ENDEMICITY OF NEWCASTLE DISEASE VIRUS IN VILLAGE INDIGENOUS CHICKENS AND THE ROLE OF CARRIER DUCKS

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DECLARATION

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

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DEDICATION

To my beloved husband Dr. John Muriithi; son Kelvin Macharia and daughter Christine Gathomi for their support and encouragement and my parents Ephantus Njagi Rugea and Kellen Gichuku Njagi for their unconditional love and support.

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ABBREVIATIONS

ACTH	Adrenocorticotrophin hormone
ADCC	Antibody dependent cell – mediated cytotoxicity
AEZ	Agro - ecological zone
APMV	Avian paramyxovirus
cDNA	Complementary deoxyribonucleic acid
CEF	Chicken embryo fibroblast
CIA	Chicken infectious anemia virus
CMI	Cell mediated immunity
CNS	Central nervous system
CPE	Cytopathic effect
ECE	Embryonated chicken eggs
EDTA	Ethylenediamine tetraacetate
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
F	Fusion (F) glycoprotein
HA	Hemagglutination assay
HI	Hemagglutination inhibition
HE	Hematoxylin eosin
HMA	Heteroduplex mobility assay
HN	Hemagglutinin-neuraminidase
IBD	Infectious bursal disease
ICPI	Intracerebral pathogenicity index

IHC	immunohistochemistry
Ig	Immunoglobulin
IS	Immunosuppressed
IVPI	Intravenous pathogenicity index
kD	Kilodaltons
L	Large polymerase
LAT	Latex agglutination test
M	Matrix
MDT	Mean death time
MEM	Minimum essential medium
MLD	Ministry of livestock
mRNA	Messenger RNA
NDV	Newcastle disease virus
NIS	Non - immunosuppressed
NK	Natural killer (cells)
NP	Nucleocapsid protein
nt	Nucleotide
NVNDV	Neurotrophic velogenic Newcastle disease virus
OIE	Office International des Epizooties
ORF	Open reading frame
P	Phosphoprotein
PBS	Phosphate buffer saline
PHA	Passive Hemagglutination assay

P.i.	Post inoculation
PMV	Paramyxovirus
RBCs	Red blood cells
RNA	Ribonucleic acid
RT – PCR	Reverse transcription polymerase chain reaction
SPF	Specific pathogen free
TBS	Tris buffered saline
VN	Virus Neutralisation test
VVNDV	Viscerotrophic velogenic Newcastle disease virus
vNDV	Velogenic Newcastle disease virus

ABSTRACT

While the epidemiology of Newcastle disease (ND) in commercial poultry systems is very well documented, the ecology of this disease in indigenous birds, especially in tropical environments, is not adequately reported. This thesis covers work carried out to investigate the ecological and biological factors that are associated with NDV endemicity in local indigenous chickens in wet and dry areas. The results led to the development of the elements of an endemicity model. Three on-farm studies were done, namely: (1) Establishing factors associated with ND disease outbreaks in chickens for five different agro-ecological zones; (2) Establishing ND virus prevalence in chickens in these zones; and (3) Establishing the role of serum and egg-yolk antibodies as indicators of ND carrier status in chickens. The first study was carried out by interviewing 15 farmers from each zone using questionnaires. Samples were collected from chicken for viral isolation and serology from the respective farmer's flocks for the second and third studies.

Controlled studies were then designed to investigate whether a stress model can explain the dynamics of Newcastle disease virus (NDV) ecology in the duck – chicken transmission interactions. Stress was simulated by injecting birds with dexamethazone to induce immunosuppression (IS). The study design comprised experiments 4 -7, namely: (4) Cross-transmission studies between infected ducks and sentinel chickens; (5) Studies establishing the types of pathological lesions in NDV carrier ducks, (6) Determination of NDV antigen localization in various tissues of experimental ducks; and (7) Determination of persistence of the virus in experimental ducks with various levels of NDV antibodies. Various groups of NDV - seronegative ducks, raised at the university premises, were

used in the four experiments. In experiment 4, five IS-infected, five non-IS-infected and five naïve ducks were each mixed with five naïve chickens. In experiment five, 38 IS-infected, 37 non-IS infected ducks were investigated for pathological lesions and compared with respective control ducks that were penned separately. In experiment six, 23 IS-infected ducks and 22 non-IS infected ducks were tested for the location of the viral antigen and compared with 10 naïve ducks that were separately penned. In Experiment seven, 94 ducks were divided into 3 groups according to antibody status [low antibody (32), medium antibody (32) and antibody free (30)]. Each duck group had four sub-groups namely IS-infected; IS-non-infected and two respective controls. Each experiment had 12 non – IS infected chickens as positive controls.

Data showed that ND outbreaks in chickens were significantly associated with: stress – inducing factors ($p<0.05$), i.e. confinement of birds, seasons, windy conditions and temperature changes. Other factors associated with ND outbreaks were: age of birds, restocking of farms with chickens and disposal methods of infected birds and fecal matter. Dust storms, cultural ceremonies and wild birds were not significantly associated with ND outbreaks ($p>0.05$). Prevalence of Newcastle disease virus was higher (17.8%) in the dry zone (Lower midland 5, LM 5) compared to the cool wet zone (Lower highland 1, LH 1) at 9.9%. Sero-prevalence was significantly highest ($p<0.05$) in adult birds (10%) while growers had 5.1% and chicks 2.9%. The geometric mean antibody titres were significantly higher in mature eggs than in sera of the same hens ($p<0.05$). The geometric mean antibody titres of mature egg yolk were significantly higher than those in ovules in LH 1, Upper midland 2 and Lower midland 3, but the reverse was the case in Upper

midland 3 and LM 5. Hens were seronegative and infected or seropositive with antibodies in eggs and ovules or seronegative but with antibodies in eggs and ovules. The hens with high antibody levels would be infected by NDV but not die, however when antibodies waned off they would be susceptible to infection. This completed one component of the endemicity model.

Ducks showed minimal to very mild clinical signs. They did not die but transmitted the virus to in – contact sentinel chickens, resulting in 100% chicken mortality. Ducks shed the virus for 15 days post infection. Chickens mixed with IS ducks showed more clinical signs than those mixed with non - IS ducks. The NDV was more readily transmitted from IS ducks to chickens than from non - IS ducks demonstrating the second component of the endemicity model. This model simulates the potential for disease transmission scenario in rural duck – chicken mixed flocks. This phenomenon has not been demonstrated before and is being reported here for the first time.

Air-sacculitis, necrotic foci on the spleen and congestion of the small intestines were dominant pathological lesions in challenged IS ducks. Congestion of the liver, lymphoid depletion in cecal tonsils and spleen and the focal infiltration of mononuclear cells in these organs were observed more in IS ducks than in non- IS ones. The lesions (except airsacculitis) in positive control chickens were extremely severe compared to those seen in ducks. Immuno-suppression therefore exacerbated lesions in ducks completing the third component of the model. On immunohistochemistry, viral nucleo-proteins were found mainly in the large mononuclear cells of cecal tonsils and tubular epithelial cells of

infected duck kidneys. This study demonstrated for the first time that NDV localized and possibly multiplied in cecal tonsils and kidneys of the carrier ducks, where it can be excreted into feces leading to periodic outbreaks of the disease in duck – chicken mixed rural flocks. This formed the fourth component of the model.

For the low-antibody and medium-antibody maintained ducks that were challenged with virulent NDV, the IS ducks manifested more clinical signs of ND than NIS ducks. The ducks that were NIS, with no pre –challenge antibody titers had a high increase in antibody levels compared to respective IS –challenged ducks. The IS ducks had a high concentration and persistent viral levels in their tissues than NIS ones, making them better carriers. The pre – challenge antibody levels therefore affect the immune response in NDV carrier ducks and form the fifth part of the endemicity model.

In conclusion, seronegative hens harbored NDV while seropositive hens did not. Kidneys and cecal tonsils seemed to sequester the virus in carrier ducks while immunosuppression increased the intensity and frequency of lesions, clinical signs and the persistence and quantity of virus released from IS carrier ducks to chickens. Thus, a five-component endemicity model can explain the ND carrier status in duck – chicken mixed flocks and in village indigenous multi-age chicken flocks and should be taken into account when ND control strategies are being developed.

Further studies should investigate the role of egg yolk and sera antibodies in carrier ducks in addition to carrying out a prospective cohort study with large sample size and long

period of follow up in order to understand the role of the risk factors that were raised in this study in the epidemiology of ND in village indigenous chickens in Kenya. Since there is frequent transportation of birds between the two agro -ecological zones studied, a phylogenetic analysis of the NDV isolates recovered to reveal whether there are differences among them is recommended.

