SEROIMMUNE RESPONSES TO STRATEGIC VACCINATION IN CHICKENS AGAINST NEWCASTLE DISEASE USING COMMERCIALLY AVAILABLE VACCINES

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A DISSERTATION SUBMITTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF APPLIED MICROBIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE.

MOROGORO, TANZANIA.

2014

ABSTRACT

Evaluation of the Newcastle Disease (ND) antibody level after different vaccination strategies using I-2 and La Sota vaccines was conducted on day old broiler chicks. Five groups for each type of vaccine, containing sixteen (16) randomly assigned chicks each were kept in none communicating pens. The chicks were accorded standard management procedures including *ad libitum* feeds and water as well as deworming and vaccination against fowl pox and infectious Bursa disease. At three weeks of age, chicks were wing tagged and vaccination strategies employed. Vaccination strategies employed for each vaccine type were 12.5%, 25% and 50%, the remained percentage was filled by in-contact chicks. Vaccination at 100% and no vaccination (0%) served as controls. Blood samples were collected before vaccination and then after every two weeks, five times post vaccination. Sera were prepared and tested for antibodies against Newcastle Disease virus using the standard Haemagglutination Inhibition (HI) procedure. At 12.5% vaccination strategy for I-2, only 12.5% of the vaccinated chickens had protective antibodies level (HI titre results <log3 base 2) and none of the in contact chickens and the Geometric Mean Titre (GMT) of 1.13 was recorded and was not significantly different ($p>0.05$) with that of the negative control group. For La Sota vaccines at 12.5% strategy, 62.5% of the chickens had protective antibodies level and the GMT of 3.31 was recorded and was not significantly different ($p>0.05$) to the GMT of positive control group. At this strategy, La Sota vaccine has proved to do better than I-2 vaccine in activating immune response in chickens. At 25% vaccination strategy, both I-2 and La Sota vaccines showed that, 75% of the chickens had protective antibodies level. The GMT was 2.5 and 3.06 for I-2 and La Sota respectively. These were significantly different ($p<0.05$) to GMT of the negative controls.

The GMT in La Sota was not significant different ($p>0.05$) to the GMT of the positive control. At 50% vaccination strategy, for I-2 vaccine, 81% of the chickens had protective antibodies level and the GMT of 2.75 was recorded and was not significant different to the GMT of the positive control ($p>0.05$). For La Sota vaccines, 94% of chickens had protective antibodies level and the GMT of 5.56 was recorded and was not significant different to the GMT of the positive control ($p>0.05$). For the in-contact chickens, at 12.5% vaccination strategy the highest GMTs recorded were 2.07 and 3.07 for I-2 and La Sota respectively. The two GMTs were significantly different ($p<0.05$) and La Sota vaccine seroconverted more in-contact chickens to protective level than I-2 vaccine. For the vaccination strategy 25%, the highest GMTs were 3.83 and 3.17 for I-2 and La Sota vaccines respectively. The two GMTs were not significantly different ($p>0.05$) and the two vaccines had same potential in protecting in contact chickens. For the 50% vaccination strategy, the highest GMTs were 2.75 and 4.25 for I-2 and La Sota respectively. The two GMTs were not significant different ($p>0.05$) in protecting in contact chickens. For the trend of increasing antibodies level, statistics have shown that, there was no significant difference in the trend of antibodies levels with time between the two types of vaccine ($p>0.05$) when the GMTs of positive controls were compared throughout the study period. Therefore, if farmers follow properly manufacturers' direction of using vaccines, they need to vaccinate only 50% of the flock using either I-2 or La Sota to get herd immunity similar to vaccinating all and therefore save time and resources.

DECLARATION

I, Isaac Joseph Mengele, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

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ACKNOWLEDGEMENTS

I am thankful to the LORD, my GOD whose blessing, strength, and will, made me accomplish this work in my life time and I still need HIM more.

My sincere thanks and gratitude to my supervisors, Professor Msoffe, P.L. of Department of Veterinary Medicine and Public Health, Dr. Misinzo, G. of Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture for their tireless advice, encouragement, critics and positive cooperation in making this dissertation successful, they were all mentors to me.

My sincere thanks are extended to the Ministry of Livestock and Fisheries Development who sponsored my MSc. studies and this research work. The financial support is greatly appreciated.

I sincerely express my thanks to Officer Incharge, VIC Mpwapwa for allowing me to use their laboratory facility and equipments, Campus Director, LITA Mpwapwa for allowing me to use their poultry units for my research work, Mr. James Sehaba who carefully and tirelessly day and night kept the chickens up to the end of study, Mr. Jonas Fitwangile and Hashim Matimbwa for their valuable technical laboratory work assistance at VET-SUA laboratory, to all course mates and others who have assisted me to accomplish this dissertation.

Lastly, many thanks are extended to my family members for their moral support and encouragement during my study period, I greatly appreciate them all.

Thank you so much for your cooperation.

DEDICATION

This work is dedicated to my mother Mrs. Nicoline Mengele for her tireless support throughout my life. My late uncle Dismas Chapuga who took me to school for the first time in my life. Last but not least to my wife Angela Panyika, our beloved children Baraka and Glory for their patience during all the time of my absence during my study period.

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LIST OF ABBREVIATIONS

%	percentage
°C	degree celcius
µL	microliter
APMV	avian paramyxovirus
bp	base pair
CD	cluster of differentiating
cDNA	complement deoxyribonucleic acid
CFT	complement fixation test
EID	Embryo infectious dose
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FAO	Food and Agriculture Organization
FRVC	Free range village chickens
GMT	Geometric mean titre
HA	Haemagglutination
HI	Haemagglutination inhibition
IBAR	Interafrican Bureau for Animal Resources
IBD	Infectious bursa disease
IBV	Infectious bronchitis virus
ICPI	Intracerebral pathogenicity index
ID	Identity
IgA	Immunoglobulin A
IHC	Immunohistochemistry

INF	Interferon
ISH	In situ hybridization
KAR	Killer activator receptor
LITI	Livestock training institute
LoNDV	Low virulence Newcastle disease virus
mAbs	Monoclonal antibodies
MDT	Mean death time
MHC	Major Histocompatibility Complex
MLFD	Ministry of Livestock and Fisheries Development
MPAI	Mild pathogenic avian influenza virus
mRNA	messenger Ribonucleic acid
ND	Newcastle disease
NDV	Newcastle disease virus
NK	Natural killer
NLRI	National Livestock Research Institute
OIE	Office International des Epizootics
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMV	Paramyxovirus
PPMV	Pigeon paramyxovirus
RBC	Red blood cells
RLDC	Rural Livelihood Development Company
RNA	Ribonucleic acid

RNAP	Ribonucleic acid polymerase
rpm	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain reaction
SPF	Specific Pathogen Free
USA	United States of America
VIC	Veterinary Investigation Centre
WHO	World Health Organization

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background Information

It is estimated that Tanzania had about 36.2 million chickens by 2008, out of which almost 95% are Free Range Village Chickens (FRVC) and the rest are exotic breeds (RLDC, 2011) and most of these village chickens are kept mainly in the rural areas by women and children. Village poultry production plays an important contribution to household food security and income generation (Goromela, 2009). The proportion of traditionally raised birds kept in Tanzania, majorities are chicken (96 %), ducks, geese, guinea fowl and turkeys are less contributing to household food security, therefore are kept in minimal number by minimal number of household (NBS, 2008).

The rate of growth of the chicken industry has been increasing at 2.6% per year since 2003 (Msami, 2008). The rapid increase of the chicken industry has been influenced mainly by the increased demand of chicken meat and eggs as source of animal protein due to increasing fast food vendors in urban settings (Gyles, 1989) as well as population increase. In Tanzania chicken industry is divided into the traditional and the commercial sectors. Indigenous free range local chickens dominate in the traditional sector. Chickens are poorly managed, poorly housed and some roost on trees, no regular feed supplementation and are hardly given any veterinary attention and if given traditional medicines are practiced. Chicken management in a family is usually the responsibility of women and children and men show little interest in chickens except in some areas where chicken sale fetch higher selling price especially in areas where traders from big cities and town come to buy chickens for urban consumption. Village chicken supplies most of the poultry meat and eggs demands for rural people and about 20% of urban demand (Msami, 2008).

Poultry play a major role in meeting economic and social obligations for a family, especially for poor families. In addition to slaughtering for home consumption, chickens are sold to generate income to buy foodstuffs, medicine, clothes and may also be used to generate income for school fees, bride price, farm implements, fertilizers, and levies. Chickens are also kept for traditional healing and religious rituals.

Economic studies of village poultry keeping have shown that the sector is viable and promising alternative source of income for rural families. Salum *et al.* (1999) calculated that a household with10–15 chickens, at a reproduction rate of 3–4 generations per year and clutch size of 10–15 eggs, will generate an income of between 890, 000 and 1,580 000 T. Shs. (US$563–1000) per year at exchange rate of;1US$:1580Tsh).

The commercial sector is mainly found in urban and peri urban areas and is based on keeping the exotic improved breed of layers and broiler chickens for eggs and meat respectively to meet the high demand in urban areas (Msami, 2008). These chickens are kept in proper houses, highly supplemented with properly compounded feed and get routine veterinary attention to maximize their performance and production.

The effort in developing the chicken industry is directed to both the commercial and traditional sectors (Melewas, 1989). Importation of hatching eggs and day old chicks for commercial purposes and production and increased used of Thermo stable Newcastle disease vaccine for village chickens are all effort to develop the chicken industry in the country (MLD, 2008). This is because the industry provides employment to people both in urban and rural settings and provides income to government through levy. Therefore Tanzanian government through Ministry of Livestock Development (MLFD) has been encouraging chicken keeping for income generation and therefore poverty alleviation (MLD, 2006).

The major hindrance to rural chicken prosperity is diseases especially Newcastle Disease (ND). The disease may cause 90% of mortality rates and sometimes clears the whole flock during an outbreak (Buza and Mwamuhehe, 2000). Vaccination against the disease has remained the most effective means of controlling ND (Orajaka *et al.*, 1999; Usman, 2002). In Tanzania mostly available commercial ND vaccines are La Sota and I-2 vaccine. La Sota, a lentogenic live vaccine is used mainly in commercial poultry sector and having setbacks in application in rural areas due to the problem of heat intolerance, large dose presentation, affordability, reliability, transport and cold chain for effective administration of the vaccine (Dias *et al.*, 2001). The avirulent, thermostable ND vaccine strains I-2 provide rural poultry farmers with an effective, affordable and reliable means of controlling ND in their flocks (Dias *et al.*, 2001), and has been used widely and effectively in village chickens population in many Asian and African countries (Dias *et al.*, 2001). The vaccine is currently widely used in Tanzania (Wust *et al.*, 2010), the vaccine has been accepted as suitable for use in Vietnamese villages (Tu *et al.*, 1997) and has been used in Nigeria, Kaduna state (Nwanta *et al.*, 2006). However, apart from the vaccine availability, ND is still a bottleneck to local village production.

1.2. Problem Statement and Justification

1.2.1. Problem statement

In spite of the availability of vaccines against the ND, there is inadequacy of controlling the disease; this could probably be due to failure of following manufacturer's indications to vaccinate individual chicken in a flock, the free range nature of chickens and short time available due to farmers' engagement to other economic activities. Therefore this study was aimed at looking for the best vaccination strategy which will ensure highest level of flock protection and at the same time lessen the work of vaccinators or farmers.

1.1.2. Problem justification

Poultry play an important role in meeting economic and social obligations for the rural household. The major hindrance to rural and to some extent commercial chickens development is ND. This disease is one of the most important infectious diseases of poultry because of its worldwide distribution and the potential for massive loses (Patti *et al.*, 2010). Mazengia *et al.*, (2009) reported that, in some outbreaks of the highly infectious ND caused almost 100% mortality in some parts of African countries like United Republic of Tanzania, Ethiopia and Gambia.

The disease has potentials to be spread amongst chickens by direct contact, inhalation of viral contaminated aerosols and dust particles and eating of viral contaminated feed and water (Alexander, 1988b). Mechanical spread between flocks is influenced by the relative stability of the virus in the atmosphere and its wide host coverage (Fenner *et al.*, 1993). Vertical transmission does not occur but chicks may become infected in hatcheries from contaminated shells. The resource derivable from the chickens cannot be fully utilized unless the disease is controlled particularly in the village poultry flocks which act as reservoirs and carriers to themselves and the more prone exotic breeds in commercial farms (Adu *et al.*, 1990; Nwosu and Okeke 1989; Olabode *et al.*, 1992).

Vaccination so far remains the most viable and effective strategy for controlling Newcastle Disease (Orajaka *et al.*, 1999; Usman, 2002) and has been used widely throughout the world since the 1940s (Beard and Hanson, 1984a). La Sota vaccines have been used mainly in commercial poultry sector due to availability of transport and cold chain facilities, which is a setback for use in rural areas. However, the availability of avirulent, thermostable ND vaccine strains I-2 has rescued rural chickens from the disease; efforts are going on to extend their use.

Despite their use, these vaccines are relatively inexpensive, researches done shows that some chickens stayed in contact with vaccinated chickens were also immune to the respective disease when directly inoculated using a local virulent isolates of NDV (Dias *et al.*, 2001). Therefore the purpose of this study was to establish the flock immunity following strategic vaccination coverage of the flock. Vaccinating only a proportion of a flock will assist the farmers to reduce costs and save time for other income generation activities while protecting their chicken against ND induced mobidity and motality and hence increased production.

