein, 2mls of blood was aseptically collected into clean sterile plain blood bottles (free of anticoagulants). This was centrifuged at 1500 rpm for 5 minutes to allow for maximum separation of serum which was collected and thereafter stored in a freezer between 2– 8$^{o}$C.

## 3.6 Serology

3.6.1 Preparation of 1% (v/v) washed chicken erythrocyte: Blood was collected from the wing vein of three chickens free from ND, 10 ml in total into sterile universal bottles containing an equal volume of Acid citrate dextrose (anticoagulant). This was centrifuged at 1500rpm for 5minutes after which the erythrocytes were washed three times with 10ml portion of PBS at 1500rpm for 5minutes after which packed cell volume was measured and a 1% (v/v) suspension of the washed cells was prepared in clean universal bottle. The suspension was stored at 4$^{0}$C. Stored solution remained clear. Any red colour in the solution indicates haemolysis and the suspension is not suitable for use (OIE, 2004).

3.6.2 Haemagglutination (HA) Test: It is used to titrate the antigen used in the experiment to determine the HA unit i.e., lowest dilution that will cause complete agglutination of the red blood cells. Value for 4 HA unit of the control virus was then calculated. The method is as follows; (OIE, 2012b):

Aliquots of 0.025 ml of PBS was dispensed into each well of the plastic V-bottomed microtitre plate and 0.025 ml of virus suspension (La Sota strain reconstituted in 2mls PBS) was added to the first well. Doubling dilutions of 0.025 ml of the virus was made across the plate and 0.025 ml of the 1% (v/v) of the chicken red blood cell (RBC) was dispensed into each well. The plate was tapped gently to mix its content and the RBCs allowed to equilibrate for about 40 minutes at ambient temperature (23$^{o}$C). HA was determined by placing the plate on a microtitre equipment and observing the absence or presence of tear-shaped streaming of RBCs. The titration was read to the highest dilution giving complete HA i.e., no streaming. This represented 1 HA unit. Afterwards the value of 4HAU was calculated thus:

If 1 HA unit in the haemagglutination titration was the minimum amount of virus that caused complete agglutination of the red blood cells, the last well that showed complete agglutination was the one containing 1 HA unit.

The HA titre of the antigen was found to be 256, dilution factor to prepare 4 HA unit is calculated thus,

$$256/4 = 64$$

Therefore, 1ml of the original viral suspension was diluted in 63ml PBS.

The HA titre of the test sample was the reciprocal of the dilution that produced one HA unit. i.e., since the last microwell was 256,

HA titre = 1/256

3.6.3 Haemagglutination-Inhibition (HI) Test: This is used to titrate the antibody concentration in the serum of the vaccinated birds to determine the highest dilution that will inhibit haemagglutination of RBCs by specific virus. The method is as follows;

Aliquot of 0.025ml PBS was dispensed into each well of the plastic V-bottomed microtitre plate and then, 0.025ml of serum was placed into the first well of the plate. Twofold dilutions of the 0.025ml volumes of the serum was made across the plate and then, 0.025ml of the 4HAU of antigen was added to each well and the plates left to incubate at ambient temperature (23$^{o}$C) for 30 minutes. Aliquots of 0.025ml of the 1% (v/v) chicken RBC was added to each well and gently mixed by tapping the plates lightly and allowed to equilibrate for 40 minutes at ambient temperature. The HI titre was recorded as the highest dilution of serum causing complete inhibition of 4HAU of the antigen. The agglutination of RBC was assessed by viewing the settling pattern over a microtitre equipment. Wells in which RBCs stream at the same rate as the control wells (devoid of antigen but, contains 0.025ml RBCs and 0.05 PBS only) was considered to show inhibition. The validity of the result was assessed against a negative and positive control serum. Serum was used in HI test as described by Allan and Gough (1974) in section 3.6.3.

## 3.7 Experimental Parameters

### 3.7.1 Persistence of Maternal Antibodies

Blood was collected from five randomly selected unvaccinated control birds on days 1, 3, 10, 14, 24, 28 and 35. Serum was extracted and assayed for HI titre which was recorded appropriately.

### 3.7.2 Potency test

The vaccines were assayed for Haemagglutination activity (HA) as described by Allan and Gough (1974) in section 3.6.2.

### 3.7.3 Test for thermostability

To compare the heat stability, the vaccines were incubated at $4^oC$, $24^oC$ and $40^oC$ for a period of 24 hours and subsequently for six (6) days. The HA titre was then determined following the procedure described in section 3.6.2.

### 3.7.4 Efficacy test

Fifty (50) day-old broilers were divided into five (5) groups (n=10) tagged as I, II, III, IV and V. They were managed differently to prevent cross contamination or horizontal spread of vaccine virus. The birds in groups I - IV were actively vaccinated against ND on day 14 and 28 via drinking water using vaccines A, B, C, and D respectively. The birds in group V serve as unvaccinated control. The serum HI antibody titre response to the different vaccines in each group and unvaccinated control was determined at ages of 17, 21, 24, 28 and 35 days using the Haemagglutination-Inhibition test as described in section 3.6.3.

### 3.7.5 Effect of time on vaccination schedule

Twenty (20) day-old broilers were divided into two (2) groups of ten birds each (n=10) tagged as I and II. Two out of the four experimental vaccines were randomly selected; denoted as A and B respectively. The birds in group I were vaccinated with vaccine A and those in group II with vaccine B on day 14 and 35 via drinking water. The serum HI antibody titre of each group were determined on day 17, 21, 28, 35 and 42 using the Haemagglutination-Inhibition test as described in section 3.6.3.

### 3.7.6 Mortality rate

In the course of the study at week five, the birds were exposed to an environmental form of viscerotropic velogenic form of the virus and the number of mortality across the groups was noted.

## 3.8 Data Analysis

The data collected through the study was recorded as titre under corresponding days. Statistical Product and Service Solutions (SPSS 16) was used for Analysis of variance (ANOVA) of means of the data and $P \leq 0.05$ was considered significant.

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 Persistence of Maternally Derived Antibody (MDA)

Maternally derived antibody (MDA) was measured as HI titre and is shown in Table 4.1; Appendix 2. Heamagglutination-inhibition was at its peak value 64-128 (102.40±35.05) on day 1 and lowest on day 28; 0-2 (0.80±1.10). However, on day 35, there was no measurable HI titre. Day 1; 64-128 (102.40±35.05), Day 3; 64-128 (89.60± 35.05), Day 10; 16-64 (41.60±21.47), Day 14; 16 (16.00±0.00), Day 24; 4-8 (5.60±2.19), Day 28; 0-2(0.80±1.10), Day 35; 0 (0±0.00).

The titre obtained on day 1 and 3 are similar but of the five randomly selected chicks on day 1, three gave HI titre value of 128 and two gave 64. But on day 3, two gave 128 while three gave HI value of 64. On day 10, two birds gave HI titre value of 64, two gave 32 and only one gave value of 16. Day 14, all the five randomly selected birds gave HI titre value of 16. On day twenty-four, two birds gave HI titre value of 8 and three gave value 4. However, on day 28, which indicated absence or non-protective titre, two birds gave value of 2 while the last three gave titre value of 0. On day 35, all five randomly selected birds gave value of 0.

### 4.2 Potency Test of Four Live NDV La Sota Vaccines used

The haemagglutination activity titre obtained immediately after purchase was 128 for all vaccines (Table 4.2). This showed that all the vaccines used in this research were lentogenic strains.

### 4.3 Thermostability of the Vaccines

After storing the test vaccines at different temperatures for seven consecutive days; HA titre measured is shown in Table 4.3. At 4$^{o}$C of storage temperature after 24 hours and day 7, HA titre measured was 128. However, at storage temperatures of 25$^{o}$C and 40$^{o}$C, HA titre showed variation. The highest and lowest value of measured HA was 512 from vaccine B after 24 hours of storage at 40$^{o}$C and HA of 0 from vaccine C and D at 40$^{o}$C of storage at day 7.