CHAPTER 1

1.0. Introduction

Kenya is mainly an agricultural country. The agricultural sector contribution comprises about 25% of the annual gross domestic product (GDP) of which 4% is from the poultry sub – sector. There are thirty four million poultry, 70% of which comprises village chickens (Personal communication, Nairobi Provincial Livestock Officer, 2007). With ever-increasing prices of red meat, village birds have become the main source of animal protein in form of meat and eggs for the rural human population, which comprises about 80% of the Kenya's total population (Njue *et al.,* 2002). Besides supplying poultry meat and eggs for human consumption, the village birds are a source of readily available cash for smallholder farmers, especially women and children (Njue *et al.,* 2002). Improving the health and productivity of these birds would help in alleviating poverty and rendering economic empowerment for the rural farming population.

Newcastle disease (ND) is the most economically important viral poultry disease in most developing countries that limits both production and international trade. Whereas its epidemiology and control are better documented in commercial poultry systems, there are fewer studies in free – range indigenous poultry in developing countries, including Kenya (Awan *et al.,* 1994). A study of the occurrence of ND in free – range indigenous poultry can reveal the nature and maintenance factors that play a part in the sustenance of the carrier status. The results will enable development of control strategies for ND. The effects of this will be the availability of more birds, eggs and meat for sale and subsequent poverty alleviation for smallholder farmers.

Ducks, turkeys, doves, geese, and guinea fowl can harbour Newcastle disease virus (NDV) and once infected with NDV they shed the virus, and act as a source of infection for chickens (Adene, 1997). They may or may not develop clinical ND, depending upon the pathotype of the virus isolate and the bird species (Clavijo *et al.*, 2000). In Kenya ND occurs all the year round with peaks in both dry and cold seasons (Nyaga *et al.*, 1985). Elements involved in this ND endemicity in different climates and zones are not well understood. So far, there have been no studies investigating other climatic and spatial factors, e.g. agro- ecological zone (AEZ) as risk factors for ND outbreak. Studies of such factors may give more information on the endemicity of ND. The sex of the birds has been shown to affect the survival rate in ND outbreaks. This has been reported by Kutubuddin (1973) found that male birds are more affected by NDV than female birds. However, the role of age and sex in village chicken on ND prevalence in Kenya is unknown and needs to be investigated.

Capua and others (1993) detected virulent NDV in uninfected cell cultures prepared from embryonated chicken egg. The true significance of such transmission in epizootics of ND is not clear. Hens suffering from natural ND have been shown to yield infected embryos, which died during incubation (Beard and Hanson, 1984). Virus may also penetrate the shell after eggs are laid (Williams and Dillard, 1968), further complicating the assessment of true vertical or transovarian transmission. There is therefore need to evaluate the manner in which ND virus and antibody in serum, eggs and ovules of the village hens play a role in the persistence of the virus in village poultry.

In Kenya, ducks are often reared together with chickens under village conditions (MLD, 2005). Although there are, indications that ducks play a role in the maintenance of NDV and its transmission to chickens in mixed flocks of rural poultry, the mechanism of this transmission is poorly understood (Adene, 1997). When flocks and different breeds are mixed, a wide range of bacterial, viral and parasitic diseases and poor nutrition induce stress in free-range chickens; not to mention stress caused by walking long distances in search for feed. These stressful conditions may render village poultry more susceptible to Newcastle disease either by increasing shedding of the virus by ducks or by increasing the susceptibility of the chicken to the virus. Since stress is associated with immunosuppression, a study of the effect of immunosuppression on ducks and chickens in the transmission of NDV from ducks to chickens will reveal whether stressful situations in the villages play a role in the persistence of ND in the flocks. There are no reports on whether the ducks that are symptomless but harbour ND virus revert to clinical disease under stress. Therefore an evaluation of clinical signs, gross and microscopic lesions and location of NDV antigens in various organs and tissues in immunosuppressed and non – immunosuppressed NDV carrier ducks was planned to be undertaken in this study.

Study hypothesis

Endemicity of Newcastle disease virus in village indigenous chickens is not influenced by diverse climate, management practices and immunosuppression in carrier ducks.

1.1. General objective

To investigate the endemicity of Newcastle disease virus in village indigenous chickens under different agro-ecological conditions, and to examine the role of immunosuppression in carrier ducks.

1.1.1. Specific objectives:

(1) Establish factors associated with ND outbreaks in chickens in five different agro-ecological zones.

(2) Establish ND virus prevalence in chickens in the five agro -ecological zones.

(3) Establish the role of serum and egg-yolk antibodies as indicators of ND carrier status in chickens.

(4) Determine the effect of immunosuppression on ND virus transmission from non – immune ducks to chickens.

(5) Establish the types of pathological lesions found in NDV carrier ducks.

(6) Determine the target organs for NDV antigen localization in experimentally infected ducks

(7) Determine the effect of immunosuppression on the virus persistence in ducks with different levels of NDV antibodies.

1.2. Justification

Village indigenous chickens and ducks comprise the highest number of poultry in Kenya, and with very little input from the owners, they contribute significantly to the socio-economic welfare of the village communities. Any efforts towards increasing production of these birds will help in poverty alleviation. Newcastle disease is the greatest constraint to the production of village chickens because of its high mortality rate, rapid spread, high contagiousness and marked economic impact. Factors that play a role in maintaining ND in village poultry are not well documented in Kenya. This study will establish the nature and components of ND endemicity model and explain the nature of its occurrence and carrier status in duck – chicken mixed flocks and in village indigenous multi-age chicken flocks. This study will provide data that can be used in designing effective control measures for this disease in Kenya, leading to higher poultry production, more meat, eggs, and better livelihoods for the smallholder village farmers.

CHAPTER 2

2.0. Literature review

2.1. Newcastle disease virus

2.1.1. Classification

Newcastle disease virus is a member of the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae* and genus *Avulavirus* (Mayo, 2002). Newcastle disease virus is the only member of the genus *Avulavirus* that is pathogenic to chickens (Mayo, 2002). Other important members of the family *Paramyxoviridae* are the mumps virus, simian virus 5 and parainfluenza virus type 2.

2.1.2. Virion

The ND viral particles are pleomorphic in nature and range from 150-400 nm in size. These virions contain a long helical nucleocapsid structure, which is 1,000 nm long, and 17-18 nm in diameter. Their envelope is covered with spike glycoproteins, which are 8-12 nm in diameter. The genome of NDV is a single strand of ribonucleic acid (RNA) of negative sense, and has a molecular weight of 5.2 to 5.7 $\times 10^6$ daltons (Alexander, 1997). Its genomic RNA consists of 15,186 nucleotides (nt) (Krishnamurthy and Samal, 1998; De leeuw and Peeters, 1999). Nucleocapsid protein (NP) and genome RNA together form a core structure to which the phosphoprotein (P) and the large polymerase protein (L) are attached (Lamb and Kolakofsky, 1996). This core forms the ribonucleoprotein (RNP) or the transcriptive - replicative complex and serves as the minimum infectious unit. The

viral envelope contains two surface glycoproteins: the hemagglutinin-neuraminidase (HN) protein responsible for attachment of the virus to host cells and release of maturing virus from infected cells and the fusion (F) protein required for fusion of the virus into the host cell membrane. The F and HN proteins are also the main targets of the immune response of NDV (Morgan *et al.*, 1992). Internal to the envelope is the matrix (M) protein, which is thought to be important in viral assembly (Peeples, 1991). Figure 2.1 shows a schematic diagram of the NDV and Figure 2.2 depicts an electron micrograph of the virus particle.

2.1.3. Genome organization

The NDV genome consists of six genes (3' NP-P-M-F-HN-L 5') (Steward *et al.*, 1993). Its RNA contains a 3' extracistronic region of 55 nucleotides, known as the leader, and a 5' extracistronic region of 144 nucleotides, known as the trailer (Krishnamurthy and Samal, 1998). These regions are essential for replication of the genome, and they flank the six genes. At the beginning and end of each gene are conserved transcriptional control sequences, known as the gene start and gene end sequences, respectively. Between the gene boundaries are intergenic regions, which vary in length from 1- 47 nucleotides (Krishnamurthy and Samal, 1998).