1.3. Objective

1.3.1. Main objective

To investigate the extent of horizontal transmission of vaccine virus and protective antibody response to non-vaccinated in contact chickens following strategic vaccination of chickens against Newcastle disease.

1.3.2. Specific objectives

i. To determine the lowest proportion of vaccinated birds that provide high sero-conversion in the non-vaccinated in-contact chickens using I-2 and La Sota vaccines

ii. To determine the Highest Geometric Mean HI titre for unvaccinated in contact chickens following strategic vaccination.

iii. To compare the trend of Mean antibody titre levels between the two types of vaccines in vaccinated chickens.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Newcastle Disease

2.1.1. Background information

This disease is one of the most important infectious diseases of poultry because of its worldwide distribution and the potential for devastating loses, other species can be infected including mammals occasionally, example conjunctivitis in man working in the laboratory, vaccination team and to crews eviscerating poultry in processing plants (Clarence *et al.*, 1991). It occurs on at least six of the seven continents of the world and is enzootic in many countries (Patti *et al.*, 2010).

The disease was first reported in 1926 in Java, Indonesia and in Newcastle-upon-Tyne, England (Doyle, 1927), Beard and Hanson (1984) and Alexander (1990) reported that the etiology of the disease was first recognized as being a virus by Doyle in 1927. The name "Newcastle Disease" was named by Doyle as a short term measure because he wanted to avoid a descriptive name that might be confused with other diseases (Doyle, 1935). However the name has been used to date, although when referring to ND virus (NDV), the synonym "avian paramyxovirus type 1" (APMV-1) is now often employed. However, APMV-1 has been used to describe ND strains of low virulence, as the definition used by the World Organization for Animal Health (Alexander and Senne, 2008) and other international agencies reserve NDV for virulent viruses.

Since the discovery of Newcastle disease virus (NDV) in 1927, nine genotypes of class I viruses and ten of class II have been identified by comparing the sequences isolated over time (Ballagi-Pordany *et al.*, 1996; Czegledi *et al.*, 2006; Kim *et al.*, 2007b), they are representing a diverse and continually evolving group of viruses (Patti *et al.*, 2010). The emergence of new virulent genotypes from global outbreaks and frequent changes observed in the genomic sequence of NDV of low and high virulence implies that distinct genotypes of NDV are simultaneously evolving at different geographic locations all over the world. This significant genomic diversity may be favored by the wide range of avian species susceptible to NDV infection and by the availability of highly migrating wild bird reservoirs (Cann, 2005). Because of multiple species susceptibility and genetic potential of NDV, these may lead to diagnosing unidentified infections (Patti *et al.*, 2010). Therefore it is very important to keep following the circulating NDV throughout the world for the effective diagnosis of the NDV (Patti *et al.*, 2010).

2.1.2. Clinical manifestation

Clinical form of the disease is rapid, and signs start to appear throughout the flock within 2-12 days (average 5) after aerosol exposure (Clarence *et al.*, 1991). Beard and Hanson (1984b) found that, incubation period vary from 2 to 15 days or longer regardless of the portal of entry of the NDV. Clinical signs are widely variable depending on the strain of virus, species and age of bird, concurrent disease, and status of the immunity. Clinical manifestation of ND in chickens varies significantly among isolates. Infection with NDV has been groped into five pathotypes due to their clinical manifestations seen in infected chickens (1) viscerotropic velogenic: a highly pathogenic form in which hemorrhagic intestinal lesions are frequently seen (OIE, 2012).

In some cases greenish diarrhea can be noted (Jordan, 1990). Mortality is variable but can be as high as 100% (Clarence *et al.*, 1991). (2) neurotropic velogenic: a form that presents with high mortality following respiratory and nervous signs, (3) The mesogenic: a form that manifests with respiratory signs and infrequently nervous signs but low mortality. (4) Lentogenic or respiratory, a form that presents with mild or subclinical respiratory infections and (5) Asymptomatic enteric, a form that usually manifests with subclinical enteric infections (OIE, 2012). For this disease the severity of an infection depends on virus virulence, the age, immune status, and host species susceptibility. Chickens are the most and waterfowl the least susceptible of domestic poultry and infection with low virulence NDV contribute to low productivity (Clarence *et al.*, 1991).

2.1.3. Newcastle Disease distribution and transmission

Newcastle disease (ND) is the most important viral disease of poultry in the all over the world (Nawathe *et al.*, 1975; Spradbrow, 1997). Some countries in Africa named it as one of the notifiable disease which need special attention as an effort toward eradication of the disease. Thirty one African countries covering west, east and southern Africa regions reported ND to the AU-IBAR in 2011. The three countries with the highest number of outbreaks were Ghana (216), Benin (152) and Uganda (120). Generally all other African countries have consistently reported ND during (AU-IBAR, 2013).

Newcastle disease virus (NDV) is an important pathogen that causes disease and death not only in domestic and commercial poultry, but also in wild bird populations around the world. NDV causes disease in more than 250 species of birds and typically affects the respiratory, gastrointestinal, and/or nervous system (Yu *et al.*, 2009).

Virulent NDV strains are endemic in poultry in most of Asia, Africa, and some countries of North, Central, and South America. Other countries, including the USA and Canada, are generally free of those strains in chickens and maintain that status with import restrictions and eradication by stamping out diseased poultry (Merk and Sharp, 2011). Low virulence NDV is prevalent in poultry and wild birds, especially waterfowl. Occasionally human being infection occurs as an occupational disease manifesting as an inflammation of the conjunctiva (Pink eye) and a mild fever (Fenner *et al.*, 1993), recovery is usually rapid, and the virus is no longer present in the eye fluids after 4-7 days. Clarence *et al.* (1991) explained the potentials of egg shell and carcass from acutely infected bird in transmitting the ND to human beings who work in slaughter houses and hatcheries.

Infected birds shed virus in exhaled air, respiratory discharges, and feces. Viruses are shed during incubation, clinical stage, and at a varying degree during convalescence (Clarence *et al.*, 1991). Chickens are readily infected by aerosols and by ingesting contaminated water or food. Infected chickens are the primary source of virus, but other domestic and wild birds may be sources of NDV. Transfer of virus, especially in infective feces, by the movement of people and contaminated equipment is the main method of spread between poultry flocks. Mechanical spread between flocks is encouraged by viral relative stability to the environment and its wide host range (Fenner *et al.*, 1993). The virus survives for long periods at ambient temperature, especially in feces, however it is quite sensitive to disinfectants, fumigants and sunlight. It is inactivated by temperatures of 56 °C for 3 hours or 60 °C for 30 min, acid pH, formalin and phenol, and is ether sensitive (Paul, 2004).

Vertical transmission has not been proven (Spradbrow, 1987), but chicks may be infected in hatcheries through viral contaminated egg shells (Paul, 2004), egg infected with lentogenic and avirulent pathotypes of NDV may develop and hatch, but this is not true in case of virulent NDV strains as the developing embryo is killed before hatching (Alexander, 1988b). ND can be spread through contaminated vaccines as a result of vaccine mishandling, laboratory errors, and quality control failures in the vaccine production process (Alexander, 1988b).

The role of wild bird to the spread of the ND can either be by infection or mechanical transfer, Garnett and Flanagan (1989) did not find any ND antibodies in any of the wild birds which were surveyed. Ezeifeka *et al.* (1992) revealed some serum antibodies against NDV in Bearded barbet, Scaly fronted Weaver, Gray canary, Village weaver, Grey headed sparrow, Red bishop and Guinea fowls. Low virulence NDV is prevalent in poultry and wild birds, especially waterfowl.

2.1.4. The disease in Tanzania

In Tanzania there is no enough history regarding the ND although importation of exotic breeds of chickens has been executed since 1926 (Yongolo, 1996). In between 1926 and 1928 an outbreak of fatal disease of chickens sweeping the whole country occurred which was characterized by high mortality (Yongolo, 1996). There was serious talk to whether the disease was fowl cholera or fowl typhoid. The occurrence of the described epidemic coincided with the first imports of exotic chicken breeds in Tanzania, and the signs described was highly suggestive of ND than any other disease although the disease was never confirmed (Yongolo, 1996).

The molecular studies of Yongolo *et al.* (2011) found that NDV were persistently present among chicken populations and spread through live chicken markets or migration of wild birds. Presence of circulating NDV in chicken populations suggested the existence of ND, the outbreak occurred in between 1926 and 1928 characterized by fatal disease could have been due to ND apart from failure to confirm the disease.

2.1.5. Classification of Newcastle disease Virus, Structure and replication strategy

In 2000 the International Committee on the Taxonomy of Viruses reclassified the Family *Paramyxovirinae* into two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae*. Newcastle disease virus, also known as avian Paramyxovirus serotype-1 (APMV-1), is a member of the genus Rubulavirus within the Paramyxoviridae family (Fauquet and Fargette, 2005; Mayo, 2002), including some of the most prevalent viruses known (measles, respiratory syncytial, parainfluenza). The family, is a negative-sense, single stranded, non-segmented, enveloped RNA virus (Alexander and Senne, 2008). Newcastle disease virus (NDV) and other avian paramyxoviruses have been included in the Rubulavirus genus, primarily because of their nonconserved intergenic junctions and lack of a C-protein open reading frame (ORF), hallmarks specific to the Rubulavirus genus (Robert and Daniel, 2007).

Newcastle disease virus (NDV) contains a lipid bilayer envelope that is derived from the plasma membrane of the host cell in which the virus is grown (Choppin *et al.*, 1975) and replicate entirely in the cytoplasm. Their genomes are 15 kb (15,186kb) in length (de Leeuw and Peeters, 1999) and the genomes contain six tandemly linked genes in the order 3'- NP-P-M-F-HN-L-5' (Fig. 1b) (Wilde *et al.*, 1986). Now it is believed that fusion (F) protein is a major determinant for virulence (Peeters *et al.*, 1999). A lipid envelope containing two surface glycoproteins (F and HN), which mediate the entry and exit of the

virus from its host cell, surrounds the virions. Inside the envelope lies a helical nucleocapsid core containing the RNA genome and the nucleocapsid (N), phospho- (P), and large (L) proteins (Fig. 1a), which initiate intracellular virus replication. Residing between the envelope and the core lays the viral matrix (M) protein which is important for virion architecture and which is released from the core during virus entry (Fig. 1a).

For the virus to enter a host cell, a molecule containing sialic acid (CD 150) (sialoglycoconjugates) serve as cell surface receptors while viral surface glycoproteins (HN, or H or G) serves as ligand for attachment and the fusion (F protein) is occurring at the neutral pH, the consequence of which is the release into cytoplasm a helical nucleocapsid (Fig. 1c). The driving force for this virus uncoating is believed to be the difference in acidic pH in endosome and that of the plasma membrane, just like in Influenza a virus (Robert and Daniel, 2007).

Schaper *et al.* (1988) said that HN and F protein are important to the host immune response as arrangement of their amino acids form the antigenic sites (epitopes). NDV like any other PMVs possesses a hemolysin, which gives the virus capability of lysing and agglutinate erythrocytes (Beard and Hanson, 1984), Alexander (1989) suggested that haemolysis is a result of cell fusion and mediated by the F protein present on NDV, this may also bring about syncytial formation when virus particles are budding from cells in which they are produced. The rigid membrane of RBCs usually results in lysis of the cell at the fragile area caused by the fusion protein on the virus membrane and this is the evident by the leakage of haemoglobin from the ruptured RBCs.

Newcastle disease virus replicates entirely in the cytoplasm of an infected cell and require no nuclear function (Fig. 1c) (Alexander, 1988b). Once the genomic RNA of negative-strand RNA viruses entered a host cell, has to serve two functions: first as a template for synthesis of mRNAs, and second as a template for synthesis of the antigenome (+) strand. The newly synthesized antigenome (+) strand serves as the template for further copies of the (–) strand genomic RNA. Negative-strand RNA viruses encode and package their own RNA polymerase (RNAP), but mRNAs are synthesized only after the virus has been uncoated in the infected cell. Viral replication occurs after synthesis of the mRNAs and requires the continuous synthesis of viral proteins (Fig. 1c).

The intracellular site of nucleocapsid assembly is the cytoplasm. The nucleocapsids are assembling in two steps: first, association of free N subunits with the genome or template RNA to form the helical RNP structure, and second, the association of the P-L protein complex. It has been assumed that the (+) leader (5' end of the antigenome) and (–) trailer (5' end of the genome) regions contain specific sequences for initiating encapsidation (Basak *et al.*, 1992).

The assembly of the second part of the virus, the envelope, is at the cell surface in polarized epithelial cells. The viral integral membrane proteins are synthesized in the ER and undergo a step-wise conformational maturation before transported through the secretory pathway and then buds only from the apical surface and released (Fig. 1c) (Robert and Daniel, 2007).