**Table 4.1: Persistence of maternally derived antibody (MDA)**

| Age of birds (days) | No. of birds | GR of HI titres | GM±SD |
|---|---|---|---|
| 1 | 5 | 64-128 | 102.40±35.05 |
| 3 | 5 | 64-128 | 89.60±35.05 |
| 10 | 5 | 16-64 | 41.60±21.47 |
| 14 | 5 | 16 | 16.00±0.00 |
| 24 | 5 | 4-8 | 5.60±2.19 |
| 28 | 5 | 0-2 | 0.80±1.10 |
| 35 | 5 | 0 | 0 |

SD=standard deviation, HI=heamagglutination inhibition, GR=group range, GM=geometric mean

**Table 4.2: Comparative Haemagglutination Activity (HA) of Four Live ND La Sota Vaccines Commercially Sold in Kano State.**

| Vaccine source | $EID_{50}$ | HA titre |
|---|---|---|
| **A** | NA | 128 |
| **B** | NA | 128 |
| **C** | $10^6$ | 128 |
| **D** | $10^6$ | 128 |

$EID_{50}$=embryonic infective dose 50, HA=haemagglutination, NA= not available

**Table 4.3: Effect of temperature treatment on the Haemagglutination activity (HA) of the test ND La Sota vaccines**

| | 24 hours | | | 7 days | | |
|---|---|---|---|---|---|---|
| **Vaccine** | **4°C** | **25°C** | **40°C** | **4°C** | **25°C** | **40°C** |
| **A** | 128 | 128 | 16 | 128 | 64 | 4 |
| **B** | 128 | 128 | 512 | 128 | 128 | 32 |
| **C** | 128 | 128 | 0 | 128 | 128 | 0 |
| **D** | 128 | 128 | 0 | 128 | 8 | 0 |

°C = degrees celsius

### 4.4 Comparative Haemagglutination Inhibition (HI) Titre of Chickens Following Vaccination with Four different brands of NDV La Sota vaccine

The relative HI antibody titers of sera samples obtained from birds in vaccinated groups A-D together with that of the non-vaccinated group E is shown in Table 4.4; Appendix 3,4,5,6. The mean HI titers of the four vaccinated groups of birds are of similar order when measured at 17, 21, 24, 28, 35 days old. Haemagglutination inhibition measured in all the groups was highest on day 17; three days post vaccination (DPV) and lowest on day 35. Although the ranges appear similar, on day 17, out of five randomly selected birds in group A, two gave HI titre of 512 while three gave value of 256. Similarly, on the same day among group B, four of the birds gave titre value of 512 and only one gave a titre of 256. Whereas, among group C, two gave 512 and three was 256. In group D, three of the results are 512 and the rest two, 256. This shows that all test vaccines provoked a high level of antibody production. On the different days each tests were conducted, p-values calculated was significant ($p<0.05$).

However, across the days; p-values calculated shows that there was no significant difference among the HI titres obtained in all five groups. The range obtained from day 17 is 256-512. On day 35; HI titre obtained was as low as 8-16 in all the groups. It means therefore that though vaccinated, chickens may be at risk of ND at ages above 5 weeks.

**Table 4.4: Comparative Haemagglutination Inhibition (HI) Titre of Chickens Following Vaccination with Four different brands of NDV La Sota vaccine**

| Vaccine | Day17<br>GR<br>GM±SD | Day21<br>GR<br>GM±SD | Day 24<br>GR<br>GM±SD | Day 28<br>GR<br>GM±SD | Day 35<br>GR<br>GM±SD | P-Value |
|---|---|---|---|---|---|---|
| **A** | 256-512<br>358.40±140.22 | 128–256<br>230.40±57.24 | 64-256<br>179.20±105.16 | 16–64<br>35.20±26.29 | 8–16<br>9.60±3.58 | 0.065* |
| **B** | 256–512<br>460.80±114.49 | 128–256<br>230.40±57.24 | 128-256<br>204.80±70.11 | 16–64<br>35.20±26.29 | 8–16<br>12.80±4.38 | 0.075* |
| **C** | 256–512<br>358.40±140.22 | 128-256<br>204.80±70.11 | 128–256<br>179.20±70.11 | 16–64<br>35.20±26.29 | 8<br>8.00±0.00 | 0.068* |
| **D** | 256 – 512<br>409.60±140.22 | 128 – 256<br>230.40±57.24 | 128 - 256<br>204.80±70.11 | 16 – 64<br>44.80±26.29 | 8 – 16<br>11.20±4.38 | 0.066* |
| **Control** | 16 – 64<br>41.60±21.47 | 8 – 16<br>12.80±4.38 | 4 – 8<br>5.60±2.19 | 0 – 2<br>0.80±1.10 | 0<br>0.00 | 0.190* |
| **P-Value** | 0.011** | 0.013** | 0.015** | 0.017** | 0.020** | |

SD = standard deviation; * = no significant difference; ** = significant difference; GR= geometric range; GM= geometric mean

## 4.5 Effects of Time with Respect to Vaccination Schedule

The mean anti-ND virus antibody HI titre of the two vaccinated groups (I and II) follow a similar order when measured on days 17, 21, 28, 35 and 42. The highest titre value range in group I was obtained on day 17 as; 256 – 512 (409.60±140.22) and lowest on day 28 as; 128 – 256 (204.80±70.11). Similarly, HI titre value measured from group II, was highest on day 17; 256 – 512 (409.60±140.22) and lowest on day 21 as; 128 – 512 (409.60±140.22). The result is presented on Table 4.5.

## 4.6 Morbidity and Mortality Rate among the Different Study Groups

At five weeks of age, the total number of morbid birds (Plate I) from all the groups was 16; this accounted for 32% of the total population. Similarly, the total number of mortalities (Plates II-IV) recorded was nine indicating 18% of the total population of study birds. The result is shown on Table 4.6. However, percentage morbidity and mortality of the vaccinated groups is 27.5% and 12.5% respectively and 50% and 40% respectively in the unvaccinated group. This is shown on Table 4.7.

**Table 4.5: Effects of Time with Respect to Vaccination Schedule**

| Vaccine | Day17<br>GR<br>GM±SD | Day21<br>GR<br>GM±SD | Day 28<br>GR<br>GM±SD | Day 35<br>GR<br>GM±SD | Day 42<br>GR<br>GM±SD | P-Value |
|---|---|---|---|---|---|---|
| **A** | 256–512<br>409.60±140.22 | 128–512<br>281.60±140.22 | 128 - 256<br>204.80±70.11 | 256-512<br>307.20±114.49 | 256-512<br>307.20±114.49 | 0.001** |
| **B** | 256–512<br>409.60±140.22 | 128–256<br>230.40±57.24 | 256<br>256.00±0.00 | 256-512<br>307.20±114.49 | 256<br>256.00±0.00 | 0.001** |
| **Control** | 16–64<br>41.60±21.47 | 8–16<br>12.80±4.38 | 4<br>4.00±0.00 | 0<br>0.00±0.00 | 0<br>0.00±0.00 | 0.210** |
| **P-Value** | 0.144* | 0.168* | 0.182* | 0.184* | 0.187* | |

SD = standard deviation; * = no significant difference; ** = significant difference; GR= geometric range; GM= geometric mean

**Table 4.6: Mortality and Morbidity Rate (MMR) among the Study Groups at five weeks**

| Group | No. of morbidity | % | No. of Mortality | % | GM HI titre |
|---|---|---|---|---|---|
| **A** | $3^{s}/10^{n}$ | 30 | $1^{d}/10^{n}$ | 10 | 9.60 |
| **B** | $2^{s}/10^{n}$ | 20 | $1^{d}/10^{n}$ | 10 | 12.80 |
| **C** | $2^{s}/10^{n}$ | 20 | $2^{d}/10^{n}$ | 20 | 8.0 |
| **D** | $4^{s}/10^{n}$ | 40 | $1^{d}/10^{n}$ | 10 | 11.20 |
| **E** | $5^{s}/10^{n}$ | 50 | $4^{d}/10^{n}$ | 40 | 0.00 |
| **Total** | $16^{s}/50^{n}$ | 32 | $9^{d}/10^{n}$ | 18 | - |

s= no of sick birds; d= no of dead chickens; n= no of birds in the group; GM=group mean; HI= heamagglutination inhibition;

**Table 4.7: Compared Total MMR of Vaccinated to Unvaccinated birds in the study**

| Group | Total no. of morbidity | % | Total no. of mortality | % |
|---|---|---|---|---|
| **Vaccinated** | $11^s/40^n$ | 27.5 | $5^d/40^n$ | 12.5 |
| **Unvaccinated** | $5^s/10^n$ | 50 | $4^d/10^n$ | 40 |

s= no of sick birds; d= no of dead chickens; n= no of birds in the group

Plate I: clinical signs among control group; note anorexia and depression (birds lying down).