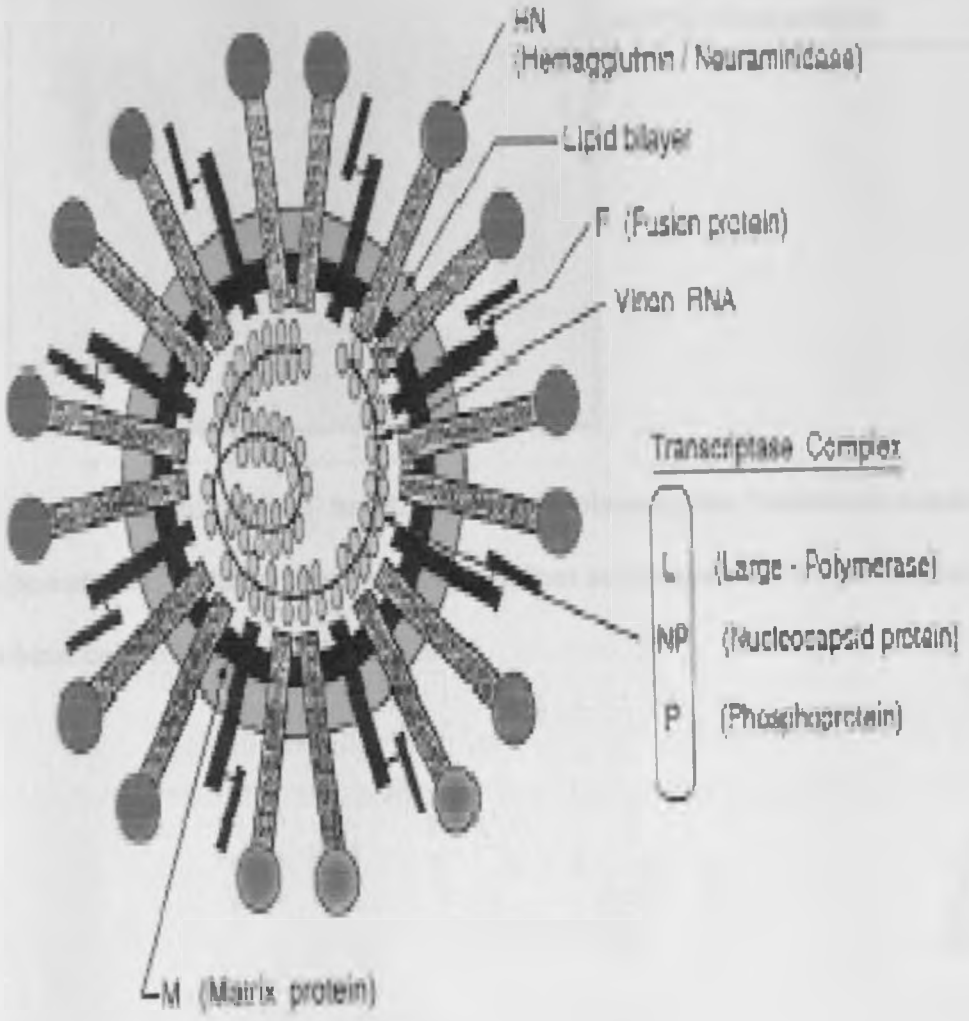

Figure 2.1. Schematic diagram of Newcastle disease virus particle (not drawn to scale)

(Courtesy of Panda, 2003).

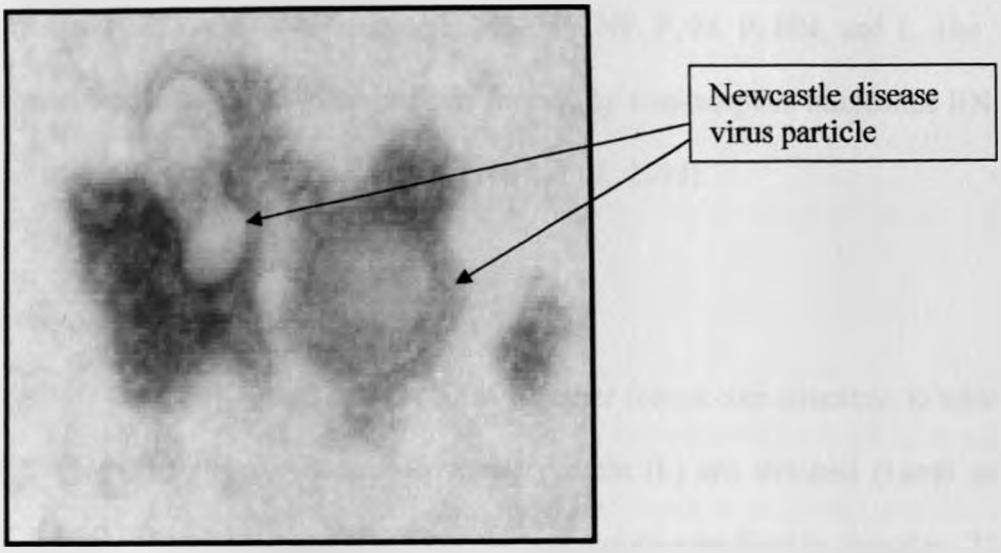

Figure 2.2. Electron micrograph of negatively stained pleomorphic Newcastle disease virus, strain Beaudette C particles (arrows) obtained from supernatant of infected chicken embryo fibroblast cells (Courtesy of Panda, 2003).

2.1.4. Viral proteins

The six genes of NDV code for at least eight proteins: NP, P, M, F, HN, and L. The V and W proteins are the two additional proteins formed by non-template nucleotide RNA editing process during P gene transcription (Steward *et al.*, 1993).

2.1.4.1. Nucleocapsid and associated proteins

The nucleocapsid protein (NP) and genome RNA together form a core structure, to which the phosphoprotein (P) and the large polymerase protein (L) are attached (Lamb and Kolakofsky, 1996). These three proteins form the transcriptive-replicative complex. The NP protein serves several functions in viral replication, including encapsidation of the genome RNA into a nucleocapsid, association with the phosphoprotein and the polymerase during transcription and replication and most likely, interaction with the M protein during virus assembly. The NP gene of NDV consists of 1747 nucleotides and a polypeptide molecular weight of 54 kilodaltons (kD) (Krishnamurthy and Samal, 1998).

The P gene of NDV is 1451 nucleotide long. The P gene ORF encodes an unedited version of mRNA, which results in formation of the P protein. RNA editing with the addition of one G nucleotide at the editing site (near the center of the ORF) produces an mRNA, which encodes the V protein. The addition of two G nucleotides produces an mRNA that encodes the W protein (Steward *et al.*, 1993; Lamb and Kolakofsky, 1996). This P protein is essential for viral RNA synthesis and is highly phosphorylated in nature. It is an essential component of the viral RNA polymerase and the nascent chain assembly complex formed during viral RNA synthesis. The P protein associates with the L protein

forming a complex and thus, functions as a transcriptive and replicative factor. It also associates with the unassembled NP forming the P- NP^0 complex (Hamaguchi *et al.*, 1983). This property of P protein has been suggested to prevent NP^0 from assembling RNA non-specifically (Masters and Banerjee, 1988). The predicted molecular mass of the P gene is 53 kD (Daskalakis *et al.*, 1992).

The L protein is the least abundant of the non - structural proteins (about 50 copies per virion). The L-gene is the most promoter-distal in the transcription map and thus the last to be transcribed. Both P and L proteins form a complex that is required for polymerase activity with NP: RNA templates (Curran *et al.*, 1994). The L protein is also responsible for capping and polyadenylation of the mRNAs. Polyadenylation is thought to result from polymerase stuttering on a short stretch of U residues. The L gene is 6704 nt long with a molecular mass of the polypeptide of 242 kD (Yusoff *et al.*, 1987).

The matrix protein (M) is the most abundant protein in the virion. The M gene of NDV is 1241 nt long. Its predicted molecular mass is 40 kD (Chambers *et al.*, 1986). It interacts with the nucleocapsid and the envelope proteins of the virion. This protein is considered the central organizer of viral morphogenesis, interacting with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer, and the nucleocapsids. The self-association of M and its contact with the nucleocapsid may be the driving force in forming a budding virus particle (Peeples, 1991).

2.1.4.2. Envelope glycoproteins

Newcastle disease virus possesses two integral membrane glycoproteins namely, the hemagglutinin-neuraminidase (HN) glycoprotein which is involved in cell attachment and the fusion (F) glycoprotein which mediates pH-independent fusion of the viral envelope with the plasma membrane of the host cell.

The HN glycoprotein of NDV is a multifunctional protein and a major antigenic determinant of the virus. It is responsible for the attachment of the virus to sialic acid-containing receptors. In addition, HN mediates enzymatic cleavage of sialic acid (neuraminidase activity) from the surface of virions and the surface of infected cells. In addition to the hemagglutinating and neuraminidase activities, HN also has a fusion promoting activity, through interacting with the fusion glycoprotein of NDV (Lamb and Kolakofsky, 1996). Previous research has indicated that for fusion to occur, a type-specific interaction between the F and HN proteins is required (Lamb and Kolakofsky, 1996). It is proposed that HN undergoes a conformational change on attachment to its ligand and thereby triggers a conformational change in the F protein to release the fusion peptide (Lamb, 1993).