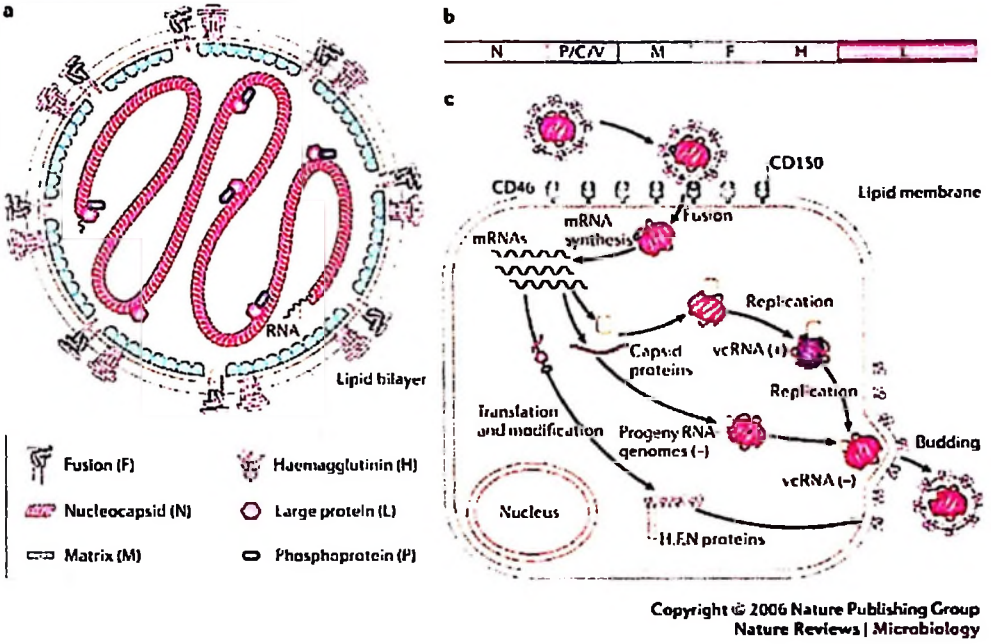

Figure 1 a, b and c: Shows Virus replication, assembly and release.

Source: www.biowiki.org

2.1.6. Pathogenesis and Histopathological manifestations

Pathogenesis of the ND in all clinical forms follows patterns of viral replication strategies and organ predilection of the virus, epithelial and endothelial cells are the targets for the necrotic changes induced by the virus (Parede and Young, 1990). The pathogenesis studies carried out have not yet tell much other than the distribution in tissues and resultant lesions from NDV infection (Brown *et al.*, 1999) The higher the levels of viral replication result in more virus production which may overwhelm the host immune response, hence causing enhanced pathogenesis (Dortmans *et al.*, 2011). Presence of viral nucleoprotein and viral mRNA in the affected tissues was confirmed by immunohistochemistry (IHC) and in situ hybridization (ISH) respectively; these findings are similar to those findings of Piacenti *et al.*, (2006), as they were explaining the molecular aspects of pathogenesis for NDV.

However, regarding the virulence and pathogenesis of NDV, mesogenic isolates do cause some gross and histologic lesions that are considerably less extensive than those caused by a velogenic virus infection (Brown *et al.*, 1999 and Kommers *et al.*, 2003). Wakamatsu *et al.* (2006) reported lymphocellular necrosis and apoptosis in lymphoid organs such as spleen, bursa, and thymus in addition there was a drop-out of cerebellar Purkinje cells in experimental pathogenesis caused by NDV.

Viscerotropic velogenic form, Brown *et al.* (1999) found extensive necrosis of lymphoid areas in the spleen and intestine. Piacenti *et al.* (2006) and Brown *et al.* (1999) went further in their findings saying that viral nucleic acid was detected in multiple tissues but most prominently in macrophages associated with lymphoid tissue. Susta *et al.* (2014) found not only the extensive necrosis of lymphoidal tissues but also the gastrointestinal necrosis and hemorrhages resulting from viral replication strategies seen following immunohistochemical staining. Rue *et al.* (2011) suggested that the host immune response contribute to the pathogenesis of the highly virulent strain in chickens after seeing robust induction of the key host response genes, alpha interfron, beta interferon, and interleukin 1β and interleukin 6, in splenic leukocytes.

Neurotropic velogenic form, in this form the ND is manifested by respiratory and nervous signs. El-Mubarak *et al.* (1990) described the lesions in gastro intestinal tract and trachea as manifested by marked congestion, catarrhal inflammation, haemorrhagic, ulcerative and necrotic. The findings of El-Mubarak *et al.* (1990) were complemented by the findings of Shaheen *et al.* (2005) who reported the lesions found mainly in the brain, liver, kidneys and spleen but kidneys were more frequently involved and histopathological changes were also observed in the lungs, liver, kidneys, brain and spleen. Nakamura *et al.* (2008) found both the respiratory and nervous changes in the histopathological sections;

they saw perivascular cuffing, neuronal degeneration and necrosis, and glial proliferation in the cerebrum, cerebellum, and medulla oblongata. They found extensive rarefaction and malacia in the parenchyma of severely affected brains. They also found proliferation of macrophages in the lungs with congestion, in addition macroscopically; there was bursa atrophy, white spots on the pancreas, and discoloration and enlargement of kidneys and spleen in the broilers.

Mesogenic form, causes respiratory and neurological signs but low mortality (Patti *et al.*, 2010). Pathogenesis and development of disease follows same patterns as in velogenic neurotropic form, the major difference is that this strain is less virulent and has sometimes been used as vaccines candidate to immunize birds (Sharp and Dohme, 2011).

Asymptomatic enteric forms, this form is also referred to as Lentogenic form, is of low virulence. Lentogens replication is occurring primarily in the gut without respiratory signs (Patti *et al.* 2010). Parade and Young (1990) found viral nucleic acid of both mesogenic and lentogenic in myocardium and air sac epithelium when they were studying the replication strategy of the virus in the predilection organ.

2.1.7. Immunity against Newcastle Disease Virus (APMV-1)

Newcastle disease virus isolates are of a single serotype, but have a wide range of naturally occurring pathogenicities from highly virulent (velogenic), to mildly virulent (mesogen) and avirulent (lentogen) (Alexander and Senne, 2008). Newcastle disease viruses are known to infect more than 250 species of birds in 27 orders (CFSPH, 2008). Both innate and adaptive immunity are important in clearing virus infection.

The host innate response to NDV infection is an immediate reaction designated to retard virus growth and aid the host in developing pathogen-specific protection from the adaptive immune response (Rue *et al.*, (2011); Kapczynski *et al.*, (2013)).

The innate immune response comprises factors that exist prior to the advent of infection, and are capable of exclusion or rapid response to microbes. The primary components of innate immunity of poultry against NDV are (1) physical and chemical barriers, such as feathers and skin, epithelia and production of mucus; (2) phagocytic cells, including macrophages and natural killer cells; (3) complement proteins and mediators of inflammation; and (4) cytokines (Kapczynski *et al.*, 2013).

Physical and chemical barriers are primarily the first line of defense before the virus gets into the body. The virus is shed into the environment where it encounters new susceptible host. Though NDV can be demonstrated in skin and feathers, this does not appear to be a significant source of viral infection due to its mechanical protection support (Tyler and Nathanson, 2007).

Phagocytic cells like macrophages and other cells like T lymphocytes are called antigen presenting cells have germ-line encoded receptors known as pattern recognition receptors (PRRs) like Toll Like receptors (TLR)which recognize evolutionarily conserved molecular markers of infectious microbes, known as pathogen-associated molecular patterns (PAMP's) like H and HN on NDV (Kapczynski *et al.*, 2013). The virus is first recognized by host sentinel proteins, including TLR proteins, producing rapid signaling and transcription factor activation that lead to production of soluble factors, including interferon and cytokines, designed to limit and contain viral replication (Kapczynski *et al.*, 2013).

Sick *et al.*, 1998 and 2000 found that NDV induces Nitric oxide (NO), alpha interferon (α IFN) and beta interferon (β IFN) in macrophages. Early production of interferon is important for resistance to NDV infection (Kapczynski *et al.*, 2013). The chemical mediators of inflammation interferons and cytokines set up a state of inflammation in the tissue and bring neutrophil and plasma protein to the infection site to initiate adaptive immune response (Janeway *et al.*, 2001). Macrophages and neutrophils have granules called lysosomes, that contain enzymes, proteins, and peptides that can mediate an intracellular antiviral response. The phagosome fuses with one or more lysosomes to generate a **phagolysosome** in which the lysosomal contents are released to destroy the pathogen (Janeway *et al.*, 2001). The significant increase in the level of serum NO is potentially destructive innate immune response of chickens to NDV infection (Rue *et al.*, 2011).

Natural killer cells (NK) are evoked early in the immune response and do not keep immunological memory (Whitton and Aldostone, 2007). Mostofa *et al.*, (2009) commented that the exact mechanism of NK cell activation is not known but the Haemagglutinin-neuraminidase (HN) of the NDV serve as a ligand structure for NKp44 and NKp46 receptors to activate the killing function of the NK cells. Again NK cells work by recognizing chicken body cells that have downregulated MHCI (infected cells) and killing them is done by Perforin and Granzyme which leave behind perforated viral infected cells (Cann, 2005). Natural killer cells have another pathway for killing viral infected cells called antibody dependent cell mediated cytotoxicity (ADCC). In this case viral infected cell is coated with Fab region of specific antibodies mostly IgG, the Fc region to the Fc receptor on the NK cell activates the killing function of the NK cell (Whitton and Aldostone, 2007). Killer activation receptors (KARs) found on the NK cells trigger the killing (Cann, 2005).

Adaptive immunity response is a cell mediated immunity and specifically mediated by T lymphocytes and has been suggested to be an important factor to the development of protection in chickens vaccinated against NDV and contribute to viral clearance (Sharma, 1999). Cell-mediated stimulation following NDV infection is detected as early as 2–3 days post infection (Ghumman *et al.* (1976), Reynolds and Maraqa, (2000)). The two major classes of T lymphocytes are cytokine-secreting CD4+ T helper cells and CD8+ cytotoxic T lymphocytes (CTL) (Kapczynski *et al.*, 2013). The CD4+ and CD8+ molecules recognize the Major histocompatibility (MHC) class II found in nucleated cells and MHC class I found in antigen presenting cells (APCs), respectively (Janeway *et al.*, 2001). The CD4 or CD8 molecules associate on the T-cell surface with the T-cell receptor and bind to invariant sites on the MHC portion of the composite **MHC- peptide** complex on the APCs (Janeway *et al.*, 2001). Activation of CD4+ T helper causes production of cytokines which is necessary for activation and clonal proliferation of NDV specific CTL (Cann, 2005). Kapczynski *et al.* (2013) found the correlation about increase in the level of IFN-γ and that of T lymphocytes and therefore increase in the level of cytotoxicity by NDV specific CTL to NDV-infected cells. The killing mechanism of NDV infected cells by CTL is similar to that of NK cells (Cann, 2005).

B cell activation and antibody production follows the signals from the bound MHC I-peptide complex to the CD4+ T helper cell. The CD4+ T helper produces cytokines which induce the B cell to proliferate and differentiate into mature plasma cell secreting specific antibody and memory B cell (Whitton and Aldostone, 2007). There are three classes of Antibodies, immunoglobulin G (IgG), IgM, and IgA and IgG is the most important class of antibody for direct neutralization of virus particles in serum and other body fluids into which it diffuses (Cann, 2005). In the chicken, IgM, IgY (avian IgG equivalent) and IgA antibodies are produced as part of the immune response (Jeurissen *et al.*, 2000).

Antibodies are detected at the site of infection and in the blood starting at six days after infection or live virus vaccination and peaks 21–28 days after infection (Al-Garib *et al.*, 2003a). The protection of antibodies is directed to viral proteins involved with attachment and fusion (Kapczynski *et al.*, 2013). Al- Gharib *et al.* (2003a) found that, the primary function of the antibodies is to neutralize the NDV particles and denying them from attachment to host cells and therefore no disease development.

Vaccination remains to be the key alternative to prevent the disease. NDV vaccines induce an immune response that reduces or completely prevents clinical disease and mortality from ND, decreases the amount of vNDV shed into the environment, and increases the amount of virus needed to infect the vaccinated animal (Marangon and Busani, 2006; Miller *et al.*, 2009). Both live and inactivated NDV vaccines induced antibodies other than IgA, not only in serum, but also in tracheal and intestinal washes to prevent ND (Chimeno *et al.*, 2008).

2.1.8. Disease control and prevention

Vaccination is the only way of controlling endemic ND all over the world (Orajaka *et al.*, 1999; Usman, 2002). Vaccination relies on commercially available vaccines derived from pathotypes of NDV. Includes thermo labile F strain, L strain, B1 strain and La Sota strain. The thermo stable vaccine includes V4 strain, V4-HR strain and I-2 strain (, 2002).

Genetically engineered recombinant vaccines (vectored vaccine) have been developed and tested and found to confer specific protection to ND (Meulemans *et al.*, 1987. Ogawa *et al.*, 1990; Vegad *et al.*, 2010) found that combination of live and killed cloned vaccines yielded higher titres than the combination of live cloned and non-cloned killed vaccines.

Genetic engineering involves splicing of HN and F genes and inserted into a vector virus (example fowl pox virus) or bacteria (Boursnell *et al.*, 1990). AviPro ND C131 is suitable for broilers, layers and breeders; it is produced from a cloned strain of the La Sota virus produced by Lohmann Animal Health. This new vaccine not only reduces mortality and severity of ND symptoms in poultry, but it also decreases the amount of virulent virus shed from vaccinated birds (Yu *et al.*, 2009).

There have been advances in passive immunization by using protective effect of monoclonal antibodies (mAbs) to NDV (Umino *et al.*, 1990a). It has been found that those antibodies with high virus neutralization activity directed to one antigenic site of the HN protein delayed virus growth and prolonged host survival time, but all chickens eventually succumbed to infection (Umino *et al.*, 1990a). However monoclonal antibodies (mAbs) directed to two antigenic sites HN and F proteins, completely suppressed virus growth and prevented death of the chickens (Umino *et al.*, 1990a). Iorio *et al.* (1989) found some viral particles were capable of binding antibody while retaining their infectivity. The implication of this finding to the mechanism of neutralization have been found and explained by Umino *et al.* (1990a), when he said that, this may occur if the antibodies directed to HN only and leave the F protein on virus free.