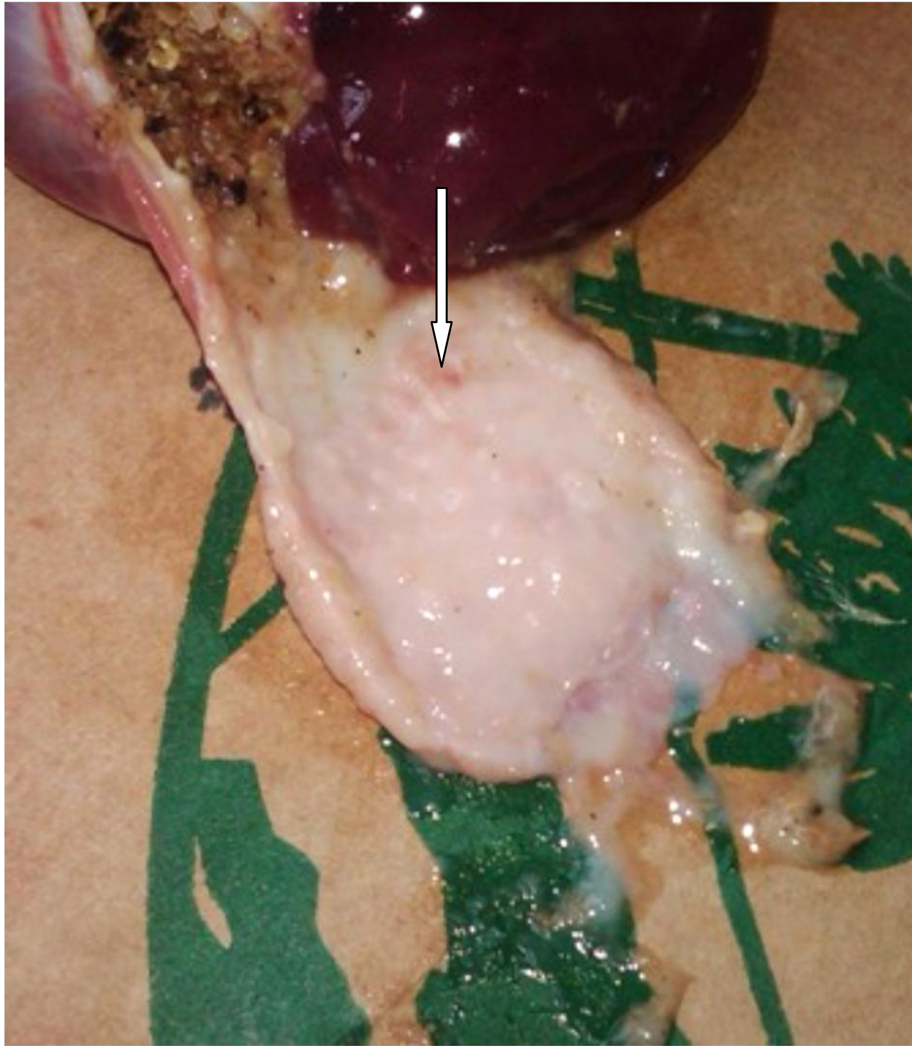

Plate II: Ulcer and pin-point hemorrhage in the proventriculus of a dead chicken from group C.

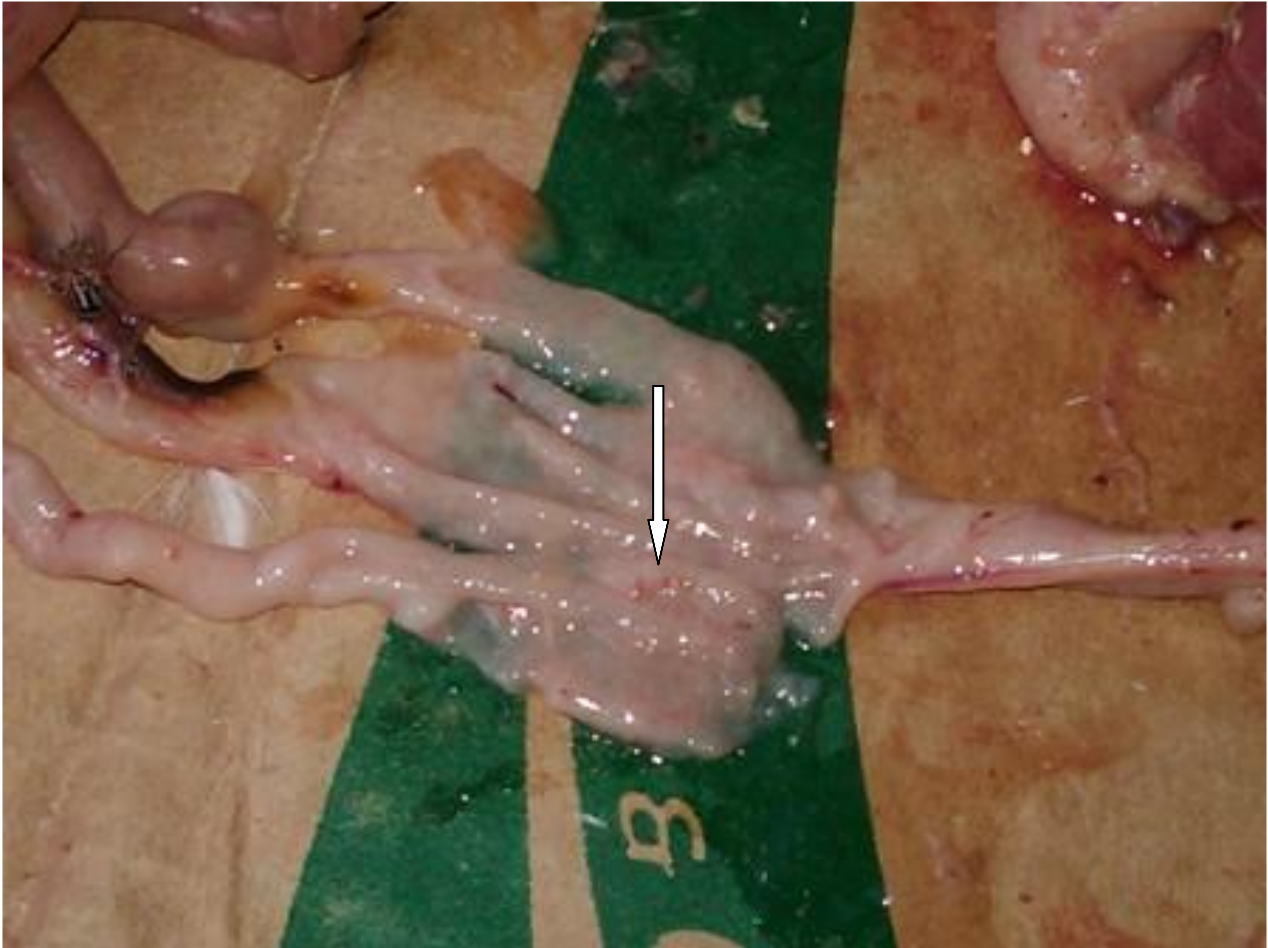

Plate III: Hemorrhagic ulcer in caeca and caecal tonsils of a dead chicken from group A.

Plate IV: Breast muscle of a dead chicken from group E showing congestion.