The F protein of NDV mediates viral penetration by fusion between the virion envelope and the host cell plasma membrane, in a pH-independent manner. After fusion, the nucleocapsid is delivered to the cytoplasm. Later in infection, the F protein expressed at the plasma membrane of infected cells can mediate fusion with neighboring cells to form syncytia (giant cells). Syncytia formation is a hallmark of NDV infection in host cells. It

is a typical cytopathic effect caused by the virus and can lead to tissue necrosis and might be a mechanism of viral spread. The F protein is a type I integral membrane protein and is synthesized as an inactive precursor (F_0) that is cleaved by a host-cell protease. This cleavage releases a new N-terminus of F_1, thus forming the biologically active protein, consisting of disufide-linked chains F_1 and F_2 (Scheid and Choppin, 1974). The cleavage of F_0 is a key determinant for pathogenicity of paramyxoviruses. Viruses that have multiple basic residues in the cleavage site of the F protein have proteolytic cleavage of the F molecule intracellularly by subtilisin-like proteases such as, furin, during transport of the protein through the trans Golgi network. Paramyxoviruses that have single basic proteins in the F cleavage site cannot be cleaved intracellularly and require exogenous proteases for cleavage activation (Ortmann *et al.*, 1994).

2.1.5. Newcastle disease virus pathotypes

Newcastle disease virus is grouped into five pathotypes on the basis of predominant signs in affected chickens (Beard and Hanson, 1984). These are: (i) Doyle's form – viscerotrophic velogenic ND (VVND)- an acute lethal infection of all age groups characterized by haemorrhagic lesions of the digestive tract (Doyle, 1927); (ii) Beach's form – neurotrophic velogenic ND (NVND) – an acute lethal infection of all age groups characterized by respiratory and neurological signs (Beach, 1942); (iii) Mesogenic (Beaudette's form)– is a less pathogenic form of NVND with mortalities usually in young birds. It is caused by mesogenic virus strain often used as secondary live vaccines (Beaudette and Black, 1946); (iv) Lentogenic (Hitchner's form) - a mild or inapparent respiratory infection caused by lentogenic virus strains commonly used as live vaccines

(Hitchner and Johnson, 1948); and (v) Asymptomatic enteric form – mainly involves infections with lentogenic virus which causes no overt disease (McFerran and Mc Cracken, 1988).

2.2. Epidemiology of the Newcastle disease virus

2.2.1. Occurrence of Newcastle disease virus

In Kenya, Newcastle disease (ND) was first encountered on the Mombasa island and later spread throughout the country (Musiime, 1992). Newcastle disease outbreaks were reported to occur in Kenya during the cold and dry periods of the year with peaks in April, June – July and September – November periods (Nyaga *et al.*, 1985). The nature of where the virus remains during the periods between outbreaks has not been fully studied.

Studies carried out by Kasiti (2000) who sampled live bird markets in the city of Nairobi indicated that Newcastle disease virus (NDV) strains are widespread in village chickens in Kenya. However, the study did not cover ducks. In the United States of America, low virulence NDV strains have been recovered in live bird markets (King and Seal, 1997), while velogenic NDV strains were recovered from chicken in Southern Eastern United States and Puerto Rico (Marin *et al.*, 1996); velogenic viscerotropic Newcastle disease virus (VVNDV) was isolated from domestic psittacine birds in six states: Illinois, Indiana, Michigan, Texas, California, and Nevada; and from quarantined birds (Panigraphy *et al.*, 1993). The isolation of virulent NDV from healthy – appearing village indigenous chickens and wild birds, indicates presence of the virulent virus in these birds, from which it may emerge to infect susceptible birds; a clear situation of a carrier status for NDV (Schelling *et al.*, 1999; Clavijo *et al.*, 2000).

The appearance and rapid spread of neurotropic velogenic NDV in double – crested cormorants and other wild birds in widely disparate geographical areas of Michigan, Minnesota, North Dakota, South Dakota, and portions of Canada indicate the potential of wild birds as source of virulent virus to domestic chicken (Neetles, 1991). Studies on the role of non- chicken poultry (such as ducks) as carriers of NDV are a good study model that provide data on factors involved in the carrier status of this disease.

Village free -range indigenous chickens may be exposed to virulent virus shed from recovered vaccinated birds that have various levels of antibodies in their blood. Such birds are infected, carry virulent virus but are not susceptible. On the other hand, such non – susceptible birds may under stressful conditions come down with disease and release virulent virus. This would infect susceptible chickens (Carter, 2005). There is also a possibility that, during inter- epidemic period, there are factors that lead to the attenuation of virulent strains as they remain in the birds (without causing disease). When excreted, these attenuated viral strains may infect susceptible birds and, through passage among the birds, may revert to virulence. None of these possibilities have been investigated.

Various circumstances may therefore play a role in maintaining the ND virus carrier status and allow the virus to exist in its different pathotypes in the carrier birds. The factors that lead to the maintenance or the release of virulent virus and eventual cause of disease in the respective carrier states need to be investigated. The conceptual framework derived from literature on the carrier status is as proposed in **Figure 2.3.**

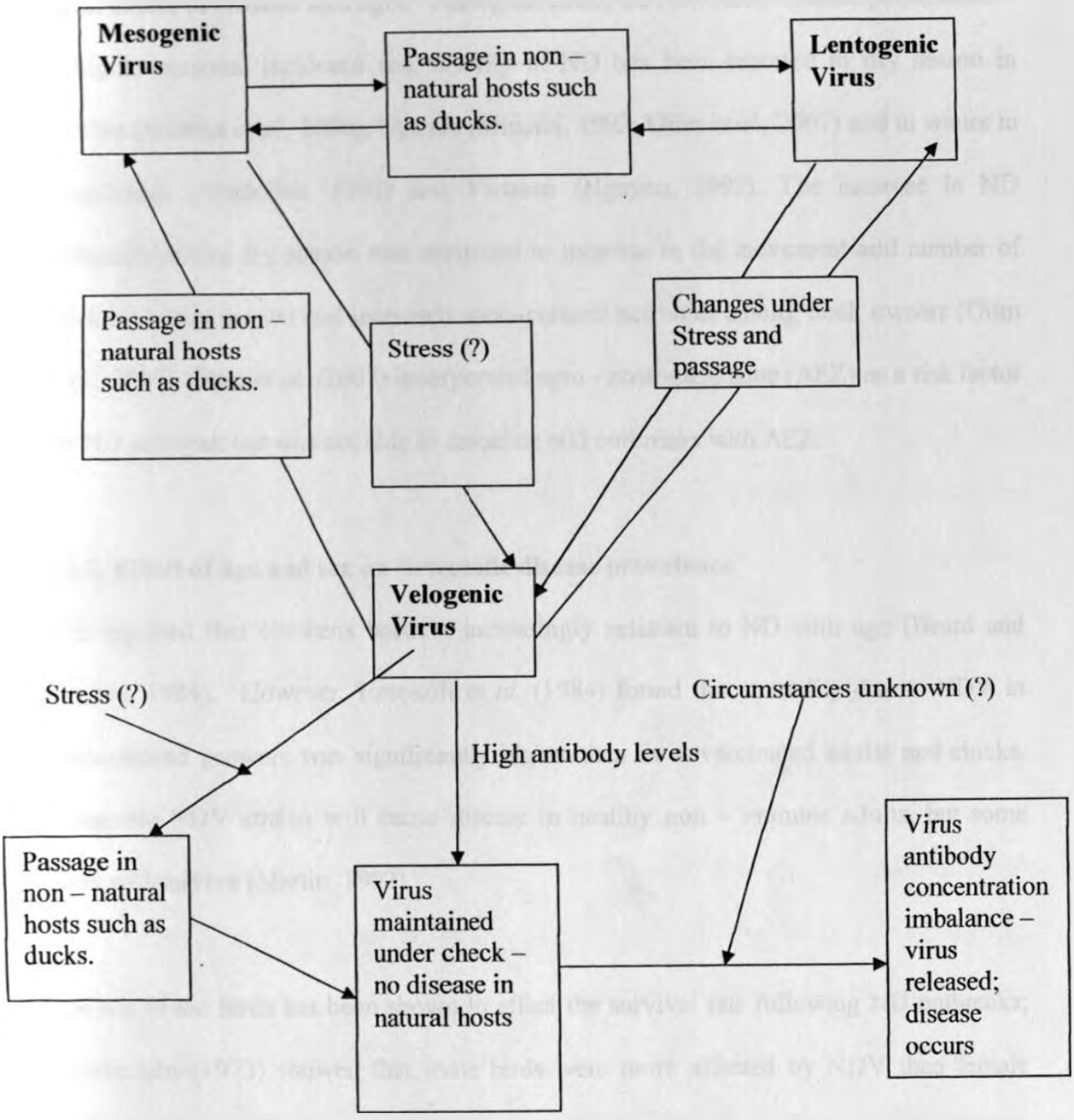

Figure 2.3: Proposed conceptual framework of the Newcastle disease virus carrier status in village chickens as derived from literature (Njagi *et al.*,2003)

2.2.2. Effect of seasons and agro - ecological zones on Newcastle disease prevalence

A higher seasonal incidence and severity of ND has been reported in dry season in Zambia (Sharma *et al.*, 1986), Uganda (Mukiibi, 1992; Otim *et al.*, 2007) and in winter in Bangladesh (Asadullah, 1992) and Vietnam (Nguyen, 1992). The increase in ND outbreaks during dry season was attributed to increase in the movement and number of chickens in the market and increased socio-cultural activities among flock owners (Otim *et al.*, 2007). Otim *et al.* (2007) incorporated agro - ecological zone (AEZ) as a risk factor for ND outbreak but was not able to associate ND outbreaks with AEZ.