The mode of the vaccine delivery varies according to type of vaccine (Allan *et al.*, 1978). Live lentogenic may be given by mass application in drinking water, aerosol spray, or individual by eye drop (conjunctival sac), or beak dipping. Healthy chicks are vaccinated as early as day 1-4 of life. However, delaying vaccination until the second or third week avoids maternal antibody interference with an active immune response (Sharp and Dohme, 2011).

Application of lentogenic vaccine in feed pellets has been developed and found to be suitable for the village chickens due to its heat stability (Spradbrow and Samuel, 1991). Mesogenic vaccine require intradermal inoculation via the wing web, while acqueous or oil based inactivated vaccines must be given individually by intramascular or subcutaneous injection (Jordan, 1990). Apart from using vaccines alone, some researches revealed that natural plant products and tonics can influence increased antibodies level production following vaccination. Qiu *et al.* (2012) reported four Chinese herbal polysaccharides which were given in chicken at vaccination and results indicated that the 4 polysaccharides possessed significant immune-enhancing properties in chickens and suggested that this finding may have direct application in vaccine design and other strategies designed to potentiate immune system development and function in chickens. Mazija *et al.* (1990) also found that Vitamins and minerals have potency in boosting up antibody production levels.

Prevention of the disease is accomplished through a combination of sanitary management practices aimed at reducing chances of exposing birds to the infective virus (Alexander, 1991). Prevention can be targeted at different levels. At international level, it is aimed at prevention of infection from infected to non-infected country and local level it involves prevention of spread from farm to farm, village to village, district to district and region to region. In Tanzania by law, there are animal check points and animal movement permit for serving the purpose. The establishment of a strict quarantine and destruction of all infected and exposed birds with financial indemnification for losses followed by thorough cleaning and disinfection of premises were the main features necessary for eradication of VND virus from the poultry producing area. For the individual farmer/farm the principles which can be applied to prevent propagation and spread of infections are: all in - all out, one age on a farm, one production purpose, one genetic basis and a long-enough pause between

cycles so a radical break in transmitting biological contamination from the processing cycle of previous poultry generation is possible (Mazija *et al.*, 1990). However these practices are very difficult to implement under the free ranging production system. According to Hanson (1984), the following are potential sources of infection and need to be taken care;

1. Movements of clinically unapparent carriers.
2. Movements of staff from contaminated flocks to uninfected ones.
3. Movements of different vehicles from flock to flock.
4. Migration of mice that are (for seven days or more) the carriers of ND virus. During harmless removal of the flock and cleaning and disinfecting the pens, mice and wild birds looking for feed transmit N D virus to other poultry farms.
5. Mechanical propagation of ND virus by flies.

In Tanzania ND control depends on the use of both thermolabile vaccines (La Sota) and thermostable vaccine I-2. La Sota vaccines have been widely used in the commercial poultry sector (Wust *et al.*, 2010). Apart from the widely use in commercial sector, these vaccines have limitations on their use to the rural chickens because they need cold chain from the manufacturer, distributor to the farmer (Wust *et al.*, 2010). Local and international researches have been done for years before and after 2000 to find the solutions to control ND for the rural chickens. Yongolo *et al.* (2006) isolated local NDV and for the first time found the existence of a potential local vaccine candidate in Tanzania. The first thermostable vaccine to be produced was the NDV4-HR vaccine and proved to be effective for the rural chickens not only in Tanzania but also other countries of Africa and Southeast Asia (Alders and Spradbrow, 2001). Foster *et al.* (1999) used the NDV4 thermostable vaccine in village condition and found that chickens were protected by 70 per cent after being challenged by virulent strains of NDV. However this vaccine

seemed to be expensive due to its commercial ownership and therefore the Australian Centre for International Agricultural Research (ACIAR) developed a thermostable vaccine, I-2 strain, which is able to spread from vaccinated to unvaccinated chicken (Bensink and Spradbrow, 1999). This vaccine is free of commercial ownership and has been produced and used in Tanzania (Alders and Spradbrow, 2001, Msami, 2005). Studies have been done to explore the potentials of I-2 vaccine in rural chickens in Tanzania. Wambura *et al.* (2000) studied the potential of I-2 vaccine to protect not only the rural chickens but also commercial poultry as well, they also studied the potential of the vaccine to be applied through different route of administration like oral route via drinking water and as an eye drop. In all studies I-2 vaccine has shown good results. Again Wambura (2009) studied the potential of I-2 being administered orally via oiled rice and found that it is efficacious as it protects chickens from ND. Mwakapuja *et al.* (2012) studied the efficacy of I-2 vaccines in different age groups of free range chickens and found that I-2 vaccine is more efficacious in growers and adult than chicks, and insisted that for flock protection chicks need special vaccination program by harmonizing hatching. In Tanzania there is no use of the genetically engineered vaccine. Msoffe *et al.* (2010) recommended that vaccination against ND remains the best and cost effective means of controlling ND and the use of I-2 vaccine led to an increased average flock sizes and decreases mortality incidences.

2.1.9. Diagnosis

For a definitive diagnosis of ND, both virus isolation and laboratory characterization are necessary. Nevertheless, if the disease is known to be present in a given area, signs and lesions may be considered highly suggestive but not pathognomonic, especially for village chickens. However a number of direct and indirect methods are available for the diagnosis of the ND (Belak and Pordany, 1993).

The direct methods which involve identification of the agent (NDV) includes; the virus isolation, the detection of NDV antigen by immunodiagnostic methods like ELISA, immunohistochemistry, virus (plaque) neutralization, complement fixation (CFT), immunofluorescence, immunoperoxidase orperoxidaze- immunoperoxidaze staining methods and nucleic acid hybridization (Paul, 1990).

The indirect methods like clinical signs, mobidity and mortality rates and differential diagnosis are useful for screening and to have expected positive results. Typical clinical signs are: a state of prostration and depression in the birds, with ruffled feathers; greenish white diarrhea; and, in survivors, the head turned to one side, a condition known as torticollis is very often seen, as are paralysis of the legs, wings or other neurological signs. Other typical characteristics of the disease include: rapid spread; death within 2-3 days; a mortality rate of over 50 percent in naïve populations; and an incubation period of 3-6 days or, on rare occasions, 2-15 days (Beard and Hanson, 1984). On necropsy, a typical lesion is mucus in the trachea, and usually haemorrhages in the intestine, particularly in the proventriculus. It should be borne in mind that all the preceding signs and lesions can be caused by other diseases.

The differential diagnosis for velogenic Newcastle disease includes other causes of septicemia, enteritis, respiratory disease and/or neurologic signs. In poultry, these diseases include fowl cholera, highly pathogenic avian influenza, laryngotracheitis, the diphtheritic form of fowl pox, psittacosis, mycoplasmosis, infectious bronchitis, aspergillosis, and management problems such as deprivation of water or feed and poor ventilation (CFSPH, 2008).

2.1.9.1. Identification of Agent (NDV)

The definitive diagnosis of ND is done through isolation and identification of the virus (Alexander, 1998). Hasan *et al.* (2010) also commented that NDV detection and differentiation are based on virus isolation using embryonated chicken eggs followed by an in vivo determination of pathogenicity in chickens.

2.1.9.1.1. Samples for Virus Isolation

From a living bird, virus isolation can be done by using tracheal and cloacal swabs. A cotton-covered stick is inserted into the trachea or cloaca slowly rotated from left to right and then put into a vial containing virus transport media (phosphate buffered saline plus penicillin (2000 units/ml) and streptomycin (2mg/ml) (OIE, 2012). Cloacal swabs coated with faeces must be kept cool during transport to the laboratory where they should be stored at 4°C if they are to be processed within 48 hours or frozen at least at -20°C until the isolation attempt.

From dead birds, virus can be isolated from homogenised organs like liver, lungs, kidney intestine, spleen, brain and heart from dead birds. These samples may be collected as a pool or separately although intestines and their contents are processed separately (OIE, 2012).

2.1.9.1.2. Virus isolation and Identification

For virus isolation, supernatant fluid of faeces or tissue suspensions obtained by centrifugation at 1000g for about 10 min at a temperature not exceeding 25°C are inoculated in 0.2ml volumes into the allantoic cavity of each of at least five embryonated SPF fowl eggs of 9-11days incubation.

The eggs are candled twice daily. As dead eggs occur, they are chilled to 4°C then allantoic fluid is harvested and the virus identified by testing its ability to haemagglutinate chicken red blood cells (OIE, 2012).

For cell culture, the supernatant fluid from homogenized organs, faeces or swabs may also be used for virus isolation in cell cultures. NDV can replicate in variety of cell cultures of avian and non-avian, examples are chicken embryo kidney, chicken embryo fibroblast and African green monkey kidney. For virus recovery, trypsin is added, example 0.05µg/ml of porcine trypsin to chicken embryo fibroblast. Viral growth is accompanied by cytopathic effects represented by disruption of the monolayer and formation of syncytia (OIE, 2012).

Virus identification is based on the inhibition of haemagglutination by specific anti-NDV serum and pathogenicity index. According to Allan and Gough, (1974) what happens in the HA is that, after addition of the RBCs, a suspension of RBCs will settle at the bottom of the well of plate as a tight button or stream in a V well plate as a tear if there is no agglutinating agent. But if there is an agglutinating agent in the suspension then, the RBCs receptors are linked by the virus ligands (agglutinated) forming a mat of cells which do not settle as a button nor do they stream as a tear. Inhibition of agglutination proves infection of the bird by the virus, but does not indicate whether the virus is a pathogenic or avirulent, however there is problem of cross reaction in HI to other paramyxovirus serotype with NDV especially PMV-3 (Jordan, 1990; Beard and Hanson, 1984), this problem can be eliminated by using specific NDV antiserum or monoclonal antibodies (mAbs) (Alexander, 1991).

For pathogenicity index, Intracerebral pathogenicity index (ICPI) is determined by using 24-40 hours old chicks from a SPF flock. Fresh allantoic fluid with HA titre $>2^4$ is diluted to 1/10 in a sterile isotonic saline with no additives such as antibiotics. An amount of 0.05ml of the diluted virus is injected intracerebrally into each of ten chicks (OIE, 2012). Each bird is examined at every 24-hour intervals for eight days and graded zero if normal, one if sick and two if dead. The index is the mean score per bird per observation over the 8-day period. The most virulent viruses give ICPI values approaching the maximum score of 2.0, while lentogenic viruses give values of or close to, 0.0. (OIE, 2012).

2.1.9.1.3. Molecular techniques (NDV nucleic acid detection)

There has been increasing use of molecular techniques to detect NDV in clinical and postmortem specimens as mentioned above in virus isolation and identification. The development of Polymerase Chain Reaction (PCR) and other nucleic acid amplification techniques overcame the sensitivity barrier and have led to the development of nucleic acid–based diagnostic tests for many viruses. Initially reverse transcription PCR was used to confirm the presence of the NDV (Cattoli *et al.*, 2012). But it is essential not only to reveal the NDV but also to know the pathogenicity of the virus involved (Cattoli *et al.*, 2012). RT-PCR systems have been used to amplify a specific portion of the NDV genome that will give added value; for example F gene which can also be used to determine virulence (Glickman *et al.*, 1988; Creelan *et al.*, 2002).

The most critical challenge 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 (OIE, 2012). To avoid post-amplification processing, real-time RT-PCR (rRT-PCR) technique has been developed to detect virus in (Fuller *et al.*, 2009; OIE, 2012). The advent of real-time PCR using fluorogenic hydrolysis probes

provided highly sensitive and rapid testing procedures (Cattoli *et al.*, 2012). To date there are various NDV primers which determine different amplification programs for diagnosing NDV by using rRT-PCR.

For diagnosis of the disease, PCR generated cDNA fragment of F gene (amplicon) must be ran in a agarose gel electrophoresis to determine the band size by comparing with a standard DNA ladder ran alongside the amplicon on the same gel (Kyzsystztof *et al.*, 2006). The PCR product has been studied and become the pillar in restriction enzymes analysis (Nanthakumar *et al.*, 2000), probe hybridization (Aldous *et al.*, 2001) and nucleotide sequencing for cleavage site analysis and molecular epidemiological studies (Seal *et al.*, 1995).

2.1.9.3. Sequencing

Sequencing methods for diagnostic purposes is becoming essential due to the high capacity for mutation in RNA viruses (genetic drift) and the large diversity of NDV genotypes which makes it difficult to predict the genetic composition of new isolates (Miller *et al.*, 2010). Therefore sequencing cDNA is important to determine the sequence of individual genes, highly conserved genes like Matrix (M) for diagnosis and Fusion (F) gene for virulence differentiation (Wise *et al.*, 2004b) or the entire genomes of virus (Xiao *et al.*, 2012). Sequencing provides the order of individual nucleotides in DNA or RNA.

For the NDV to be virulent, inactive precursor fusion glycoprotein, F0, has to undergo post translational cleavage to F1 and F2 and remain linked by disulphide bond (Rott and Klenk, 1988). The cleavage is mediated by host cell proteases (Nagai *et al.*, 1976a).