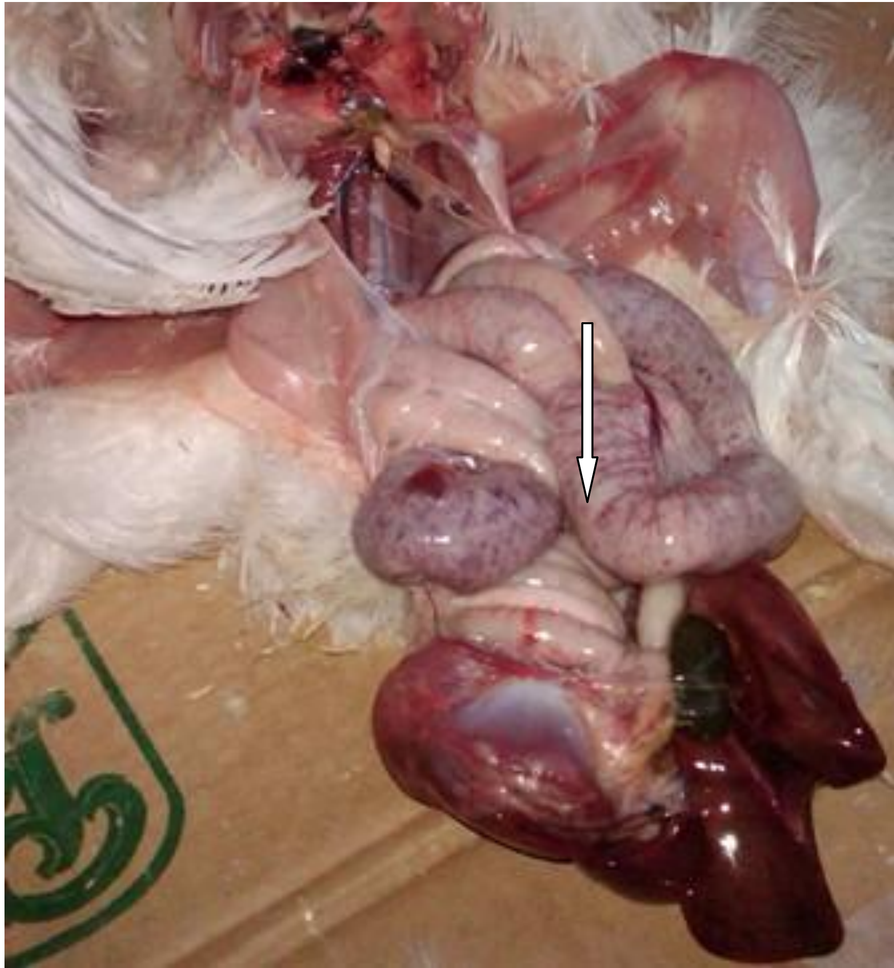

Plate V: Petechial hemorrhage in the colon of a dead chicken from group C.

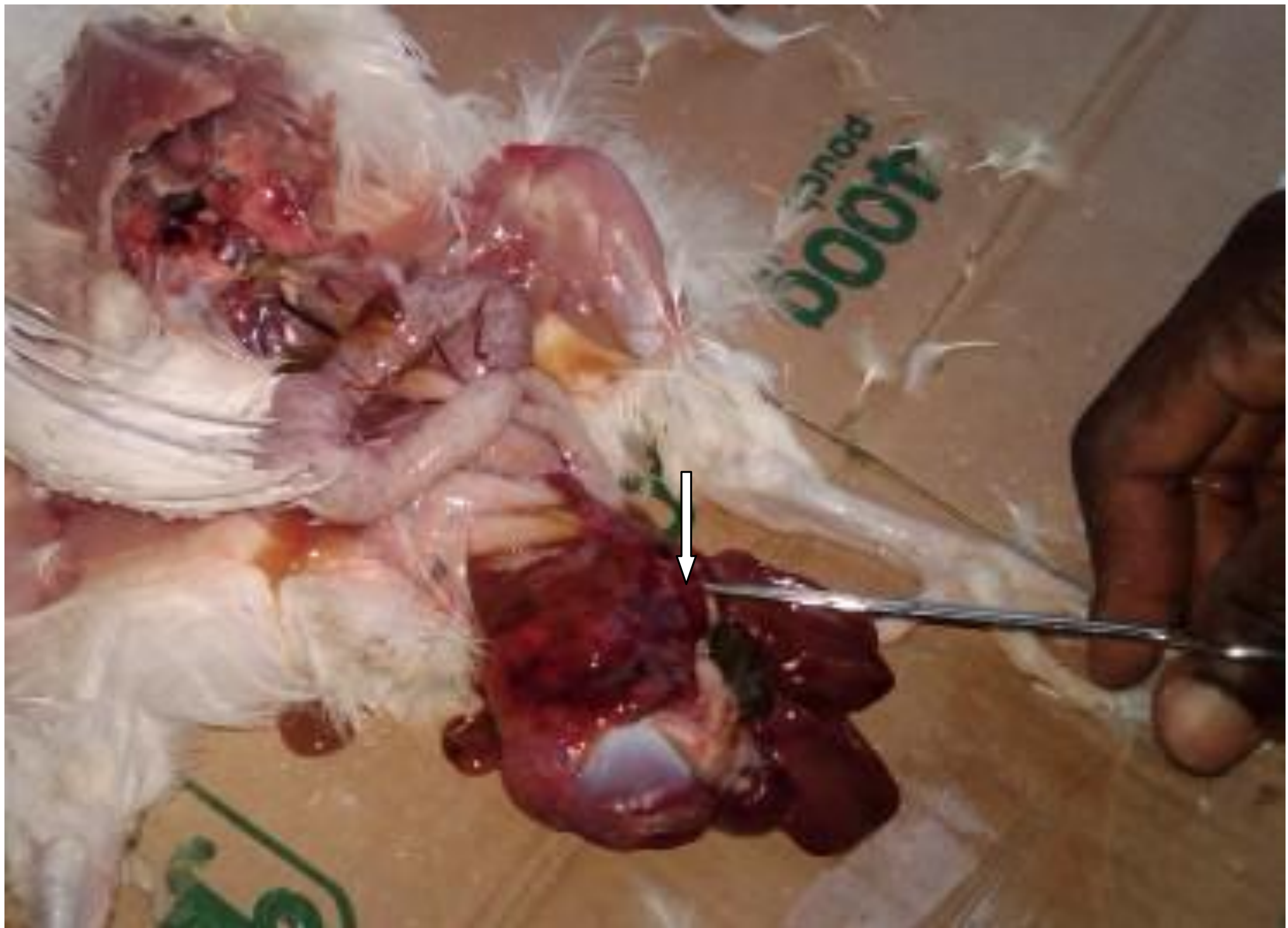


Plate VI: Severe congestion of the lungs (note the tip of the scissors) of a dead chicken from group E.

# CHAPTER FIVE

## 5.0 DISCUSSION

The mean serum Haemagglutination inhibition (HI) antibody titre measured at day old for the uncontrolled group was 102.40 which indicate a high protection of the chickens against Newcastle Disease. This titre obtained can be described as maternally derived antibody (MDAs) because active immunity against ND develops within a week of age or older. The level of MDA HI titre measured was protective throughout the first two weeks of age in correlation with the findings of Jalil *et al*. (2009) that MDAs at day old measured up to 198.60 (geometric mean) and this high levels of MDAs provide protection of chickens for up to two weeks of age and this protected all chicks after challenge with virulent ND virus.

From this research, MDA titre obtained at day 1 was high enough to confer complete protection; this declined gradually till maternal protection was completely lost on day 28. However, the result obtained from the study showed that MDA remained protective till day 14 but persisted till day 24. This is similar to reports by Balla (1986) and Mahmud (2006) which both reported that MDA persists for 27days. Barua *et al*. (2008) and Begum *et al*. (2006) reported 20 days and 18 days respectively. Chowdury *et al*. (1982) reported 12 days, Islam *et al*. (2003) reported 15 days and Shil *et al*. (2011) reported 17 days. It is important to note that the rate of declination of MDA is half every seven days (Jalil *et al*., 2009).

These results although similar in trend, still exhibit a few disparities which could be as direct description of the quality of poultry stock available in the commercial market. The level of MDA is an indication of the immunity level of the brooder stock as passed down vertically to the

chicks. Day-old chicks should be of optimum quality with remarkable passive immunity against common infections that pose severe economic consequences. The priming time is dependent on the level of MDA (Barua *et al.,* 2008). These results show that primary vaccination against ND is best at two weeks of age of birds; a little earlier or later may not yield the desired results. The former is because of high titre of MDAs which can interfere with vaccine virus multiplication leading to low level of sero-conversion resulting in weak immunity (vaccine failure) as reported by Awang *et al*., 1992 that vaccine virus can be neutralized in the presence of high level of MDA. Furthermore, the latter is because after 14 days, the flock is weakly protected against ND as a result of dawning level of MDAs and Exposure to the environmental form of ND virus can result in infection which can become magnified by the stress of vaccination leading to severe post-vaccination response leading to mortality among the flock. This is supported in a report by OIE (2012a) that one of the most important considerations affecting vaccination programs is the level of maternal immunity in young chickens which may vary considerably from farm to farm, batch to batch and among individual chickens.