2.2.3. Effect of age and sex on Newcastle disease prevalence

It is reported that chickens become increasingly resistant to ND with age (Beard and Hanson, 1984). However, Ezeokoli et *al.* (1984) found that mortality due to NDV in unvaccinated growers was significantly higher than in unvaccinated adults and chicks. Velogenic NDV strains will cause disease in healthy non – immune adults, but some birds will survive (Martin, 1992).

The sex of the birds has been shown to affect the survival rate following ND outbreaks; Kutubuddin (1973) showed that male birds were more affected by NDV than female birds. In addition, Huchzermeyer (1993) noted that brooding hens and hens with clutches of chicks that were kept segregated could also escape infection. It is not known whether it is the physical separation or the sex of the birds that made them survive ND outbreaks. The status of the influence of age and sex in the Kenyan village chicken on ND prevalence is unknown and needs to be evaluated.

2.2.4. Survival of Newcastle disease virus in the village poultry flocks

Newcastle disease virus is thought to be maintained in the village by cycling of virus in chickens, other domestic species, and wild birds (Hanson, 1976). Although NDV is readily isolated during an active infection, the duration of virus persistence in birds has not been clearly defined (Alexander, 1997). Species differences in viral shedding also occur, from very little in blackbirds to prolonged shedding in sandhill crane and parrots (Erickson *et al.*, 1977). Earlier studies have not differentiated prolonged virus shedding following an acute infection from recurrent shedding of virus (Seal *et al.*, 2000a). Recurrent shedding of the virus, if it exists, is of great concern in designing a successful NDV control program, particularly if resources are not available to eradicate the disease (Seal *et al.*, 2000a). This shedding and persistence for varying periods is another state of NDV carriage that is not clearly understood.

2.2.5. Newcastle disease virus host range

Indigenous breeds of village poultry are probably as susceptible to ND as commercial breeds. Besides the chicken, other poultry are susceptible to Newcastle disease virus, and about 30 species of wild birds, including some migrant waterfowls have been shown to be infected with the virus, although infection does not necessarily lead to overt disease. In Nigeria, velogenic, mesogenic and lentogenic strains of NDV have been found in wild birds, which were considered a reservoir and a source of virus to susceptible rural poultry (Olabode *et al.*, 1992). Alexander *et al.* (1979) recovered NDV of low virulence from a wild mallard duck, which could be distinguished from the vaccinal viruses. Lipkind *et al.* (1995) isolated a mixed population of viruses of Avian paramyxovirus serotype 1

(APMV -1) and Avian paramyxovirus serotype 2 (APMV -2) in wild and domestic birds in Israel. The isolates were recovered from the apparently healthy birds and turned out to be virulent for chickens (Lipkind *et al.*, 1995). Lipkind *et al.* (1995) did not establish whether the sequence at the fusion protein cleavage site of the NDV, that later proved virulent in chickens, changed after passage in the chicken or remained the same motif as in the wild birds; however, De Leeuw *et al.* (2003) have shown that changes do occur in the fusion protein cleavage site after passage in chicken brains. The NDV has also been isolated from dead ostriches and emus (Jørgensen *et al.*, 1998). Rosenberger (1974) obtained four isolates of NDV that were lentogenic from free – flying Canadian geese in the Atlantic flyway. Stallknecht *et al.* (1991) isolated APMV1, 4, 6 and 8 from migrating and resident ducks in coastal Louisiana USA. These were typical PMV's commonly associated with free – flying waterfowls.

2.2.6. Transmission of the Newcastle disease virus

Infection of NDV takes place by either inhalation or ingestion and spread from sick to healthy birds (Alexander, 1988a). In natural infections, large and small droplets containing virus are released from the respiratory tract of infected birds or carried in virus laden dust, faeces and other particles, leading to infection upon inhalation (Meulemans, 1988). Egg transmission of Newcastle disease virus has been reported in few incidents. Embryos that survived NDV strain - 4 infections *in ovo* hatched and the progeny were NDV positive (French *et al.*, 1967). More recently, virulent NDV was detected in uninfected cell cultures prepared from embryonated chicken eggs (Capua *et al.*, 1993). The true significance of such transmission in epizootics of ND is not clear. Virus may

also penetrate the shell after eggs are laid (Williams and Dillard, 1968), further complicating the assessment of true vertical or transovarian transmission. There is, therefore, need to evaluate the ND viral and antibody status in eggs and ovules of the village hens to demonstrate any state of endemic situation.

2.3. Immune responses to Newcastle disease virus

The initial immune response to infection with NDV is cell mediated and it may be detected as early as 2-3 days after infection with live vaccine strains (Benedict and Berestecky, 1987; Meulemans *et al.*, 1988). This has been thought to explain the early protection against challenge that has been recorded in vaccinated birds before a measurable antibody response is seen (Allan and Gough, 1976).

2.3.1. Cell mediated and humoral immunity

Newcastle disease virus infected target cells are coated with antibodies for the virally coded surface antigens and are destroyed by the cytotoxic T – cells by the mechanism of antibody dependent cell mediated cytotoxicity (ADCC). Natutral killer (NK) cells also kill the target virus infected cells while the cytotoxic T cells release gamma interferon, making the surrounding cells resistant to viral spread (Sharma, 1997). Protection due to cell mediated immune (CMI) response following vaccination in the presence of low levels of antibodies or in their absence has been reported (Allan and Gough, 1976; Sharma, 1997). The role of T – cell populations in immunity against ND has been demonstrated in a study involving cyclophosamide and cyclosporine A (Russel *et al.*, 1997). Expansion of cytotoxic T cells with CD8 surface protein ($CD8^+$ T cells) relative

to T helper cells or effector cells ($CD4^+$ T cells) in treated chickens with clearance of NDV was observed following vaccination, indicating that the $CD8^+$ cells may play a protective role against ND infection. The significance of CMI in protection against ND was shown when *in ovo* bursectomized chickens with depleted B – cell response, vaccinated against ND and challenged with virulent ND did not develop disease (Marino and Hanson, 1987). This is due to cell-mediated immune response, which together with humoral immunity plays a role in acquired immunity to ND (Agrawal and Reynolds, 1990).

Antibodies can be detected in chicks that survive NDV infection within 6 – 10 days, depending on the infecting strain but the titres generally peak about 3 – 4 weeks post infection (Alexander, 1997). The antibodies are directed against the HN and F viral glycoproteins. Commonly used vaccines such as Hitchner B1 and La Sota induce high levels of immunoglobulin (Ig) A, IgY and IgM antibodies (Russel and Koch, 1993; Seal *et al.*, 2000b). They remain detectable up to 1 year in birds recovered from mesogenic virus strain infection or a series of immunizations. The antibody titre of log mean $2^{5.2}$ has been reported to provide 100% protection against challenge (Allan *et al.*, 1978)

2.3.2. Local and passive immunities

Antibodies are detected in the upper respiratory and intestinal tracts about the time humoral antibodies are first detected in NDV infections. The immunoglobulins produced in the respiratory tract appear to be predominantly IgA with some IgG. Similar secretions occur in the Harderian glands following ocular inoculation and this gland is the main site

for IgA – antibody formation in chicken (Russel and Koch, 1993; Russel and Ezeifeka, 1995).

The main type of Ig isolated from egg yolk is generally referred to as "IgY"; other Ig classes are present, but only in negligible amounts (Schade *et al.,* 1996). Structurally, IgY is identical to the major Ig found in serum, but it is different from mammalian Ig G. Hens with NDV antibodies pass them to their progeny via the egg yolk. These maternal antibodies confer protective immunity and ought to be taken into account when timing primary vaccination of chicks (Allan *et al.*, 1978).

2.3.3. Immunosuppression

Under field conditions, immunosuppression may occur due to infection with other viruses such as infectious bursal disease (IBD) virus. The subsequent immunodeficiency may result in a more severe disease caused by some NDV strains and a failure to respond adequately to vaccination (Rosenberger and Gelb, 1978). Immunosuppression from chicken infectious anemia virus (CIA) also has been implicated in the failure of chickens to respond well to secondary inactivated NDV vaccine (Box *et al.*, 1988). Other sources of immunosuppression for the village chickens other than IBD and CIA may include stress, mineral deficiencies, malnutrition, aflatoxicosis moulting, environmental factors such as too much heat or cold and reproduction cycles (Box *et al.*, 1988). It is not clear what role immunosuppression may play in the persistence of virulent virus in immune birds or in the emergence of avirulent or virulent NDV mutants.