When Collins *et al.* (1993) studying the pathogenicity and antigenicity of NDV using F gene, they found the amino acids sequence^{112}R/K-R-Q-K/R-R^{116} for the C-terminus of the F2 protein and phenylalanine (F) at the N-terminus of the F1 protein, residue 117 in a virulent and mesogenic strains. They also deduced amino acid sequence in the corresponding region of all viruses of low virulence was^{112}G/E-K/R-Q-G/E-R-L^{117}. The nucleotide sequencing is the fundamental source of information for phylogenetic analysis. Phylogenetic analysis not only can be used to study the pathotype prediction of NDV isolates but can also be used to determine topotype of NDV isolates (Seal *et al.*, 1995).

2.1.9.4. Serological Test

There are wide ranges of serological tests which can be used to detect NDV antigen, these tests are enabling neutralisation or enzyme linked immunosorbent assays (ELISA) and HI to be used for assessing antibody levels in birds. Sample to be collected for the serological tests is serum. These tests identify immunoglobulins produced by lymphocytes in the course of infection during acute disease and chronic disease represented by IgG and IgM respectively (Kudesia and Wreghitt, 2009).

Apart from the advantages, there is a challenge of using serological tests for diagnosis of NDV due to wide use of vaccine in controlling the disease (Copland, 1987).

2.1.9.4.1. The HI test

Newcastle Disease virus particles (Paramyxovirinae) have an envelope protein called the hemagglutinin, or HA, which binds to sialic acid receptors on host cells (Lamb and Kolakofsky, 2007). The virus can also bind to chicken erythrocytes (red blood cells), causing the formation of a lattice. This property is called hemagglutination, and is the basis of a rapid assay to determine levels of Newcastle Disease (ND) virus present in a sample.

The HI is the simple method that use the ability of the specific antibodies to inhibit the agglutinating potential of NDV, this method is commonly used for the diagnosis of ND (Allan and Gough, 1974). Identification of birds that have been exposed to NDV (natural infection/vaccination) is usually based on monitoring seroconversion and rise in titre (Alexander, 1991). The principle behind the HI test is that all NDV pathotypes do agglutinate chicken RBCs, but similar phenomenon can be seen in other virus and bacteria (Jordan, 1990); this problem has been eliminated by using specific NDV antiserum. The details and procedures are explained clearly by Allan and Gough (1974).

2.1.9.4.2. Enzyme Linked Immunosorbent Assay (ELISA)

A number of methods have been published for the detection of antibodies against NDV by means of enzyme-linked immunosorbent assays (Adair *et al.*, 1989). Commercial kits are available for the most commonly used test worldwide. ELISA and HI have been found to have equal sensitivity when compared (Brown *et al.*, 1990) and show similarity in the levels of cross reactivity (Brown *et al.*, 1990) but ELISA was found to be more sensitive and specific than HI (Reddy and Srinivasan, 1992). Alexander (1991) stated that the advantage of ELISA over the HI lies in the possibility of the test being semi-automated and multiple samples being conveniently assayed. ELISA is a colourimetric assay unlike HI and requires the use of a sophisticated instrument to read the optical density of the reactions (Alexander, 1991).

2.1.9.5. Immunohistochemical techniques (Monoclonal antibody)

Production and maintenance of monoclonal antibodies (mAbs) to NDV has been well explained (Alexander, 1990). The specificity of the mAbs makes it possible to distinguish NDV stains (Alexander, 1990).

Panels of mAbs can be used in detection of the relationships or differences between isolates which can be used to establish the epidemiology of ND (Aldous *et al.*, 2001). Specificity of mAbs has also been used to evaluate NDV isolates and rapid detection of NDV in chicken tissue by immunohistochemical techniques (Ahmad and Matsumoto. 1990). The introduction of the molecular-based techniques supersede the use of monoclonal antibodies, for characterizing viruses for molecular epidemiological purposes because they are able to cover the three aspects of ND diagnosis which are; detection of virus, characterization which include inference of virulence, and epidemiology, which are done quickly, accurately and definitively in a single test (Aldous *et al.*, 2001).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study site

The experimental site was located at latitude 6° 20'66''South and longitude 36^0 30' 60'' E. with an altitude of 948 m above sea level (GPS. geko 101, Hampshire, UK). This study site was chosen because of availability of poultry units which needed little modification to suit the present experiment at LITI poultry units, to disseminate the knowledge about chickens rearing, sample collection and processing to LITI students using VIC laboratory facilities, the area is accessible to Dodoma where chickens inputs can be fetched.

3.2. Experimental Study Design and Methodology

3.2.1. House preparation and chicks managements

Cleaning and disinfection of the housing using broad spectrum disinfectants was done using Rhino White disinfectant containing tar acids at 7-8 % (v/v), (SAPA Chemical Industries, Dar es Salaam, Tanzania). Disinfection was done twice, a month before and two weeks before stocking the chicks. Chicken house was divided into non communicating compartments (Fig. 2) and spacious enough (90 cm wide x 240 cm long x 210 cm high) to accommodate 16 chickens until the end of study period. The none communicating compartments where enough to prevent cross contact amongst chickens of different experimental groups. Foot bath carrying tar acids 8% (v/v) was placed at the entry point to prevent contaminants brought in by the attendant. Attendant had to wear overcoat and gumboot when attending chickens and not allowed to work for chickens/animals anywhere else to avoid introduction of contaminants.

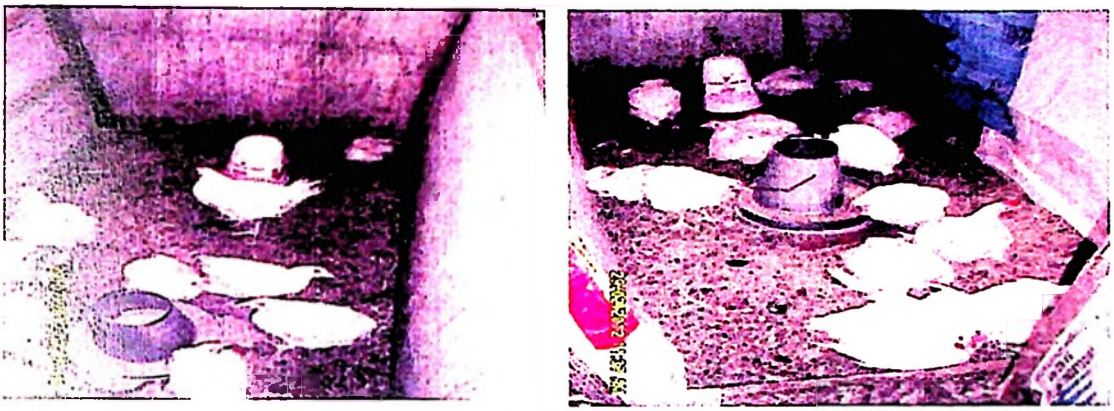

Figure 2: A section of chickens in their none-communicating pen

Feed and water were given *ad libitum* using commercially prepared feed sourced in the market (Igo products, Dar Es Salaam, Tanzania), commercial vitamins supplements (A, B, C, D, E and K) were provided to the chicken through drinking water. Light source was made available to provide heat and light twenty four hours for the first three weeks during brooding (Fig. 3) and only during night hours thereafter until the end of the study period. Anticoccidial products (Ancoban, Ipswich, UK) were provided to chickens as prophylaxis and treatment during the study period. The deep litter (saw dust) 3 inch was set at the beginning of study and was not changed till the end of the study period.

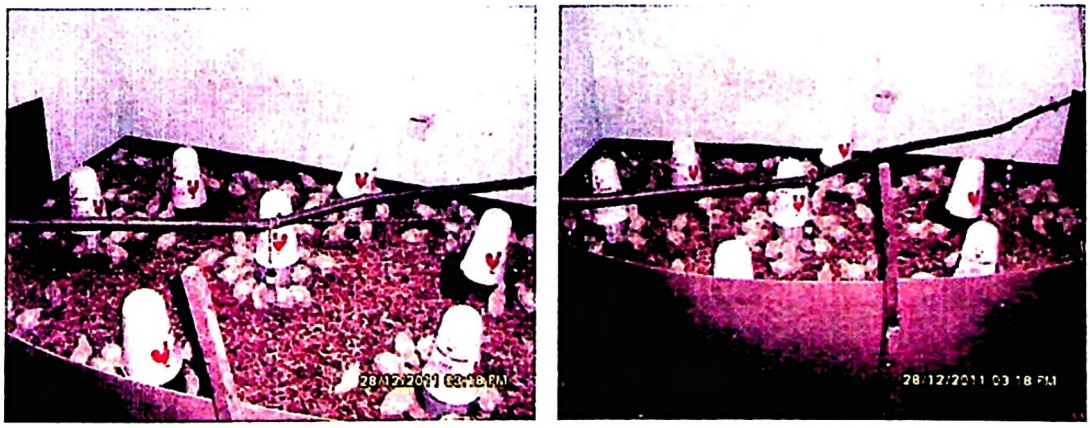

Figure 3: A Section of chicks during brooding stage

3.2.2. Chickens source and vaccination

About two hundred day old broiler chicks (Fig. 3 above) were purchased from a local supplier who sourced them from Interchick® company limited, Dar Es Salaam, Tanzania, these chicks of similar age sex and breed came from the parent stock with a history of being vaccinated against ND using live vaccine (Nobilis Clone 30, a product of Intervet, South Africa). The chicks were brooded in the isolation unit for three weeks before being transferred to the experimental site. All chicks were vaccinated against Infectious Bursa Disease (IBD) at the age of two weeks and against Pox at the age of four weeks.

3.2.3. Experimental design

At the age of three weeks, 160 chickens were randomly selected and divided into ten groups of 16 chickens each to be vaccinated using commercially available vaccines either I-2 or La Sota. The first five groups were for I-2 vaccine and the remained five groups were for La Sota vaccine. Each chicken was wing tagged and identified by type of vaccine, group number and specific number of chicken example GL. 1. 1-16 where GL stood for Group La Sota vaccine, 1 stood for 12.5% vaccinated and 1-16 refer specific number of chick within the group. Yellow colored tags were for none vaccinated and red colored tag for vaccinated ones.

In this experimental trial, the first five groups were vaccinated using I-2 vaccine (CVL product, Dar Es Salaam, Tanzania) batch ND 1107VD111). From group 1 to 5 strategic vaccinations were 12.5% (2 chicks vaccinated), 25% (4 chicks vaccinated), 50% (8 chicks vaccinated), 100% (16 chicks vaccinated) and 0% (None is vaccinated) respectively (Figure 4). I-2 vaccine was given as an eye drop (Young and Alders, 2002).

The same strategic vaccination was applied for the La Sota vaccine (BIOVAC product, Israel, batch 101414) and was given through drinking water. For both vaccines groups, 100% and 0% vaccination was used as positive and negative controls respectively.

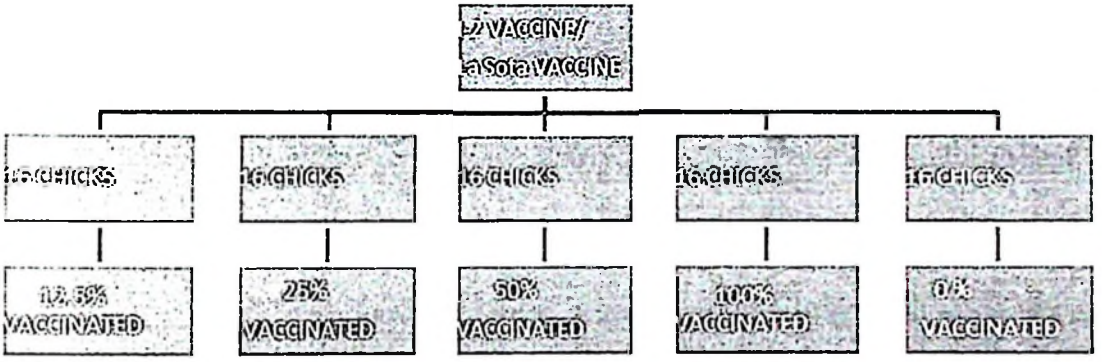

Figure 4: The design of the experimental groups per vaccine used, number of chickens in each group and strategic vaccination in per centage of chickens in a group.

3.2.4. Blood Sample collection

From all the chickens, the first blood sample were collected at the age of three weeks before vaccination and thereafter after every two weeks five times post vaccination (Appendix 1). Using 2mls sterile syringe and needle, 1-2 mls of blood was collected from each chicken through wing vein (Brachial vein), before blood collection, feathers were removed and the site was disinfected using cotton wool soaked in 70% alcohol (Yongolo, 1996) and put in the plain vacutainer tubes and the tubes were labeled.

Blood was left to coagulate in a refrigerator at 4°C overnight (Allan and Gough, 1974) and centrifuged the following day at 1500 rpm for 2 minutes for clear serum collection. Each serum was kept in a labeled cryovial and stored in the deep freezer at -20°C until HI testing (Allan and Gough, 1974).

Wing tag number (ID number of chicken) and date of blood sample collection were marked on the vacutainer tube and corresponding cryovial.

3.2.5. Serum Testing Procedure

The Haemaglutination Inhibition (HI) titre of sera from experimental chickens was measured by using standard procedure of microplate HI test (Allan and Gough, 1974). HI test was performed using four HA units (4HA) of ND virus and a 1% suspension of chicken red blood cells (Allan and Gough, 1974) in V shaped well microtitre plates. All titres were recorded as $\log_2$ of the reciprocal of the end point dilution. In this study the HI titre ≥ 3 (Log_2) was considered positive based on the findings of Allan and Gough (1974) and Bell *et al.* (1991a) who reported that birds with HI titre ≥ 3 ($\log_2$) were protective against challenge with a virulent strain of ND virus. The end point dilution forming a stream following tilting the plate was recorded as a true positive.