The Haemagglutination (HA) titre measured for all four test vaccines used in the study was 128 shown in Table 4.2. This follows the report that La Sota vaccine is a lentogenic live virus vaccine. Haemagglutination titre is used to evaluate immune response after vaccination (OIE, 2012b); therefore, the ability of birds (in study groups A-D) to produce measureable anti-ND virus HI titre post vaccination indicated that the vaccines provoked immune response and antibody production in the birds. According to history of ND vaccination, vaccinated birds appeared to be more affected by ND than unvaccinated birds. This could be as a result of ND vaccines circulating in the market as some might not be potent rather virulent due to loss of

attenuation resulting from bad handling or more likely, due to the fact that vaccination administration regime need to be revised to suit the specific need of the Nigerian poultry farm. Okwor *et al*. (2009) reported that at common fluctuating storage temperatures obtained in Nigeria, the potency of foreign La Sota vaccines as measured by HA titre declines appreciably as storage progressed. In this study, storage at high temperature of 40$^{o}$C showed what can be termed as antigenic reversion. This is supported by report by Gould *et al*. (2001) that on the issue of reversion to virulence by live attenuated vaccine, virulent ND virus can emerge by mutation from virus of low virulence. The vaccines used for this study showed no thermostability as measured HA titre after storage under high temperatures indicated loss of viability (HA=0) and loss of attenuation (HA=512). The HA titre of the different test vaccines under different storage temperature and time is shown in Table 4.3.

Anti-ND virus antibody HI titre measured post vaccination for all study groups is shown in Table 4.4. Measured HI titre at day 17 (three days post primer vaccination) was in the range of 256-512 for all vaccinated groups. This show decline in value measured till day 28 but the values were still within protective range suggesting that vaccinated chicks responded well and showed notable sero-conversion. Administration of booster dose of vaccine on day 28 did not produce any notable increase in measured HI titre values obtained on day 28 and 35. Though, one may assume that time is required for sero-conversion. However, when HI titre was measured at thirty-five days (five weeks) of age, levels obtained indicated weak protection and vulnerability of birds to ND virus infection. This low HI titre obtained at five weeks geometric mean 9.60, is very similar to results obtained by Shil *et al*. (2011) at age thirty-two of birds after a single vaccination.

One deduction from this result is the possibility that though the birds did not show higher HI antibodies production after booster dose was administered, one can assume that the level of virus neutralizing (VN) antibodies had increased. This is so because Lancaster (1981) reported that serological response of chickens to ND virus either from natural infection or vaccination is manifested by appearance of both HI and VN antibodies. Hossain (1989) and Haplin (1978) stated that HI and VN antibody production followed a similar pattern but VN antibodies persist longer and in relatively higher titers. HI test provides a measurement of the ability of serum from an exposed bird to inhibit agglutination of chick RBC by ND virus, on the other hand, VN antibodies indicates the ability of serum to neutralize infective property of ND virus and therefore, provides more precise information about protection. This is in agreement with Spradbrow (1993) whose work showed that oral vaccination against ND virus can prevent disease but not infection. Among the various causes of endemic Newcastle disease despite vaccination are the age of birds and the time of vaccination (Barua *et al*., 2008). It is a fact that although vaccination is thoroughly followed, incidence of ND with varying degree has been reported by different investigators such as 18.65% by Kamal (1989), 4.80% by Bhattacharjee *et al*. (1996), Islam *et al*. (1998) reported by 17.20%, 10.24% by Talha *et al*. (2001), Saleque *et al*. (2003) reported 9.80% and 19.50% was reported by Das *et al*. (2004).

Administration of vaccine through water has earlier been described as the best and cheapest method of immunizing chickens. Live vaccines prepared with lentogenic strains of ND virus are commonly used in broilers than vaccines prepared from chemically inactivated strains of ND virus mixed with adjuvant (Alexander, 1997; Biggs *et al*., 1998). This is because live freeze-

dried vaccines can be produced on a large scale and they rapidly stimulate humoral, cell mediated and mucosal surface immunity (Van Eck, 1987). Low HI titre value as measured in chicks vaccinated via water in the study and even on field maybe as a result of peck order as reported by Halle *et al*. (1999) that the inability of some birds to take in adequate vaccine-in-water during mass vaccination might contribute to the high susceptibility of chicken to ND. This plays an important role in determining which bird had access to the drinker during vaccination as available drinkers may not be enough. Also, the time taken by chicks to consume reconstituted vaccines play important role in the immunological response of vaccinated flock (Halle *et al*., 1999).

Another explanation for this decline in mean HI antibody titre obtained in all groups is the interval between the primary and booster administration of vaccine. Allan *et al*. (1978) reported that if the time interval between primary and secondary vaccination is less than 21 days, the antibodies produced by the first vaccination are likely to interfere with the multiplication of the second dose of vaccine virus. In this study, birds primed at day 14 and boosted at day 35 produced more measurable HI titre with consistence in value measured ranging from 256-512 in all groups up until day 42 as shown on Table 4.5. This is similar to reports by Nasser *et al.* (2000) that there is significant rise of HI antibody titre following booster dose given three weeks after first vaccination. Jalil *et al*. (2009) reported that birds vaccinated twice produced better humoral response to the vaccine; also birds primed on seven days of age and boosted on 28 days of age (three-week interval) produced better immune response. Iederis *et al*. (1990) and Nasser *et al*. (2000) reported that birds vaccinated against ND virus orally only once died from challenge.

Flock immunity reported by van Boven *et al*. (2008) is the only means to prevent the transmission of ND virus and this is in agreement with Young *et al*. (2002) that if over 70% of the flock are immune, there will not be enough susceptible to propagate an epidemic. In this study, morbidity and mortality rate recorded among the vaccinated group (27.5% and 12.5% respectively) is low compared to the unvaccinated group (50% and 40% respectively). This shows that oral La Sota vaccine is effective in protection of birds against Newcastle disease; oral ND vaccines have been reported to primarily provoke mucosal immunity and it is accepted that this is the first line of defence against ND virus infection which occurs via inhalation, ingestion or both (Spradbrow and Samuel, 1991).

As regards vaccination of chicks against ND virus in earlier days, use of lentogenic strain is advised (Shil *et al*., 2011). Cornax *et al*., (2012) reported that live heterologous La Sota vaccine was not effective in preventing replication of ND virus genotype VIId at clinically protective doses ($10^3$-$10^5$ $EID_{50}$/birds). However, if vaccine dose was high ($10^6$-$10^8$ $EID_{50}$/birds), virus replication measured indirectly as high HI post vaccination titre was prevented. Asplin / F strain is superior to use as primary vaccine based on ICPI of 0.25. La Sota strain causes considerably greater problems in young susceptible birds than Hitchner $B_1$ strain, although it induces a stronger immune response (OIE, 2000). Newcastle disease outbreaks continue even in vaccinated areas possibly because coverage is low or the vaccination does not provide perfect immunity which could arise due to antigenic divergence between the vaccine strains and the circulating field strains (Boven *et al*., 2008; Dortmans *et al*., 2012) farmers therefore sell many of the sick birds prior to such disease occurrence in order to avoid losses from outbreaks (Ndidde *et al*., 2014).