There are few reports on specific effects of different drugs on immune response against ND. Such substances include; sulphadimetoxine and ormetoprim. After applying the live vaccines, both drugs inhibit the immune response (Derieux, 1977). Polychlorinated biphenols at the dose of 20 ppm reduce the hatchability and chick growth rate and the weight of the bursa of Fabricius (Ringer and Polin, 1977; Harris *et al.*, 1976). The direct effect of various pesticides on the immune response of chickens to ND has not been investigated completely. However, it is known that polycyclic organochlorides in the amount of 100 ppm decrease the concentration of IgG in chicken blood serum (Subba and Glick, 1977). Investigations of Tetrametiltiuram disulfide effects on haemagglutination inhibition ND antibodies indicate a decrease in the concentration of IgG in serum and induction of the specific clinical signs in chicks. Treatment with glucocorticoids such as dexamethasone increases virus yield. Glucocorticoids also decrease interferon (IFN) production (Gessani *et al.*, 1988). Further studies on parenteral administration of adrenocorticotropin (ACTH) to birds led to an increased level of glucose in the serum; involution of lymphatic organs and a simultaneous change of the lymphocyte distribution in peripheral lymphatic tissues, including the spleen (Piquer *et al.*, 1995; Puvadolpirod and Thaxton, 2000a, b).

Dexamethasone is a synthetic glucocorticoid known to induce cell – mediated immunosuppression and lower resistance to infection in various animal species, including the chicken (Corrier and Deloach, 1990; Isobe and Lillehoj, 1992; Huff *et al.*, 1998). Glucocorticoids may decrease the amount of specific mRNAs by post – transcriptional regulation, as shown by dexamethasone inhibition of accumulation of granulocyte –

macrophage colony – stimulating factor mRNA in murine macrophages (Thorens *et al.*, 1987). Dexamethasone has been shown to inhibit interferon (IFN) production thus, increasing the virus yield (Gessani *et al.*, 1988).

2.4.Clinical signs, morbidity and mortality of Newcastle disease

2.4.1. Newcastle disease in chickens

Initially NDV replicates in the mucosal epithelium of the upper respiratory and intestinal tracts. Shortly after infection, virus spreads via the blood to the spleen and bone marrow, producing a secondary viraemia, which leads to infection of lung, intestine, and central nervous system (CNS). Respiratory distress and dyspnoea result from congestion of the lungs and damage to the respiratory centre in the brain (Alexander, 2003).

Highly virulent viruses may produce peracute infections to fully susceptible chickens, with sudden appearance of the disease and high mortality without any other clinical signs. In VVNDV infection, clinical signs often begin with listlessness, respiratory distress, and weakness ending with prostration and sudden death. Other typical signs may be depression, diarrhoea, circulatory disturbances and impairment of the central nervous system leading to lameness and ataxia, inappetance, somnolence, coughing, dyspnoea, and oedema around the eyes and head. Greenish diarrhoea is frequently seen in birds that do not die early in the infection, and prior to death, muscular tremors, torticollis, paralysis of the legs and wings and opisthotonos may be apparent. Morbidity is usually high and mortality varies 0-100% (Alexander, 2001b). The neurotropic velogenic ND, mainly reported in the U.S. in chickens is marked by sudden onset of severe respiratory disease,

followed a day or later by neurological signs but without diarrhoea. Morbidity may reach 100% but mortality is lower, upto 50% in adults and 90% in young birds (Alexander, 2003).

Mesogenic NDV strains usually cause severe respiratory disease followed by nervous signs, with 50% mortality or more, particularly in very young susceptible birds. Exacerbating conditions may dramatically affect mortality (Alexander, 2003). Lentogenic strains usually cause no disease in adults though young susceptible birds may develop serious respiratory problems resulting in death in case of complicating infection. Poor management and the presence of other organisms may produce disease comparable to that seen with virulent strains (Alexander, 2001b).

2.4.2. Newcastle disease in ducks

Ducks may be infected with NDV and show few or no clinical signs even with strains lethal to chickens but are capable of spreading the virus (Spradbrow, 2000; Alexander, 2001a). Nishizawa *et al.* (2006 and 2007) who worked on commercial breeds of ducks, mainly pekin, found that they did not show any signs of ND. Otim *et al.* (2006) also reported similar findings. However, Roy *et al.* (1992) reported that in unvaccinated commercial duck farm, the mortality due to ND was 10% and the affected birds showed anorexia and greenish - white diarrhoea. Thus, it seems that there are other factors, which play a role in the pathogenesis of Newcastle disease in ducks and the release of the virus to the environment.

Other domestic birds, including ducks, turkeys, doves, pigeons, geese, guinea fowls and ostriches have been found to harbour and shed the virus (Higgins, 1971; Hiroki *et al.*, 1998) and therefore act as a source of NDV to susceptible chickens. Over 3% of cloacal and oral swabs taken from healthy ducks in Hongkong yielded NDV (Higgins and Shortridge, 1988). Although disease has been reported in ducks and geese Awan *et al.* (1994) reported that ducks and geese were more resistant to ND infection.

2.5. Pathology caused by Newcastle disease virus

There are no pathognomonic lesions associated with any form of Newcastle disease. Gross lesions may also be absent. Nevertheless, the presence of hemorrhagic lesions in infected chickens has been used to distinguish VVNDV from NVNDV, a distinction important for the diagnosis of ND in the United States (Hanson, 1988). These lesions are often particularly prominent in the mucosa of the proventriculus, caecum, and small intestine. They are markedly hemorrhagic and appear to result from necrosis of the intestinal wall or lymphoid tissues such as caecal tonsils and Peyer's patches (Alexander, 2003). The histopathology of NDV infections is as varied as the clinical signs and gross lesions (Alexander, 2003). The pathology in NDV carrier birds (chickens and ducks) is not documented.

2.6. Diagnosis of Newcastle disease

2.6.1. Conventional diagnosis of Newcastle disease

2.6.1.1. Newcastle disease virus isolation

Newcastle disease virus is readily cultivated in 10 to 12 day – old specific pathogen free (SPF) embryonated eggs, inoculated into the allantoic sac. Although virulent ND viruses can be propagated in cell cultures, embryonated chicken egg are more preferred since they are more sensitive and convenient (Alexander, 2003). Isolation can be made from tracheal and cloacal swabs, faeces, bone marrow and spleen. The samples are normally transported on ice or frozen (Alexander, 1988a). Bone marrow may be a useful sample for virulent viruses as the viruses have been demonstrated to be present after several days at 30^0C (Omojola and Hanson, 1986). Many strains of NDV inoculated in embryonated chicken eggs will kill the embryos in 24 – 72 hours, causing haemorrhagic lesions and encephalitis. The infected allantoic fluids will agglutinate chicken red blood cells (RBCs). Most NDV strains will multiply, produce haemagglutinins, haemadsorb and cause cytopathic changes in a wide range of secondary cultures including those of rabbit, pig, calf, monkey kidney, chicken tissues and HeLa cells (Alexander, 1997).

2.6.1.2. Serology

Numerous serological tests may be used to detect antibodies, but the most commonly used one is the hemagglutination – inhibition test (Alexander, 2003). The OIE states that a titre may be regarded as positive if there is inhibition at a serum dilution of 2^4 or more against 4 HA units, or 2^3 or more against 8 HA units (OIE, 2000). Positive serology and clinical signs in unvaccinated birds are strong diagnostic evidence of ND especially in

situations where virus isolation is not possible. For the use of HI and other tests in measuring immune status of vaccinated birds, mean levels of HI titres ranging from 2^4 – 2^6 after a single live vaccine to 2^9 – 2^{11} with multiple programs are expected (Alexander, 2001a).

Other tests used to detect antibodies to NDV in poultry sera include: single radial immunodiffusion (Chu *et al.*, 1982), single radial haemolysis (Hari, 1986), agar gel precipitation (Gelb and Cianci, 1987), virus neutralisation (VN), using chick embryos (Beard, 1980), enzyme – linked immunosorbent assay (ELISA) (Snyder *et al.*, 1984), passive hemagglutination test (PHA) (Roy and Venugopalan, 2000) and plaque neutralization (Beard and Hanson, 1984). Enzyme – linked immunosorbent assay (ELISA), which can be automated, has become popular, especially as part of flock screening procedures (Snyder *et al.*, 1984). Good correlation has been reported between ELISA and HI tests (Cvelic – Cabrilo *et al.*, 1992).