3.2.6. Preparation of Newcastle disease virus antigen for use in HI tests

Antigen was prepared by inoculating embryonated chicken eggs free from NDV obtained from Minimum Disease Flock (MDF) at SUA poultry farm. The driller was used to open the allantoic cavity and 100 µL of sample of NDV from I-2 vaccine was inoculated, the opening was sealed by wax and then eggs were incubated for four days before harvesting allantoic fluid. A volume of 60 ml of allantoic fluid was harvested and centrifuged at 1,200 *g* to clarify and remove contaminating red blood cells. The working antigen was stored in a refrigerator at 4°C and the stock antigen kept at -20°C (Allan and Gough, 1974).

3.2.7. Preparation of Washed Red Blood Cell Suspension

About 6 mls of blood was collected from 3 chickens found in SUA poultry farm by using 2mls syringe and needles and then transferred to a vacutainer tubes impregnated with an anticoagulant (EDTA), then blood was gently mixed. The blood was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the tube was refilled with PBS and centrifuged again at 1500 rpm for 5 minutes, again the supernatant was discarded. This step was repeated three times. The last round of centrifugation was done without adding PBS and afterwards the supernatant was discarded. The small part of RBC was diluted to a 1% solution, by adding PBS to prepare a working RBC solution (1ml RBCs: 99ml PBS).

3.2.8. The control sera

For control purposes, positive secondary laboratory standard serum from SUA VET Virology Laboratory (SUA) was used. This secondary laboratory standard serum was developed following comparative testing with the standard serum from Veterinary Laboratory Agency (Ministry of Agriculture. Weybridge, Surrey, UK). This secondary standard serum was used to confirm our prepared antigen by HI test and had HI titre of $\log 2^5$ and therefore used as control positives, the consistency of results when tested with 4HA units of antigen was observed (Allan and Gough, 1974). For negative control PBS was used. The positives and negative controls were run simultaneously in the plates and acted as a golden standard for the results.

3.2.9. Preparation of 4HA units of Newcastle disease virus antigen

The standard amount of Newcastle disease virus used in the haemagglutination inhibition (HI) test is 4HA units (OIE, 2012). It was necessary to prepare and test a suspension of Newcastle disease virus containing 4HA units in order to carry out the HI test. This involved a series of following steps.

The antigen prepared was tested by Haemagglutination test (HA).

Procedure (OIE, 2012).

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

ii) 25 µL of the virus suspension was placed in the first 4 wells of the first column of the microtitre plate.

iii) Two fold dilutions of 25µL a volume of the virus suspension was made across the plate

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

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

vi) The solution was then mixed by tapping the plate gently. The RBCs were allowed to settle for 40 minutes at room temperature.

vii) HA was determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming) (Fig. 5); this represents 1 HA unit (HAU). The found to have end point titre at 2^{10} (1:1024) (Figure 5).

Therefore, to get 4HA Units = Titre/4

=1024/4= 256

Therefore 1ml of Antigen was mixed with 255ml of PBS to make an antigen working solution.

The results of the back titration of the diluted antigen and the HI titre of the laboratory standard positive (SUA VET Virology Laboratory) were both used to confirm the antigen if has been diluted to a concentration equivalent to the standard 4 HA units.

Figure 5: 4HA testing (HA titre of 2^{10} = 1:1024) was recorded

3.2.10. Haemaglutination Inhibition Test Procedure (Allan and Gough, 1974)

Materials required

-Thawed serum samples in racks

-V-bottom microwell plates and covers

-Phosphate Buffered Saline (PBS)

-1 percent washed red blood cells

-V-bottom reagent trough

-25 µL single and multichannel pipettes and tips

-Microwell plate recording sheet.

-Newcastle disease virus antigen diluted to 4 HA units per 25 µL

-Standard positive and negative control (PBS)

Procedure

i) Each test serum was recorded correspondingly to the well on a microtitre plate

ii) 25µL of PBS was dispensed into each well of V botton micro well plate.

iii) After shaking the cryovial of test serum, 25 µL of each test serum was added into the first well on a column and the last well of a row.

iv) By using a multichannel pipette two fold serial dilutions was done along the row until the second last well from the end and discarding the last 25 µL from the second last well.

v) Then, 25 µL of the 4HA dilution of antigen was added into each well except the control wells in the last column.

vi) Then, the loaded microwell plate was gently shaken to allow the reagents to mix. Followed by covering the plate with a lid and allowed to stand for 30 minutes at room temperature.

vii) Then, 25µL of 1 percent washed red blood cells was added into each well including the control wells in the last column.

viii) The plate was gently held and shaken to allow mixing of the reagents. The plate was then covered by a lid and allowed to stand at room temperature for 45 minutes before start reading.

Reading Results and interpretation

In the well where antibodies are present there will be haemagglutination inhibition, free red blood cells will settle down, tears of RBCs will form in a well when the plate is tilted at an cute angle, it was done so because sometimes it is not easy to determine degree of haemagglutination inhibition by looking the size of the button and control wells (Allan and Gough, 1974).

The end point of titration is the well that shows complete haemagglutination inhibition by forming tears when a plate is tilted at an acute angle (Fig. 6).

-In the well where antibodies are not present, there is agglutination and therefore no free RBCs and tears formation when the plate is tilted at an acute angle (Fig. 2).

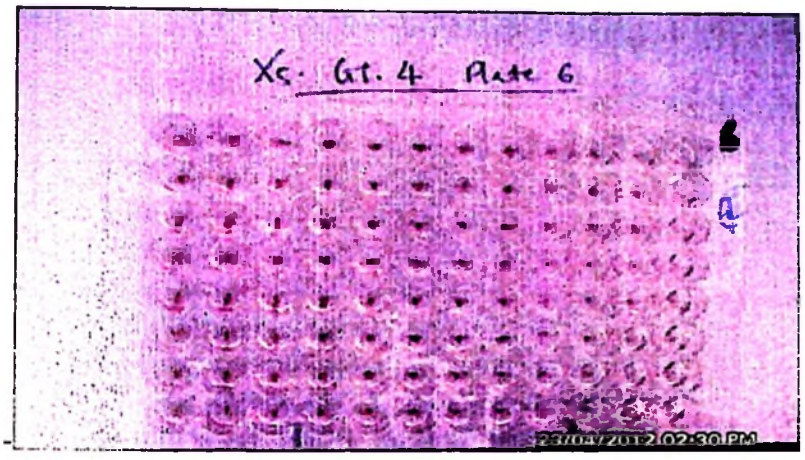

Figure 6: The HI test, Row 1 reads HI negative throughout, Row 5 read HI positive up to well 3 ($Log_2 3$).

3.2.11. Data Analysis of the test results

The microsoft office excel 2007 spread sheet was used to enter data of end point of dilution showing haemagglutination inhibition of each chicken (vaccinated and non-vaccinated) in each vaccination group (HI titre) before and post vaccination.

The excel spread sheet was used to store, summarize, analyse, design and present data. Geometric Mean titre to determine the flock immunity in each vaccination group was calculated by calculating an average of the individual chicken HI titre in a group as explained by Young *et al.* 2002 (Appendix 2).

The level of significance before and after vaccination in the same group was calculated by using **Chi square test.** The number of seropositive and seronegative chickens before and after vaccination was used to find out the effect of vaccination (Appendix 3).

The level of significance between the two vaccines in a similar vaccination strategy was calculated using paired **student's T test** by comparing their respective GMTs (Table 3). The level of significance to determine the difference in antibodies level in control positive between the two types of vaccines was calculated by using paired **student's T test** by comparing their respective GMTs before and after vaccination (Table 3). The level of significance when comparing the GMT of the vaccination strategy and the respective control was calculated by using paired **student's T test** as well.

CHAPTER FOUR

4.0. RESULTS

4.1. 12.5% vaccination strategy

For the group vaccinated using I-2. The HI before vaccination (X0) indicated that 9 chickens (44%) were seropositive (HI positive) and 56% of the chickens were seronegative (HI negative) before vaccination (Table 1). At the 8th (X4) and 10th week (X5) post vaccination only the vaccinated ones (12.5%) were seropositve and 87.5 % in contact chicken were seronegative. The highest Geometric Mean titre (GMT) of 2.00 was recorded at two weeks post vaccination (X1) and 1.13 at the 8th week (X4) and 10th week (X5) at the end of the experiment (Table 1).

For the effect of vaccination, there was no significant difference in numbers of seropositve chickens before and post vaccination ($p>0.05$). Likewise there was no significant difference ($p>0.05$) in GMT when compared to negative control group. But when compared to the positive control group (100% vaccinated), there was significant different between the two groups ($p<0.04$).

For the group vaccinated using La Sota, the HI results before vaccination (X0) indicates that 50% of the chickens were seropositive and 50% of the chickens were seronegative (Table 1). The number of seropositive and seronegative chickens keep changing, at the 8th week (X4) 63% of the chickens were seropositive and 37% of the chickens were seronegative which was maintained until the end of the experiment at the 10th week (X5) (Table 1).

The GMT of 2.75, 3.00, 2.13 and 3.19 were recorded at the 2nd week (X1), 4th week (X2), 6th week (X3) and 8th week (X4) post vaccination respectively (Table 1). The highest GMT of 3.31 was recorded 10th week (X5) post vaccination at the end of the experiment (Table 1).

For the effect of the vaccine, there was no significant difference in number seropositive and seronegative before and after vaccination ($p>0.05$). When the GMTs of this vaccination strategy were compared to the GMTs of the positive control (100%), the two groups were not significant different ($p> 0.05$) in protecting chickens.

However, at this vaccination strategy (12.5%), when the effect of La Sota and I-2 vaccines were compared using their GMT, they were significant different ($p< 0.05$) in protecting chickens and La Sota was better than I-2.

4.2 Vaccination strategy 25 %

For the group vaccinated using I-2, the HI results before vaccination (X0) indicates that 38% of the chickens were seropositive and 62% of the chickens were seronegative (Table1). At the 8th week (X4) post vaccination 56% of the chickens were seropositve and 44 % of the chickens were seronegative (Table 1). At 10th week (X5) 75% of the chickens were seropositive out of which 50% were in contact chickens and 25% of the remained in contact chickens were seronegative (Table 1) at the end of the experiment. The highest GMT of 3.25 was recorded at 6th weeks (X3) post vaccination followed by a subsequent falling in the GMT to 2.50 in the 10th weeks (X5) post vaccination at the end of the experiment (Table 1).

For the effect of vaccination, the number of seropositive and seronegative chickens before and after vaccination was found to be significant different ($p<0.05$). When the GMTs are compared to the GMTs of the positive control group (100% vaccinated) the two groups were not significant different ($p>0.05$).

For the group vaccinated using La Sota, the HI results before vaccination (X0) indicates that 44% of the chickens were seropositive and 56% chickens were seronegative (Table3). At the 4^{th} week (X2) post vaccination, 69% of the chickens were seropositive and at the 10^{th} week (X5) post vaccination, 75% were seropositive and 25% were seronegative (Table 1). The highest Geometric Mean titre of 3.25 was recorded at two weeks (X1) post vaccination and 3.06 in the 10^{th} week (X5) post vaccination at the end of the experiment (Table 1).

For the effect of vaccine, the number of seropositive and seronegative chickens before and after vaccination were statistically significant different ($p<0.05$). When the GMTs of this strategy are compared to the GMTs of the positive control the two were not significantly different ($p> 0.05$).

However, at this vaccination strategy, when the effect of La Sota and I-2 vaccines in chickens were compared themselves using their GMTs, they were not significant different in protecting chickens ($p> 0.05$).

4.3 Vaccination strategy 50%

For the group vaccinated using I-2, the HI results before vaccination (X0) indicates that 63% of the chickens were seropositive and 37% of the chickens were seronegative (Table 1). At the 2^{nd} week (X1) post vaccination 31% of the chickens were seropositive and 69% of the chickens were seronegative.

The number of seropositive was increased to 37% of the chickens at 4th and at 6th week (X3) post vaccination 87% of the chickens were seropositive followed by subsequent fall in seropositive chickens to 81% in the 8th week (X4) which was maintained to the 10th week (X5) which was the end of the experiment (Table 1). The highest GMT of 3.00 was recorded at 6th weeks (X3) post vaccination followed by a subsequent falling in the GMT to 2.75 in the 10th week (X5) post vaccination which was the end of study period (Table 1).

For the effect of the vaccine, number of seropositive and seronegative chickens before and post vaccination were not significantly different ($p>0.05$). When the GMTs of this vaccination strategy were compared to those of positive control the two groups were not significantly different ($p>0.05$) in protecting chickens.

For the group vaccinated using La Sota, the HI results before vaccination (X0) indicates that 31% of the chickens were seropositive and 69% of the chickens were seronegative (Table 1). At the 2nd week (X1) and 4th week (X2) post vaccination 63% of the chickens were seropositive and 37% were seronegative. The number of seropositive chickens at 6th week post vaccination (X3) was increased to 81%. The number of seropositive chickens was increased to 94% at the 10th week (X5) post vaccination at the end of the experiment (Table 1). The high Geometric Mean titre of 4.38 was recorded at 6thweeks (X3) post vaccination and followed by subsequent rising to the highest 5.56 in the 10th week (X5) post vaccination at the end of the experiment (Table 1).