In this experiment, the mortality observed among the vaccinated and unvaccinated groups showed correlation of immune status and the observation of clinical signs post exposure. At five weeks of age generally, the HI titre obtained among all groups can be considered as non-protective (geometric mean obtained across groups are all lower than 16). Clinical signs observed among the study groups include; anorexia, dullness, hurdling followed by respiratory signs similar to those described for viscerotropic Newcastle disease by Echeonwu *et* al., 1993 and Sa'idu *et al*., 2006. These includes; anorexia, dullness, hurdling followed by respiratory signs. Plate 1 shows the clinical symptoms shown among sick birds in the study which are anorexia and loss of appetite. Most commonly observed post-mortem lesions were ulcer and pin-point hemorrhages at the tip of the proventriculus (Plate II), hemorrhagic ulcers in the caeca and caecal tonsils (Plate III), congestion in breast muscles and lungs (Plates IV and VI) with catarrh exudates, petechial hemorrhage in colon (Plate IV). These findings agree with the findings of Hasan *et al*. (2010) and Ndidde *et al*. (2002).

For the control of ND in Nigeria, La Sota vaccines are produced by the National veterinary research Institute (NVRI), VOM (although, this brand is not available commercially in Kano metropolis during the time this research was conducted). Besides this, vaccine containing La Sota, komarov (killed and live), $B_1$ Hitchner strains are imported and sold vastly. These are administered under different schedule of vaccination depending on the flock such as broilers, layers, and parent and grandparent stock. In the course of this research, personal interactions with poultry farmers in some selected locations in Kano state revealed their incessant battle with ND despite adhering strictly to vaccination regime. They recounted experiencing symptoms such

as respiratory rales, paralysis, cyanosis (blue coloration of comb) among their flock at late ages (from about five weeks on). They can be quoted saying "broilers are not profitable because they are fragile and die all too easily". The symptoms recounted above are indicative of ND. Considering the financial implications, one would expect that these birds should be near table size and as such, any mortality is a loss of investment. Commercial broiler medication regime in Kano metropolis (study area) includes administration of antibiotics and anti-stress at five weeks of age onward. This may infer the need to curb secondary infection that may increase the burden of ND.

From results obtained in this research, one can say that the poultry farmers in Kano are fighting an incessant battle with Newcastle disease among their flock; managing their losses and quickly selling off sickly birds or even worse, prompt humane slaughter of almost dying birds. It is important to note that farmers spend a lot of money to buy drugs to combat infections whose vaccines are available. This consistence in vaccination failure against ND virus may be the main reason why this disease has become endemic in Nigeria.

# CHAPTER SIX

## 6.0 CONCLUSION AND RECOMMENDATIONS

### 6.1 CONCLUSION

This study has shown that maternally derived antibodies confer weak protection on broiler chickens and does not last longer than twenty-four days.

All four test La Sota vaccines are lentogenic strains and are not thermo-stable. They provoked immune response in the study birds which was measurable as anti-ND virus HI titre. The value of HI titre (512) measured post sero-conversion indicated high protection levels.

The protection rates conferred on vaccinated chicken after primer vaccination at day 14 is as high as 512 indicating strong protection. However, booster dose vaccination on day 28 did not improve HI titre measured and the value obtained waned before five weeks age of birds. When the birds were primed on day 14 and boosted on day 35, HI titre value measured remained as high as 512 till seven weeks age of birds.

Morbidity and mortality rate post exposure to the environmental from of ND virus was less among the vaccinated groups than among the unvaccinated control groups.

### 6.2 RECOMMENDATIONS

In deciding how best to reduce the burden and risks of ND and the economic loss associated with it, it is recommended that strategies to reduce the risk of transmission of the causative agent within the community be effected. These should involve the measures to avoid infection,

measures to be employed in event of disease occurrence and those post infection and death of the birds as follows;

1. Farmers should source for their birds from reliable outlets and never purchase sickly looking birds.
2. All routine vaccination must be strictly adhered to whether the farm has a history of ND outbreak or not.
3. Vaccines must be procured from reputable agro-vet outlets and cold chain storage maintained till administration.
4. Reconstituted vaccines should be used up within 30 minutes to one hour of administration and left-over discarded.
5. All cases of death of birds should be reported to the veterinary doctor and post-mortem carried out to ascertain the cause. Then, carcasses should be properly disposed and not thrown into the drainages or refuse dump as commonly practiced.
6. Further research should be carried out to establish more reliable optimum time interval between primer and booster ND vaccine administration for a successful broiler chicken farming.

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# APPENDICES

## Appendix 1: Preparation of 1 liter isotonic Phosphate buffered saline (pH 7.2)

| | |
|---|---|
| • Sodium chloride (NaCl) | 40gm |
| • Potassium chloride (KCl) | 1gm |
| • $KH_2PO_4$ | 1gm |
| • $Na_2HPO4$ | 7.2gm |
| • Sterile distilled water | 5000mls |
| • First pH reading | 8.4 |
| • Volume of 5M HCl added | 6mls |
| • Final pH reading | 7.2 |
| (Sterilized at 121$^{o}$C for 15 minutes) | |
| pH reading after sterilization | 7.215 |

**<u>Appendix 2: Results of persistence of MDA HI titer of the control chicks</u>**

| SAMPLE | DAY1 | DAY3 | DAY10 | DAY14 | DAY24 | DAY28 | DAY35 |
|---|---|---|---|---|---|---|---|
| **1** | 128 | 128 | 64 | 16 | 4 | 2 | 0 |
| **2** | 128 | 128 | 64 | 16 | 4 | 2 | 0 |
| **3** | 64 | 64 | 32 | 8 | 4 | 0 | 0 |
| **4** | 64 | 64 | 16 | 16 | 8 | 0 | 0 |
| **5** | 128 | 64 | 32 | 8 | 8 | 0 | 0 |
| **Mean ± SD** | 102.40± 35.05 | 89.60±35.05 | 41.60±21.47 | 12.80±4.38 | 5.60±2.19 | 0.80±1.10 | 0.00 |

## Descriptive Statistics

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| DAY1 | 5 | 64 | 128 | 102.40 | 35.054 |
| DAY3 | 5 | 64 | 128 | 89.60 | 35.054 |
| DAY10 | 5 | 16 | 64 | 41.60 | 21.466 |
| DAY14 | 5 | 8 | 16 | 12.80 | 4.382 |
| DAY24 | 5 | 4 | 8 | 5.60 | 2.191 |
| DAY28 | 5 | 0 | 2 | .80 | 1.095 |
| DAY35 | 5 | 0 | 0 | .00 | .000 |

**Appendix 3: HI titer of chicks vaccinated with JOVAC La Sota**

| SAMPLE | DAY17 | DAY21 | DAY24 | DAY28 | DAY35 |
|---|---|---|---|---|---|
| 1 | 512 | 256 | 256 | 64 | 8 |
| 2 | 256 | 256 | 256 | 16 | 8 |
| 3 | 512 | 256 | 64 | 64 | 8 |
| 4 | 256 | 256 | 64 | 16 | 16 |
| 5 | 256 | 128 | 256 | 16 | 8 |
| Mean ± SD | 358.40±140.22 | 230.40±57.24 | 179.20±105.16 | 35.20±26.29 | 9.60±3.58 |

**Descriptive Statistics**

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| DAY17 | 5 | 256 | 512 | 358.40 | 140.217 |
| DAY21 | 5 | 128 | 256 | 230.40 | 57.243 |
| DAY24 | 5 | 64 | 256 | 179.20 | 105.163 |
| DAY28 | 5 | 16 | 64 | 35.20 | 26.291 |
| DAY35 | 5 | 8 | 16 | 9.60 | 3.578 |

**Appendix 4: HI titer of chicks vaccinated with ABIC La Sota**

| SAMPLE | DAY17 | DAY21 | DAY24 | DAY28 | DAY35 |
|---|---|---|---|---|---|
| 1 | 512 | 256 | 256 | 16 | 8 |
| 2 | 512 | 256 | 256 | 16 | 8 |
| 3 | 512 | 256 | 256 | 64 | 16 |
| 4 | 256 | 128 | 128 | 64 | 16 |
| 5 | 512 | 256 | 128 | 64 | 16 |
| Mean ± SD | 460.80±114.49 | 230.40±57.24 | 204.80±70.11 | 44.80±26.29 | 12.80±4.38 |

**Descriptive Statistics**

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| DAY17 | 5 | 256 | 512 | 460.80 | 114.487 |
| DAY21 | 5 | 128 | 256 | 230.40 | 57.243 |
| DAY24 | 5 | 128 | 256 | 204.80 | 70.108 |
| DAY28 | 5 | 16 | 64 | 44.80 | 26.291 |
| DAY35 | 5 | 8 | 16 | 12.80 | 4.382 |