In passive hemagglutination test (PHA), once the quantified virus is tagged to the 1% fixed chicken red blood cells, the cells can be stored at 4^0C for a longer period and a large number of samples can be tested for the antibodies, thus minimizing any variation in results and rendering the test quick and easy. Results could be obtained by the PHA test in 40 minutes. Thus, the PHA test is an easily adoptable test for serological monitoring for NDV in commercial flocks (Roy and Venugopalan, 2000).

The latex agglutination test (LAT) involves sensitising latex particles with globulins and then using them for antigen detection. Positive diagnosis of the samples was first based on the agglutination of chicken erythrocytes and inhibition of the agglutination by specific antisera. The tissue samples were then coded before testing by LAT and assayed at least twice. One drop of 0.6% of the coated beads was mixed with one drop of the clarified supernatant of the suspected material on a glass slide. Positive and negative controls were included in the test. Agglutination of the beads indicated the positivity of the sample. The LAT is easy to carry out and the results are available within a few minutes. This test has been successfully applied for the detection of other antigens, e.g. rotavirus (Hughes *et al.*, 1984), rinderpest virus (Bansal *et al.*, 1988); infectious bursal disease virus (Birnavirus) (Nakamura *et al.*, 1993) and NDV (Thirumurugan *et al.*, 1997).

2.6.1.3. Rapid detection test

Since laboratory services for NDV are not always available in rural areas, a sensitive, simple, inexpensive and specific field test for rapid and accurate diagnosis is necessary for immediate control measures to combat the disease and avoid further dissemination.

In recent years, the enzyme-linked immunosorbent assay (ELISA) has gained widespread application for rapid viral diagnosis of both antigens and antibodies (Yolken, 1982). The high sensitivity of the technique made this type of assay very attractive. Numerous enzyme- linked immunoassays for detecting ND antibodies in serum have been reported (Snyder *et al.*, 1983; Russel *et al.*, 1983). In addition, a new ELISA- modified assay-

ImmunoComb-that permits the visual detection of ND antibodies in tracheal mucus as well as in whole blood has also been developed (Rivetz *et al.*, 1985).

Snyder *et al.* (1983) developed an ELISA in which a single serum dilution was used for determining antibody titer, based on a linear relationship between the log of absorbance and the titers determined by standard serial dilution method. A similar approach was adopted in the ImmunoComb kit. However, none of the ELISAs developed for ND described were used for detecting local immunity. Nonspecific binding of avian immunoglobulins as described by Slaght *et al.* (1979), attributed to their high affinity for plastics, was found to be negligible in the ImmunoComb test in both soluble and insoluble color-reaction systems.

The use of the ImmunoComb makes the test versatile, and its evaluation visually is accurate enough for practical purposes, so the test can be performed without a special laboratory. However, it can be easily used for photometric reading. Recently, as a further development of the ImmunoComb concept for field use, the standard curve was applied on the upper part of the comb. The standard curve spots develop color simultaneously with sample spots. All the reactions are carried out in a developing dish constructed from compartments for each step of test. The sealed dish contains the test reagents in a ready-for-use form. The coated ImmunoComb and the processing reagents can be stored at 4^0 C for at least 6 months. These properties endow the ImmunoComb kit with field applicability (Rivetz *et al.*, 1985).

2.6.1.4. Direct detection of Newcastle disease virus antigen – Immunoperoxidase histochemistry

Immunofluorescence and immunoperoxidase techniques applied to thin tissue sections or impression smears demonstrate the presence of NDV in tissues (King, 1999). Immuno histochemistry is a potential alternative to virus isolation or serology (Lockaby *et al.*, 1993). This technique offers a rapid means of identifying viral antigens (Brown *et al.*, 1999b). Immunohistochemical examinations of viral antigens can be carried out on frozen and formalin - fixed paraffin – embedded tissue sections (Jonsson and Engstrom, 1986). A major limitation to immunoperoxidase labelling is potential degradation of antigenic sites by formalin fixation on tissue processing (Elias, 1982; Polak and Van Noorden, 1987). These effects become critical when dealing with monoclonal antibodies, as the loss of a single epitope can prevent binding of primary antibody and subsequent staining (Polak and Van Noorden, 1987).

2.6.2. Molecular based techniques in the diagnosis of Newcastle disease

Molecular techniques like polymerase chain reaction (PCR) and sequencing have led to analysis of the various NDV proteins, and sequence data have been used in phylogenetic analyses of the virus. The F protein has been shown to host virulence markers and considerable genetic diversity has been detected, with viruses sharing temporal, geographical, antigenic, or epidemiological parameters (Aldous and Alexander, 2001). Newcastle disease viruses have been classified into specific lineages and this has helped in understanding the epidemiology of ND by tracing outbreak origins and assessing the degree of relatedness among the various NDV isolates (Alexander *et al.*, 1999; Herczeg

et al., 1999; Abolnik *et al.*, 2004). Strain comparison by restriction site analysis has also proved to be a useful tool for grouping NDV strains into distinct categories in which strains share epizootiological relationships or possibly common descent (Ballagi – Pordany *et al.*, 1996; Wehmann *et al.*, 1997).

2.6.2.1. Reverse transcriptase – polymerase chain reaction (RT - PCR)

Reverse transcriptase – polymerase chain reaction has been used to amplify a fragment of the cleavage site of F protein gene and to characterize the resulting DNA fragment by direct sequencing (Kant *et al.*, 1997; Yang *et al.*, 1997; Lomniczi *et al.*, 1998) or by restriction site analysis (Ballagi –Porday *et al.*, 1996). Marin *et al.* (1996) used RT – PCR, sequencing and conventional methods to identify 9 field NDV isolates from the USA. Although both methods could identify the isolates as B1 type, RT –PCR and sequencing detected minor genetic heterogeneity in the F gene of lentogenic field strains.

Kant *et al.* (1997) described RT –PCR that was performed on RNA isolated directly from tissue homogenate. They were able to differentiate 15 NDV reference strains, 11 of which were virulent and 4 non – virulent. They also detected virulent NDV in samples of seven flocks and non – virulent NDV in two out of three flocks. This was in agreement with conventional methods. They used two oligonucleotide primers representing the sequence of either virulent or non – virulent strains at the cleavage site of the F protein for differentiation. This work concluded that the RT – PCR could be used to confirm ND within 24 hours using RNA isolated directly from tissue homogenate.

Gohm *et al.* (2000) established a RT – PCR with RNA that was directly extracted from tissue samples and faeces, making their method a more rapid technique of ND diagnosis than the isolation of the NDV in embryonated chicken eggs. Conjunctiva, lung, ceacal tonsil and kidney were found to be the most suitable organs for extracting RNA samples. A 182 base – pair region of the F gene including the cleavage activation site was amplified using universal primers. The study demonstrated that NDV could be detected by RT – PCR over a longer period after infection than virus detection using embryonated eggs, suggesting that NDV neutralized by antibodies could be detected by RT – PCR but not by virus isolation. The specificity of RT – PCR was also found to be high. However, to achieve a higher diagnostic sensitivity, they recommended the use of more than one set of primers to exclude false negative results caused by genome variability.

The RT – nested PCR coupled with an ELISA for the detection of Newcastle disease virus was described by Kho *et al.* (2000). This method involves two steps of amplification reaction, sensitivity and specificity determination, and analysis and colorimetric (ELISA) detection of PCR products. Briefly, the method uses two nested pairs of primers, the outer and inner primers that are considered specific to all the different pathotypes of NDV from the consensus F gene sequence. The first amplification reaction and cDNA synthesis are carried out on a standard PCR mixture. The PCR mixture is incubated initially to synthesise the first strand of cDNA and then the incubation temperature is raised in order to inactivate the reverse transcriptase and denature cDNA. The PCR reaction is carried out in the same RT – PCR tube to minimize risk of contamination following cDNA synthesis. In the second PCR amplification,

another volume of reaction mix containing biotinylated and Dig labelled primers is added to the top reaction tube held at 94^0C. The PCR profile is then continued. To determine sensitivity, RNA extracted from a serial (10 – fold) dilution of a reference virus strain is tested simultaneously by both the developed nested RT – PCR and non – nested RT – PCR (using outer primers only). Specificity of the PCR is evaluated with other NDV strains and other infectious avian viruses e.g. infectious bronchitis virus, infectious bursal disease virus, influenza virus, and fowl pox virus (Kho *et al.*,2000).