For the effect of vaccine, the numbers of seropositive and seronegative chickens before and after vaccination were compared and found to be significant different ($p < 0.05$).

When the GMTs of this vaccination strategy were compared to those of positive control, the two groups were not significantly different ($p>0.05$) while significant different to the control negative ($p<0.05$).

However, at this vaccination strategy, when La Sota and I-2 vaccines were compared using their GMTs in this vaccination strategy (50%), they were not significant different in protecting chickens ($p>0.05$).

Table 1: HI titre and GMT results for each vaccination strategy and controls

Vaccination Strategy	Vaccine Type	HI titre results												Comparison of GMTs (Means) (p=0.05)
		X0		X1		X2		X3		X4		X5		
		+	-	+	-	+	-	+	-	+	-	+	-	
12.5%	I-2	44%	56%	44%	56%	6%	94%	13%	87%	13%	87%	13%	87%	
	GMT±1.01	2.6		2.00		0.94		0.93		1.13		1.13		
	La Sota	50%	50%	56%	44%	50%	50%	31%	69%	63%	37%	63%	37%	
	GMT±1.16	2.19		2.75		3.00		2.13		3.19		3.31		p<0.05
25%	I-2	38%	62%	38%	62%	38%	62%	50%	50%	56%	44%	75%	25%	
	GMT±2.03	1.81		2.25		2.56		3.25		2.31		2.50		
	La Sota	44%	56%	62%	38%	69%	31%	25%	75%	31%	69%	75%	25%	
	GMT±1.9	2.25		3.25		3.00		1.44		1.75		3.06		p>0.05
50%	I-2	63%	37%	31%	69%	63%	37%	88%	12%	81%	19%	81%	19%	
	GMT±1.44	2.63		2.00		2.75		3.00		2.73		2.79		
	La Sota	31%	69%	63%	37%	63%	37%	81%	19%	69%	31%	94%	6%	
	GMT±2.29	2.00		3.38		2.94		4.38		3.50		5.56		p>0.05
100%	I-2	56%	44%	12%	88%	88%	12%	81%	19%	81%	19%	81%	19%	
	GMT±1.46	2.63		1.53		3.00		3.25		3.06		3.00		
	La Sota	25%	75%	100%	0%	69%	31%	88%	12%	94%	6%	100%	0%	
	GMT±1.78	1.69		3.94		2.50		5.00		4.56		4.13		p>0.05
0%	I-2	69%	31%	19%	81%	12%	88%	6%	94%	0%	100%	0%	100%	
	GMT±1.11	2.50		1.69		0.69		0.88		1.38		1.69		
	La Sota	44%	56%	44%	56%	19%	81%	6%	94%	0%	100%	0%	100%	
	GMT±1.30	2.06		2.31		1.88		1.19		1.50		1.58		p>0.05

+ = ≥3Log base 2, - = <3Log base 2 GMT= Geometric Mean Titre, GMT± SD

4.4. The control positive (100% vaccinated)

For the group vaccinated using I-2, the HI results before vaccination (X0) indicates that 56% of the chickens were seropositive and 44% were seronegative (Table 1). At the 2nd week (X1) post vaccination 13% of the chickens were seropositive and 87% were seronegative. At the 4th week post vaccination (X2) 88% of the chickens were seropositive and 12% were seronegative. Seropositive chickens were reduced to 81% at 8th week (X4) and maintained until the end of the experiment at 10th week (X5) (Table 1). The highest Geometric Mean titre of 3.25 was recorded at 6th weeks (X3) post vaccination and followed by fall in the Geometric Mean titre to 3.00 in the 10th week (X5) post vaccination at the end of the experiment (Table 1).

For the effect of the vaccine, when the number of seropositive and seronegative chickens before vaccination were compared to the number of seropositive and seronegative chickens post vaccination and found to be significant different ($p < 0.05$).

For the group vaccinated using La Sota, the HI results before vaccination (X0) indicates that 25% of the chickens were seropositive and 69% were seronegative (Table 1). At the 2nd week (X1) post vaccination all 100% of the chickens were seropositive. The percentage of chickens who were seropositive at the 2nd week gradually reduced in the 4th week (X2), 6th week (X3) and 8th week (X4) to 69%, 88% and 94% respectively before peaking up again to (100%) in the 10th week (X5) at the end of the experiment (Table 1). The Geometric Mean Titre recorded before vaccination (X0) was 1.69 and 3.94 two weeks (X1) post vaccination and peaked up in the 6th week (X3) post vaccination to 5.00 before drop down to 4.56 and 4.13 in the 8th week (X4) and 10th week (X5) respectively. For the effect of vaccine, the number of seropositive and seronegative chickens before vaccination

when compared to the number of seropositive and seronegative chickens post vaccination was significant different ($p< 0.05$).

When the two vaccines I-2 and La Sota were compared at this positive control vaccination by using their GMTs, the two vaccines were not significantly different ($p>0.05$) in protecting chickens.

4.5. The control negative (0% vaccination)

For the control negative group stayed in the I-2 vaccinated chickens. 69% of the chickens were tested seropositive and 31% tested seronegative before vaccination (X0) (Table 1). The seronegative chickens were sharply increased to 81% two weeks later (X1) and there was slight increase to 88% at 4th week later (X2). Seronegative chickens were then increased to 94% and 100% at the 6th week (X3) and 8th week (X4) respectively and maintained until the end of the experiment at 10th week (X5) (Table 1). The highest GMT of 2.5 was recorded during the first sampling (X0) and decreased to 1.69 and 0.69 at the 2nd week (X1) and 4th week (X2) respectively before starts to increase to 0.88, 1.38 and 1.69 at the 6th week (X3), 8th week (X4) and 10th week (X5) respectively at the end of the experiment (Table 1).

Statistically there was significant difference ($p<0.05$) in the number of seropositive and seronegative chickens at the beginning and at the end of the experiment. For the negative control group in the La Sota vaccinated group, 44% of the chickens were seropositive and 56% were seronegative at first sampling (X0) and second sampling two weeks later (X1) (Table 1). Then there was a steady increase in seronegative chickens to 81% and 94% at the 4th week (X2) and 6th week(X3) respectively.

Seronegative chickens were then increased to 100% at the 8th week (X4) and maintained at the 10th week (X5) the end of the experiment (Table 1). The GMT of 2.06 was observed during the first sampling (X0) which was then increased to 2.31 at the 2nd week sampling (X1). During the third sampling at 4th week (X2) the GMT was decreased to 1.88 then decreased to 1.19 at the 6th week (X3). Then there was gradual increase in GMT to 1.50 and 1.58 at the 8th week (X4) and 10th week (X5) at the end of the experiment (Table 1).

There was significant difference in number of seropositive and seronegative chickens at the beginning when compared to the number of seropositive and seronegative chickens at the end of the experiment ($p < 0.05$). When the negative control groups stayed in the I-2 and La Sota vaccination were compared by using their GMTs, they were not significant different ($p > 0.05$).

4.6. The Highest Geometric Mean Titre in Unvaccinated chickens

4.6.1. At 12.5% vaccination strategy

At 12.5% vaccination strategy, for the group vaccinated using I-2, the GMT before vaccination was 2.43 and 36% of the chickens were seropositive (Table 2). After staying for two weeks with the vaccinated chickens (X1) the GMT obtained was 2.07 and 43% of the chickens were seropositive (Table3). The GMTs obtained in the 4th week (X2), 6th week (X3), 8th week (X4) sampling were 1.07, 1.08 and 1.15 respectively (Table 2). The GMT of unvaccinated chickens obtained at the end of the experiment (X5) was 0.67 (Table 2) and no chicken had protective antibodies level (Table 3).

For the effect of the vaccine, there was no significant difference in protection level in unvaccinated in contact chickens before and after vaccination ($p>0.05$).

For the group vaccinated using La Sota, the GMT obtained before vaccination was 2.29 and 57% of the chickens were seropositive (Table 2). The GMTs obtained post vaccination in the subsequent sampling in the 2nd week (X1), 4th week (X2), 6th week (X3) and 8th week (X4) were 2.29, 2.78, 1.86 and 2.79 respectively (Table 2). The highest GMT of unvaccinated chickens was obtained during the last sampling (X5) was 3.07 (Table 2) and 57% of the chickens were seropositve (Table 3).

To study the effect of the vaccine, there was no significant difference in protection level in the unvaccinated in contact chickens before and after vaccination ($p>0.05$). When I-2 and La Sota vaccines were compared using their GMTs, the two groups were significant different ($p<0.05$) in protecting unvaccinated in contact chickens.

4.6.2. At 25% vaccination strategy

For the group vaccinated using I-2, the GMT of unvaccinated chickens obtained before vaccination was 1.75 (Table 2) and 33% (Table 3) of the unvaccinated chickens were seropositive. The GMTs obtained post vaccination was 2.17 and 2.42 at the 2nd week (X1) and 4th week (X2) respectively. The highest GMT recorded at 6th week (X3) was 3.83 before reduced to 2.42 at 8th week (X4) (Table 2). The GMT obtained at the end of the experiment on 10th week (X5) was 2.25 (Table 2) and 67% of the unvaccinated in contact chickens were positive (Table 3).

For the effect of the vaccine, there was no significant difference in protection level in unvaccinated in contact chickens before and after vaccination ($p>0.05$). For the group vaccinated using La Sota, in the 25% vaccination strategy, the initial GMT of unvaccinated chickens obtained was 2.25 (Table 2) and 42% (Table 3) of the chickens were seropositive. The GMT variably decreased to 0.92 at 8th week and the highest GMT of 3.17 was obtained at tenth week (X5) (Table 2) and 75% of the chickens were seropositive (Table3).

For the effect of the vaccine, when the number of seropositve and seronegative unvaccinated in contact chickens were compared before and after vaccination there was significant difference in protection level before and after vaccination ($p<0.05$).When I-2 and La Sota vaccines were compared at this vaccination strategy (25%) using their GMT, statistics shows that the two vaccines are not significantly different in protecting in contact chickens ($p>0.59$).

Table 2: Geometric Mean Titres (GMTs) for in-contact chickens

	Vaccination strategy						Controls			
Sampling Interval	12.5%		25%		50%		100%		0%	
	I-2	La Sota	I-2	La Sota	I-2	La Sota	I-2	La Sota	I-2	La Sota
X0	2.43	2.29	1.75	2.25	3.00	1.88	2.63	1.69	2.50	2.06
2nd week (X1)	2.07	2.29	2.17	2.67	1.75	1.88	1.53	3.94	1.69	2.31
4th week (X2)	1.07	2.78	2.42	2.92	2.25	3.00	3.00	2.50	0.69	1.88
6th week (X3)	1.08	1.86	3.83	0.58	2.75	3.88	3.25	5.00	0.88	1.19
8th week (X4)	1.15	2.79	2.42	0.92	2.12	2.13	3.06	4.56	1.38	1.50
10th week (X5)	1.15	3.07	2.25	3.17	2.74	4.25	3.00	4.13	1.69	1.58
±SD	0.67	0.50	0.65	1.05	0.34	1.22	0.63	1.27	0.65	0.41

4.6.3. At 50% vaccination strategy

For the group vaccinated using I-2, the initial GMT of unvaccinated obtained was 3.00 and 75% of the chickens were seropositive. The GMTs recorded at the 2nd week (X1) 4th week (X2) were 1.75 and 2.25 respectively (Table 2).

The highest GMT of 2.75 was obtained sixth weeks (X3) post vaccination and 63% of the chickens were seropositive. At 8th week (X4) GMT recorded was 2.12 and at 10th week (X5) the GMT recorder was 2.74 (Table 2) and 38% of the chickens were seroconverted to protective level (Table 3).

For the effect of the vaccine, when the number of unvaccinated in contact seropositve and seronegative chickens before and after vaccination were compared, statistic shows that. there was no significant difference in protection level before and after vaccination ($p>0.05$).

For the group vaccinated using La Sota in the vaccination strategy 50%. the initial GMT obtained was 1.88 (Table 2) and 25% (Table3) of the chickens had protective antibodies level. The GMT recorded at the 2nd week (X1), 4th week (X2) and 6th week (X3) were 1.88, 3.00 and 3.88 respectively. The GMT was them reduced to 2.13 at the 8th week (X4) before getting to the highest (Table 2). The highest GMT of 4.25 was recorded at 10th week (X5) post vaccination (Table 2) and 88% of the chickens had protective antibodies levels (Table 3).

For the effect of vaccine, statistic shows that there was significant difference in protection levels in unvaccinated in contact chickens before and after vaccination ($p<0.05$). When the two vaccines were compared using their GMTs at this vaccination strategy (50%), statistics shows that, the two vaccines were not significantly different ($p>0.05$) in protecting in-contact chickens.

Table 3: The Percentage of seropositive for in-contact chickens

Sampling Intervals	Vaccination strategy 12.5%		25%		50%		Controls 100%	
	I-2	La Sota	I-2	La Sota	I-2	La Sota	I-2	La Sota
X0	36%	57%	33%	42%	75%	25%	56%	25%
2nd Week (X1)	43%	50%	33%	50%	25%	25%	13%	100%
4th week (X2)	7%	43%	33%	67%	38%	50%	88%	69%
6th Week (X3)	14%	21%	58%	0%	63%	88%	81%	88%
8th Week (X4)	0%	57%	58%	8%	38%	50%	81%	94%
10th Week (X5)	0%	57%	67%	75%	38%	88%	81%	100%

4.7. The Trend of Antibodies Titre Levels

For the 100% vaccinated chickens in vaccine types, I-2 and La2 Sota, the Geometric mean titres for each vaccine before vaccination and post vaccination were compared. For the I-2, the GMT before vaccination was 2.63 which fell to 1.53 two weeks later post vaccination and rose to 3.00 at the 4th week. At the 6th week (X3) the GMT raised to 3.88 before falling again to 2.13 at the 8th week (X4). The GMT recorded at the 10th week (X5) was 4.13 and it was the end of experiment (Table 1).