**Appendix 5: HI titer of chicks vaccinated with INDOVAC La Sota**

| SAMPLE | DAY17 | DAY21 | DAY24 | DAY28 | DAY35 |
|---|---|---|---|---|---|
| 1 | 256 | 256 | 256 | 64 | 8 |
| 2 | 512 | 128 | 128 | 64 | 8 |
| 3 | 512 | 128 | 128 | 16 | 8 |
| 4 | 256 | 256 | 256 | 16 | 8 |
| 5 | 256 | 256 | 128 | 16 | 8 |
| Mean ± SD | 358.40±140.22 | 204.80±70.11 | 179.20±70.11 | 35.20±26.29 | 8.00±0.00 |

**Descriptive Statistics**

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| DAY17 | 5 | 256 | 512 | 358.40 | 140.217 |
| DAY21 | 5 | 128 | 256 | 204.80 | 70.108 |
| DAY24 | 5 | 128 | 256 | 179.20 | 70.108 |
| DAY28 | 5 | 16 | 64 | 35.20 | 26.291 |
| DAY35 | 5 | 8 | 8 | 8.00 | .000 |

**Appendix 6: HI titer of chicks vaccinated with IZOVAC La Sota**

| SAMPLE | DAY17 | DAY21 | DAY24 | DAY28 | DAY35 |
|---|---|---|---|---|---|
| 1 | 512 | 256 | 128 | 64 | 8 |
| 2 | 512 | 128 | 256 | 64 | 8 |
| 3 | 512 | 256 | 256 | 16 | 8 |
| 4 | 256 | 256 | 256 | 16 | 16 |
| 5 | 256 | 256 | 128 | 16 | 16 |
| Mean ± SD | 409.60±140.22 | 230.40±57.24 | 204.80±70.11 | 44.80±26.29 | 11.20±4.38 |

**Descriptive Statistics**

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| DAY17 | 5 | 256 | 512 | 409.60 | 140.217 |
| DAY21 | 5 | 128 | 256 | 230.40 | 57.243 |
| DAY24 | 5 | 128 | 256 | 204.80 | 70.108 |
| DAY28 | 5 | 16 | 64 | 44.80 | 26.291 |
| DAY35 | 5 | 8 | 16 | 11.20 | 4.382 |

**Appendix 7: Descriptive Statistics for (JOVAC, ABIC, INDOVAX, IZOVAC &Control)**

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| DAY 17 | 5 | 41.60 | 460.80 | 325.7600 | 164.42520 |
| DAY 21 | 5 | 12.80 | 230.40 | 181.7600 | 95.09978 |
| DAY 24 | 5 | 5.60 | 204.80 | 154.7200 | 84.33761 |
| DAY 28 | 5 | .80 | 44.80 | 32.1600 | 18.17603 |
| DAY 35 | 5 | .00 | 12.80 | 8.3200 | 4.98317 |

**One-Sample Test (JOVAC, ABIC, INDOVAX, IZOVAC & CONTROL)**

| | Test Value = 0 | | | | | |
|---|---|---|---|---|---|---|
| | T | df | Sig. (2-tailed) | Mean Difference | 95% Confidence Interval of the Difference | |
| | | | | | Lower | Upper |
| DAY 17 | 4.430 | 4 | .011 | 325.76000 | 121.5991 | 529.9209 |
| DAY 21 | 4.274 | 4 | .013 | 181.76000 | 63.6780 | 299.8420 |
| DAY 24 | 4.102 | 4 | .015 | 154.72000 | 50.0010 | 259.4390 |
| DAY 28 | 3.956 | 4 | .017 | 32.16000 | 9.5915 | 54.7285 |
| DAY 35 | 3.733 | 4 | .020 | 8.32000 | 2.1326 | 14.5074 |

**Appendix 8:**

**Paired Samples Statistics (JOVAC, ABIC, INDOVAX, IZOVAC & CONTROL)**

| | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | DAY17 | 325.7600 | 5 | 164.42520 | 73.53319 |
| | DAY21 | 181.7600 | 5 | 95.09978 | 42.52991 |
| Pair 2 | DAY17 | 325.7600 | 5 | 164.42520 | 73.53319 |
| | DAY24 | 154.7200 | 5 | 84.33761 | 37.71692 |
| Pair 3 | DAY17 | 325.7600 | 5 | 164.42520 | 73.53319 |
| | DAY28 | 32.1600 | 5 | 18.17603 | 8.12857 |
| Pair 4 | DAY17 | 325.7600 | 5 | 164.42520 | 73.53319 |
| | DAY35 | 8.3200 | 5 | 4.98317 | 2.22854 |
| Pair 5 | DAY21 | 181.7600 | 5 | 95.09978 | 42.52991 |
| | DAY24 | 154.7200 | 5 | 84.33761 | 37.71692 |
| Pair 6 | DAY21 | 181.7600 | 5 | 95.09978 | 42.52991 |
| | DAY28 | 32.1600 | 5 | 18.17603 | 8.12857 |
| Pair 7 | DAY21 | 181.7600 | 5 | 95.09978 | 42.52991 |
| | DAY35 | 8.3200 | 5 | 4.98317 | 2.22854 |
| Pair 8 | DAY24 | 154.7200 | 5 | 84.33761 | 37.71692 |
| | DAY28 | 32.1600 | 5 | 18.17603 | 8.12857 |
| Pair 9 | DAY24 | 154.7200 | 5 | 84.33761 | 37.71692 |
| | DAY35 | 8.3200 | 5 | 4.98317 | 2.22854 |
| Pair 10 | DAY28 | 32.1600 | 5 | 18.17603 | 8.12857 |
| | DAY35 | 8.3200 | 5 | 4.98317 | 2.22854 |

**Paired Samples Correlations (JOVAC, ABIC, INDOVAX, IZOVAC & CONTROL)**

| | | N | Correlation | Sig. |
|---|---|---|---|---|
| Pair 1 | DAY17 & DAY21 | 5 | .975 | .005 |
| Pair 2 | DAY17& DAY24 | 5 | .990 | .001 |
| Pair 3 | DAY17& DAY28 | 5 | .993 | .001 |
| Pair 4 | DAY17& DAY35 | 5 | .989 | .001 |
| Pair 5 | DAY21& DAY24 | 5 | .992 | .001 |
| Pair 6 | DAY21& DAY28 | 5 | .976 | .005 |
| Pair 7 | DAY21& DAY35 | 5 | .959 | .010 |
| Pair 8 | DAY24 & DAY28 | 5 | .993 | .001 |
| Pair 9 | DAY24 & DAY35 | 5 | .971 | .006 |
| Pair 10 | DAY28 & DAY35 | 5 | .985 | .002 |

**Paired Samples Test (JOVAC, ABIC, INDOVAX, IZOVAC & CONTROL)**

| | | Paired Differences | | | | | t | Df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference | | | | |
| | | | | | Lower | Upper | | | |
| Pair 1 | DAY17–DAY21 | 144.0000 | 74.70475 | 33.40898 | 51.24180 | 236.75820 | 4.310 | 4 | .013 |
| Pair 2 | DAY17 - DAY24 | 171.0400 | 81.74184 | 36.55606 | 69.54410 | 272.53590 | 4.679 | 4 | .009 |
| Pair 3 | DAY17 - DAY28 | 293.60000 | 146.38251 | 65.46425 | 111.84210 | 475.35790 | 4.485 | 4 | .011 |
| Pair 4 | DAY17 - DAY35 | 317.44000 | 159.49761 | 71.32950 | 119.39756 | 515.48244 | 4.450 | 4 | .011 |
| Pair 5 | DAY21 - DAY24 | 27.04000 | 15.68082 | 7.01267 | 7.56969 | 46.51031 | 3.856 | 4 | .018 |
| Pair 6 | DAY21 - DAY28 | 149.60000 | 77.46793 | 34.64471 | 53.41086 | 245.78914 | 4.318 | 4 | .012 |
| Pair 7 | DAY21 - DAY35 | 173.44000 | 90.32988 | 40.39675 | 61.28063 | 285.59937 | 4.293 | 4 | .013 |
| Pair 8 | DAY24 - DAY28 | 122.56000 | 66.31416 | 29.65659 | 40.22009 | 204.89991 | 4.133 | 4 | .014 |
| Pair 9 | DAY24 - DAY35 | 146.40000 | 79.50648 | 35.55638 | 47.67967 | 245.12033 | 4.117 | 4 | .015 |
| Pair 10 | DAY28 - DAY35 | 23.84000 | 13.29541 | 5.94589 | 7.33156 | 40.34844 | 4.009 | 4 | .016 |

**Appendix 9**:

**Descriptive Statistics (Day 17, Day 21, Day 24, Day 28 & Day 35)**

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| JOVAC | 5 | 9.60 | 358.40 | 162.5600 | 143.91820 |
| ABIC | 5 | 12.80 | 460.80 | 190.7200 | 178.65059 |
| INDOVAC | 5 | 8.00 | 358.40 | 157.1200 | 141.75314 |
| IZOVAX | 5 | 11.20 | 409.60 | 180.1600 | 160.19029 |
| CONTROL | 5 | .00 | 41.60 | 12.1600 | 17.22696 |
| Valid N (listwise) | 5 | | | | |

**One-Sample Statistics (Day 17, Day 21, Day 24, Day 28 & Day 35)**

| | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|
| JOVAC | 5 | 162.5600 | 143.91820 | 64.36218 |
| ABIC | 5 | 190.7200 | 178.65059 | 79.89497 |
| INDOVAC | 5 | 157.1200 | 141.75314 | 63.39393 |
| IZOVAX | 5 | 180.1600 | 160.19029 | 71.63927 |
| CONTROL | 5 | 12.1600 | 17.22696 | 7.70413 |

**One-Sample Test (Day 17, Day 21, Day 24, Day 28 & Day 35)**

| | Test Value = 0 | | | | | |
|---|---|---|---|---|---|---|
| | | | | | 95% Confidence Interval of the Difference | |
| | T | Df | Sig. (2-tailed) | Mean Difference | Lower | Upper |
| JOVAC | 2.526 | 4 | .065 | 162.56000 | -16.1380 | 341.2580 |
| ABIC | 2.387 | 4 | .075 | 190.72000 | -31.1040 | 412.5440 |
| INDOVAC | 2.478 | 4 | .068 | 157.12000 | -18.8898 | 333.1298 |
| IZOVAX | 2.515 | 4 | .066 | 180.16000 | -18.7425 | 379.0625 |
| CONTROL | 1.578 | 4 | .190 | 12.16000 | -9.2301 | 33.5501 |

**Appendix 10:**

**Group Statistics (Day 17, Day 21, Day 24, Day 28 & Day 35)**

| | CONTROL | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| JOVAC | >= 12.16 | 2 | 294.4000 | 90.50967 | 64.00000 |
| | < 12.16 | 3 | 74.6667 | 91.42895 | 52.78653 |
| ABIC | >= 12.16 | 2 | 345.6000 | 162.91740 | 115.20000 |
| | < 12.16 | 3 | 87.4667 | 102.86561 | 59.38949 |
| INDOVAC | >= 12.16 | 2 | 281.6000 | 108.61160 | 76.80000 |
| | < 12.16 | 3 | 74.1333 | 92.00116 | 53.11689 |
| IZOVAX | >= 12.16 | 2 | 320.0000 | 126.71354 | 89.60000 |
| | < 12.16 | 3 | 86.9333 | 103.44880 | 59.72619 |

**Paired Samples Statistics (Day 17, Day 21, Day 24, Day 28 & Day 35)**

| | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | JOVAC | 162.5600 | 5 | 143.91820 | 64.36218 |
| | CONTROL | 12.1600 | 5 | 17.22696 | 7.70413 |
| Pair 2 | ABIC | 190.7200 | 5 | 178.65059 | 79.89497 |
| | CONTROL | 12.1600 | 5 | 17.22696 | 7.70413 |
| Pair 3 | INDOVAC | 157.1200 | 5 | 141.75314 | 63.39393 |
| | CONTROL | 12.1600 | 5 | 17.22696 | 7.70413 |
| Pair 4 | IZOVAX | 180.1600 | 5 | 160.19029 | 71.63927 |
| | CONTROL | 12.1600 | 5 | 17.22696 | 7.70413 |
| Pair 5 | JOVAC | 162.5600 | 5 | 143.91820 | 64.36218 |
| | ABIC | 190.7200 | 5 | 178.65059 | 79.89497 |
| Pair 6 | JOVAC | 162.5600 | 5 | 143.91820 | 64.36218 |
| | INDOVAC | 157.1200 | 5 | 141.75314 | 63.39393 |
| Pair 7 | JOVAC | 162.5600 | 5 | 143.91820 | 64.36218 |
| | IZOVAX | 180.1600 | 5 | 160.19029 | 71.63927 |
| Pair 8 | ABIC | 190.7200 | 5 | 178.65059 | 79.89497 |
| | INDOVAC | 157.1200 | 5 | 141.75314 | 63.39393 |
| Pair 9 | ABIC | 190.7200 | 5 | 178.65059 | 79.89497 |
| | IZOVAX | 180.1600 | 5 | 160.19029 | 71.63927 |
| Pair 10 | INDOVAC | 157.1200 | 5 | 141.75314 | 63.39393 |
| | IZOVAX | 180.1600 | 5 | 160.19029 | 71.63927 |

**Paired Samples Correlations (Day 17, Day 21, Day 24, Day 28 & Day 35)**

| | | N | Correlation | Sig. |
|---|---|---|---|---|
| Pair 1 | JOVAC & CONTROL | 5 | .908 | .033 |
| Pair 2 | ABIC & CONTROL | 5 | .951 | .013 |
| Pair 3 | INDOVAC & CONTROL | 5 | .922 | .026 |
| Pair 4 | IZOVAX & CONTROL | 5 | .926 | .024 |
| Pair 5 | JOVAC & ABIC | 5 | .988 | .002 |
| Pair 6 | JOVAC & INDOVAC | 5 | .997 | .000 |
| Pair 7 | JOVAC & IZOVAX | 5 | .996 | .000 |
| Pair 8 | ABIC & INDOVAC | 5 | .996 | .000 |
| Pair 9 | ABIC & IZOVAX | 5 | .997 | .000 |
| Pair 10 | INDOVAC & IZOVAX | 5 | 1.000 | .000 |

**Paired Samples Test (Day 10, Day 14, Day 24, Day 28 & Day 35)**

| | | Paired Differences | | | | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference | | | | |
| | | | | | Lower | Upper | | | |
| Pair 1 | JOVAC – CONTROL | 150.40000 | 128.47537 | 57.45593 | -9.12324 | 309.92324 | 2.618 | 4 | .059 |
| Pair 2 | ABIC – CONTROL | 178.56000 | 162.35802 | 72.60872 | -23.03411 | 380.15411 | 2.459 | 4 | .070 |
| Pair 3 | INDOVAC – CONTROL | 144.96000 | 126.03923 | 56.36646 | -11.53838 | 301.45838 | 2.572 | 4 | .062 |
| Pair 4 | IZOVAX – CONTROL | 168.00000 | 144.39169 | 64.57393 | -11.28596 | 347.28596 | 2.602 | 4 | .060 |
| Pair 5 | JOVAC – ABIC | -28.16000 | 42.65733 | 19.07694 | -81.12607 | 24.80607 | -1.476 | 4 | .214 |
| Pair 6 | JOVAC – INDOVAC | 5.44000 | 11.29106 | 5.04951 | -8.57970 | 19.45970 | 1.077 | 4 | .342 |
| Pair 7 | JOVAC – IZOVAX | -17.60000 | 21.34666 | 9.54652 | -44.10538 | 8.90538 | -1.844 | 4 | .139 |
| Pair 8 | ABIC – INDOVAC | 33.60000 | 39.58181 | 17.70153 | -15.54731 | 82.74731 | 1.898 | 4 | .131 |
| Pair 9 | ABIC – IZOVAX | 10.56000 | 22.72901 | 10.16472 | -17.66180 | 38.78180 | 1.039 | 4 | .358 |
| Pair 10 | INDOVAC – IZOVAX | -23.04000 | 18.57654 | 8.30768 | -46.10583 | .02583 | -2.773 | 4 | .050 |