For La Sota vaccine, the GMT before vaccination was 1.69 which rose to 3.94 two weeks (X1) later post vaccination and fall to 2.50 at the 4th week (X2). At the 6th week (X3) the GMT was 5.00 and fall to 4.56 and 4.13 at the 8th week (X4) and 10th week (X5) respectively (Table 1).

The correlation coefficient (r^2) describes the strength of the association between time and antibodies level, the results suggest that the titre levels tended to increase with time lapsed (positive slope) for both vaccines, for I-2, the correlation coefficient (r^2) was 0.323 and for La Sota was 0.485 (Fig. 7). When the GMTs of I-2 and La Sota were compared, there was no significant difference in increasing antibodies level between the two types of vaccines ($p>0.05$).

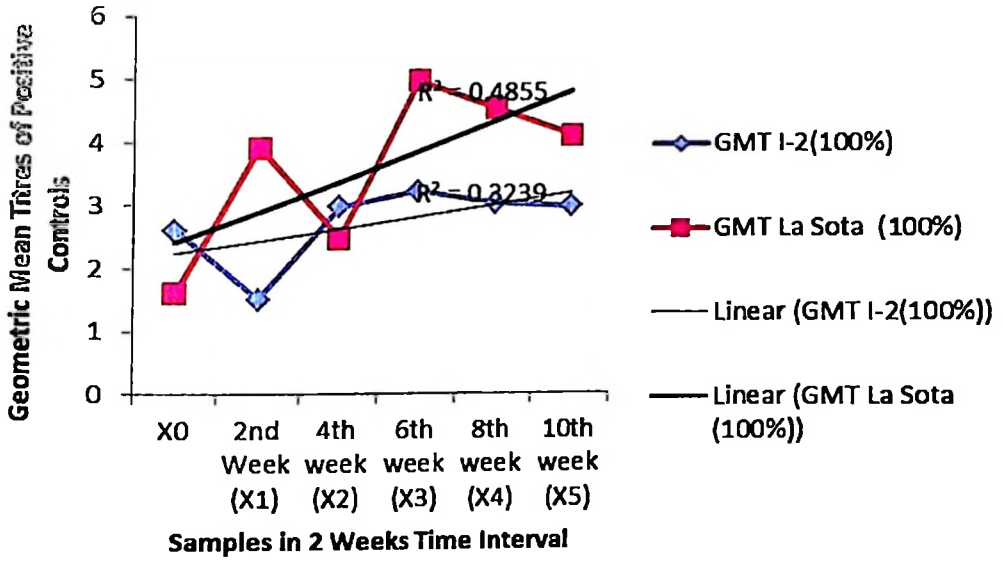

Figure 7: The trend of increasing antibodies titre levels for I-2 and La Sota vaccines

CHAPTER FIVE

5.0 DISCUSSION

This experimental trial shows that chickens vaccinated using I-2 and Lasota were protected against Newcastle disease just like many research findings Dias *et al.* (2001), Tu *et al.* (1997), Mazija (1990) and Feizi and Nazeri (2011). For each type of vaccine results shows that, vaccine viruses have the potential of horizontal spread ability from one vaccinated chicken to other in contact chicken (Dias *et al.*, (2001); Bell, (2001); Feizi and Nazeri (2011). The usefulness of vaccine in protecting chickens has been described by Nishikawa *et al.* (2007) when they challenged vaccinated chickens against ND and found no immunodepressive elements. This study used day old broiler chicks from parents with a history of being vaccinated against ND because it was not easy to acquire large number of chicks from non-vaccinated parents. This led to the use of chickens with different immune status at the beginning of the experiment due to maternal immunity. OIE recommends that to avoid interference of maternal antibody in chicks, vaccination should be done until the chickens are at the age of 2-4 weeks when most of them would have been susceptible. In this experiment vaccination was when chickens were 3 weeks of age (OIE, 2012). Al Zubeedy (2009) recommends early vaccination to enhance not only maternal derived immunity but also cell mediated immunity. The major factors affecting seroconversion and seroreversion in this study were vaccines and individual chicken's response to vaccines. Different strains of NDV used to prepare these vaccines can have effect in the immune response in vaccinated chickens and so was the objective of this study. The individual chicken's response to vaccines was taken care by using the geometric mean titre to find out effect of vaccine in immune response of the vaccinated chickens and none vaccinated in contact chickens.

For the evaluation of the effect of I-2 and La Sota vaccines, GMT obtained from each vaccination strategy were compared amongst the vaccines themselves and to the controls. In this research, at 12.5% vaccination strategy for I-2 vaccine, none of the in contact unvaccinated chickens was seropositive except for the two vaccinated ones until the end of the study. This finding is similar to the findings of Rahman *et al.* (2004) when they found only vaccinated chickens where protective. The GMTs of this vaccination strategy was not significantly different ($p>0.05$) to the GMTs of the negative control ($p>0.05$) and significantly different to the positive control ($p<0.05$). The results of having seropositive chickens following vaccination agrees to the findings of Aini *et al.* (1990) and contrast the findings of Bell *et al.* (1991b) about significant increase of positive reactors after vaccination. For La Sota vaccine 62.5% of the chickens were HI tested positive at the end of the study and the GMT of 3.31 was recorded which was not significantly different ($p<0.05$) to the control positive group (100% vaccinated) and significant different ($p>0.05$) to the negative control (0% vaccinated) group. When I-2 and Lasota were compared using their GMTs at this vaccination strategy, the two vaccines were significant different ($p<0.05$) in ability for vaccine virus spread and therefore induce antibody production and confer protection to in contact chickens and La Sota has done better than I-2. This finding agrees to the findings of Feizi and Nazeri (2011) when they compared the HI titres of Avinew and La Sota vaccines.

In the vaccination strategy 25%, I-2 vaccine showed that, 75% of the chicken were seropositive (protected) until the end of the study and the GMT of 2.5 was recorded and found to be statistically significant different ($p<0.05$) to the negative control group and not significantly different ($p>0.05$) with the positive control group. This suggests that the vaccination has done better similar to as when all chickens were vaccinated. La Sota on

the other hand, 75% of the chicken were tested positive and their GMT recorded was 3.06 which was significantly different ($p<0.05$) to the GMT of the negative control group and not significantly different ($p>0.05$) with the GMT of the positive control group. When the GMTs of both vaccines were compared, they were not significant difference ($p>0.05$) in the spread of vaccine and induction of antibody production to protective levels in the chickens.

For the vaccination strategy 50%, for I-2 vaccine 81% of the chickens were tested positive and the GMT of 2.75 was recorded at the end of the study period. When this GMT is compared to the GMT of the control negative the two are significantly different ($p<0.05$) and not significantly different with the GMT of the positive control group ($p>0.05$).This means that this strategy is as good as vaccinating all chickens. Similar results were obtained in Mozambique by Dias *et al.* (2001) when they found that 6 chickens were protected against ND when stayed in contact with 10 vaccinated chickens (62%). La Sota vaccine on the other hand, 94% of chickens tested positive and the GMT of 5.56 was recorded at the end of the study period. When this GMT is compared to the GMT of negative control group, the two are significantly different ($p<0.05$) but when compared to the positive control group, the two are not significantly different ($p>0.05$).When both vaccines at this strategy are compared using their GMT, statistics showed that they were not significantly different in stimulating antibody production in vaccinated and in contact none vaccinated chickens at this vaccination strategy.

When the GMTs of the I-2 vaccine and La Sota vaccine were compared, results and statistics showed that, the two GMTs were not significantly different ($p>0.05$). This means that the two vaccines have same potential in stimulating antibody production in broiler

chickens. Abbas *et al.* (2006) in Pakistan used five La sota vaccine strains, he primed and boosted the chicks in 7day and 21d respectively and collected serum on day 14 and 28 post vaccination. The GMT results obtained from his research were not significantly different ($p>0.05$) from the results I obtained in the La Sota positive control. Other researches show that, chickens which received booster vaccination have significantly higher antibody titres when compared to those who received only a primary vaccination and this is proved by the work of Shuaib *et al.* (2006), Feizi and Nazeri (2011) and Mazegia *et al.* (2009). However, the finding of this study contrasts the findings of Numan *et al.* (2005) when they found that the level of protection in vaccinated birds was not satisfactory in broilers.

For the unvaccinated in-contact chickens at the 12.5% vaccination strategy, for I-2 vaccine no in-contact chicken was seropositive at the end of experiment unlike La Sota which had 57% seropositive chickens. This difference was due to their difference in spread ability. Therefore, at this vaccination strategy La Sota vaccine has done better than I-2 in protecting in contact chickens.

At 25% vaccination strategy, the two vaccines were not significantly different in protecting in contact chickens ($p>0.05$). For both vaccines, more than half of the chickens had protective antibodies levels, this is a promising finding for protection of the chickens even though their antibodies level were less than those of vaccinated chickens which means that they were not protected similar to the vaccinated ones. For the vaccination strategy 50%, the two vaccines were not significant different ($p>0.05$) in protecting unvaccinated in contact chickens. Both vaccines have shown the promising future by immunizing the in contact chickens to a desirable level. But their antibodies level were not as high as vaccinated ones, which means that, vaccine virus for unvaccinated in contact

chickens cannot induce high antibody production levels similar to vaccinated ones, this phenomenon was also observed by Wust *et al.*(2010).

Vaccinating only 50% of the flock will lessen the work and save time for the busy farmer. Most chicken keeping households (94%) keep up to 30 chickens and 3% up to 40 chickens (Msami, 2008). Therefore a farmer will need to search and catch at a day time or at night only 50% of the chickens flock. This vaccination strategy will not make a farmer to think twice before deciding to vaccinate her chickens and therefore protect them from ND. Saving time and lessen the work are very important aspect for a busy farmer who has been engaged into other economic and social responsibilities.

For the trend of increasing antibodies level, results and statistics have shown that, there was no significant difference in the levels of increasing antibodies titre levels with time between the two types of vaccine ($p>0.05$) when the GMTs of vaccinated controls were compared. This suggests that, the correlation between time and antibodies titre levels for the two types of vaccines were not significantly different, and therefore the level of increasing antibodies level for the two types of vaccines was the same until end of the experiment which was two and half months. Wambura *et al.* (2000) recommended that I-2 vaccine gives protection for at least 2 months after vaccination unlike Wust *et al.* (2010) who said protection can be for at least 4 months similar to the findings of Alders and Spradbrow (2001a) in Mozambique field trials.

CHAPTER SIX

6.0. CONCLUSION AND RECOMMENDATIONS

This experimental study found that, for both vaccines, vaccine viruses have the potential of spreading from one chicken to another, as found in unvaccinated in contact chickens. For the vaccination strategies employed in this study, 50% vaccination strategy for both vaccines has shown to provide better protection when compared to the rest strategies as it give higher proportion of immunized individual 81% and 94% for I-2 and La Sota vaccines respectively. Furthermore this strategy induced highest level of GMT of 3.00 and 5.56 for I-2 and La Sota vaccine respectively the levels which ensure protection to the chickens. This levels of GMT attained were not significantly different to their respective positive controls. Therefore, instead of vaccinating the whole flock 50% vaccination is sufficient to provide flock/herd immunity similar to as vaccinating all, but field trials need to be done to comprehend this finding. It is important to be remembered that not only the humoral arm of the immunity is responsible against NDV, but also the cell mediate arm, this gives more confidence on protection against NDV at this vaccination strategy.

For unvaccinated in contact chickens, for both vaccines, 50% vaccination strategy has shown to induce better antibodies level production when compared to other vaccination strategies.

The trend of increasing the mean antibodies titre levels found to be not significant different for both types of vaccines. Therefore these two vaccines had similar potential in stimulating antibodies production in broilers, a relatively longer study period was needed to understand the peak and the point of diminishing antibodies level will start.

The general recommendations are; instead of having only prime vaccination, booster vaccinations at desired time intervals need to be done and similar variable need to be studied. Application of minerals and vitamins should also need to be done to evaluate these vaccines efficacy. The periodic change of the litter and usage litter of different nature need to be studied if they have effect in vaccine spread in a flock of chickens.

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APPENDICES

Appendix 1: Template showing organization used for sample collection

		BLOOD SAMPLE COLLECTION					
V/ strategy	ID	Pre vaccination	Post vaccination (2 weeks intervals)				
		XO	2nd week (X1)	4th week (X2)	6th week (X3)	8th week (X4)	10th week (X5)
12.5%	1-16						
25%	1-16						
50%	1-16						
100%	1-16						
0%	1-16						

Appendix 2: Shows how the GMT was calculated as per Young *et al.* (2002) Laboratory Manual

Chicken ID No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	**GMT**
HI Titre	3	3	2	4	2	2	2	2	2	3	2	3	4	3	2	2	**2.56**

Appendix 3: 2x2 Table for Chi square test (χ^2)

	Vaccine Used (I-2 or La Sota)		**Total**
	+	-	
Before vaccination	x1	x2	x1 + x2
Post vaccination	x3	x4	x3 + x4
Total	x1 +x3	x2 + x4	