Although molecular techniques such as analysis of cleavage site are such a valuable tool, studies have shown that neither highly virulent, moderately virulent NDV strains nor low – virulence / avirulent NDV strains can be fully distinguished by cleavage site motifs only. Thus, the virulence of NDV strains still requires use of pathogenicity tests such as mean death time (MDT) and intracerebral pathogenicity index (ICPI) alongside the analysis of cleavage site. Other limitations of molecular techniques of cleavage site analysis are due to the fact that RNA viruses, NDV included, exhibit genome variability and spontaneous random mutations. These factors limit the use of specific PCR detection molecular techniques reviewed above. Any variation or mutation in the region to which the primer is supposed to hybridize would lead to a false negative result. Although attempt to overcome this has been by the use of degenerate primers or alteration of reaction conditions to encourage less specific binding, these inevitably compromise the specificity of the reactions (Kho *et al.*,2000). It is important to note, that, with the application of molecular techniques, nucleotide sequence data of NDV isolates from infected chickens and other avian species worldwide have now been deposited in the

public database. Viral genomic information from these sequences can now be used to aid in further identification of molecular markers as predictors of NDV virulence (Kho *et al.*, 2000).

2.6.2.2. Heteroduplex mobility assay

Berinstein *et al.* (2001) used the GenBank viral genomic information surrounding the F protein cleavage site to develop a heteroduplex mobility assay (HMA) to aid in further identification of molecular markers as predictors of NDV virulence. The method used was modified from a protocol developed for human immunodeficiency virus. In the method modified by Berinstein *et al.* (2001), amplification products obtained from NDV isolates were mixed with equal amounts of NDV B1 or NDV Ulster. The mixture was denatured, chilled on ice and samples separated by electrophoresis, which was completed using a mutation detection enhancement matrix gel. Urea was added to the gel matrix to increase resolution. Gels were then stained in ethidium bromide and photographed over an ultraviolet (UV) transilluminator. The RT- PCR products were then sequenced by dideoxy method, the resulting sequences aligned and phylogenetic analysis done was (Berinstein *et al.*, 2001).

Berinstein *et al.* (2001) used common vaccine strains as a reference and were able to distinguish virulent viruses among NDV isolates that correlated with phylogenetic analysis of nucleotide sequence. They also examined NDV isolates not previously characterized and distinguished vaccine – like viruses from other potentially virulent strains for chickens. Comparing the nucleotide sequences encoding the F protein of NDV

B1 and the velogenic strains of NDV, they found that only 85 % of the strains shared identity. Their conclusion was that ultimately, the HMA could be used for initial screening among a large number of isolates and for rapid identification of potentially virulent NDV; this would improve diagnostics and epidemiology of Newcastle disease.

2.6.3. Pathogenicity tests of Newcastle disease virus

Virulence assessment is necessary for any NDV isolated during any investigation (Aldous and Alexander, 2001). The three *in vivo* tests used to assess the biological properties of NDV isolates are: mean death time (MDT) in eggs, intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) (OIE, 2004).

Mean death time test involves making a series of 10 – fold dilutions of virus in sterile isotonic saline and 0.1 ml of each dilution is inoculated into allantoic cavity of each of at least five 9 to 10 day – old embryonating eggs, from a specific pathogen free (SPF) chicken flock. About 8 hours later, five more eggs are inoculated at each dilution. The eggs are incubated at 37^0C and are candled twice daily (early morning and late afternoon) for 7 days. The MDT is the mean time in hours for the minimum lethal dose to kill the embryos. Hanson and Brandly (1955) placed NDV isolates into three groups based on the MDT: velogenic (<60 hours), mesogenic (60 – 90 hours) and lentogenic (> 90 hours) (OIE, 2004).

The ICPI is determined by inoculating 0.05 ml of a 1: 10 dilution of infective, bacteria – free allantoic fluid in sterile isotonic saline into the brains of each of 24 to 40 hour old

chicks from SPF chicken flock. The birds are observed daily for 8 days and at each observation, scored 0 if normal, 1 if sick, and 2 if dead. The ICPI value is the mean score per bird per observation over the 8-day period (OIE, 2000). The most virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic strains will give values close to 0.0.

The IVPI is determined by inoculating each of the ten 6 – week – old SPF chickens with 0.1 ml of a 1: 10 dilution in sterile isotonic saline, infective, bacteria – free allantoic fluid intravenously. Birds are examined at 24 – hour intervals for 10 days and scored at each observation: 0 if normal, 1 if sick, 2 if paralysed or showing other nervous signs, and 3 if dead (dead individuals must be scored as 3 at each of the remaining daily observations after death). The IVPI is the mean score per bird per observation over the 10 – day period. Lentogenic strains and some mesogenic strains will have IVPI values of 0, whereas the indices for virulent strains will approach 3.0. Some variations have been recommended in these tests. Swabbing of the cloaca and conjunctiva of 8 – week – old chickens with undiluted allantoic fluid has been substituted for the IVPI test (Hanson, 1980). The intention is to distinguish between viscerotropic velogenic and other velogenic viruses. These pathogenicity tests have proved valuable in distinguishing among vaccine, enzootic, and epizootic viruses during outbreaks (Alexander and Parsons, 1986).

Plaque formation by a NDV isolate, including size and morphology in cell culture has been used as an *in vivo* method for characterizing viruses (Hanson, 1975).

2.7. Molecular markers of pathogenicity of Newcastle disease virus

Developments in molecular techniques, especially sequencing have led to a greater understanding of the basis of pathogenicity of NDV (Rott and Klenk, 1988). Molecular techniques which elucidate the F gene cleavage site sequences are now used to determine NDV virulence (OIE, 2000). Virulent pathotypes are characterized by presence of multiple basic amino acids in the cleavage site enabling subtilisin like proteases in host cells to cleave F_0 to F_1 and F_2 proteins, enabling the virus to fuse with the host cell membrane and spread in the infected organ (Nagai, *et al.*, 1976; Rott and Klenk, 1988). The F_0 of lentogenic strains possess two single basic amino acids at positions 113 and 116 of the cleavage site, along with leucine at 117. This can only be cleaved by trypsin – like enzymes, found in a limited number of cell types mainly in the respiratory and digestive tracts (Collin *et al.*, 1996). Sequences of mesogenic strains of intermediate virulence for chicken contain two pairs of basic amino acid residues or a single arginine and a lysine / arginine pair (Collin *et al.*, 1993; 1994; 1996). The F_0 of virulent NDV has two pairs of basic amino acids at the cleavage sites: 112, 113, and 115, 116 along with phenylalanine at 117; this makes their F protein susceptible to cleavage by omnipotent proteases present in most body organs. This also helps the virulent NDV to fuse with a wide range of cells, resulting in a fatal systemic infection (Nanthakumar *et al.*, 2000; Alexander, 2003).

2.8. Newcastle disease control and prevention

The most important factors in preventing the introduction of NDV and its spread during outbreaks are the conditions under which the birds are reared and the degree of

biosecurity practiced at the farm. Although many biosecurity measures may often be regarded as costly, and time consuming by those involved, if such measures are implemented there is no doubt that the introduction of ND viruses to poultry flocks and the spread to the rest of the poultry industry will be dramatically reduced. Such measures are also likely to reduce the spread of other endemic diseases that may affect the birds and reduce their yield, and should be seen as an important investment in the profitability of poultry production (Alexander, 2003). Under industrial production conditions, ND has been successfully controlled through vaccination, biosecurity and various other tailored policies (Higgins and Shortridge, 1988).

2.8.1. Vaccination

Live and inactivated vaccines are currently used in countries that vaccinate against ND (OIE, 2000). The live vaccines have been conveniently divided into lentogenic and mesogenic groups. The lentogenic-derived vaccines include La Sota; F (Asplin); Hitchner B1 and V4 while the mesogenic vaccines include: strain H, Mukteswar, Komarov and Roakin (Alexander, 2003). The preferred mode of administration of lentogenic vaccine is by intranasal instillation, eye drop, beak dipping while mesogenic vaccines require wing – web, or intramuscular injection (Alexander, 2003). Live vaccines can also be administered in drinking water or by spray (aerosol) (Alexander, 2003).

Live vaccines sold as freeze-dried, are relatively cheap, easy to administer and can be used in mass vaccination (Alexander, 2003). Since infection by the live virus stimulates local immunity, protection occurs soon after live vaccine application. The vaccine virus

may also spread from vaccinated to non – vaccinated birds. Live virus – in – oil vaccine also leads to higher antibody response due to the escape of infective live virus from the trapping of the oil environment and replication in different tissues and initiation of immune response (Roy and Venugopalan, 1998). However, some of the disadvantages are that some vaccines may cause mild disease. It is therefore advisable to use extremely mild virus for primary vaccination and as a result, multiple applications of vaccine (s) usually are needed. Being heat – labile, live vaccines are particularly a disadvantage under village management systems where transport and cold storage facilities are often inadequate (Alexander, 2003).

Inactivated vaccines are produced from infective allantoic fluid treated with B-propiolactone or formalin to kill the virus and then mixed with adjuvant, commonly oil – emulsion. Ulster 2C, B1, La Sota, Roakin seed viruses have been used in the production of oil – emulsion vaccines. One or more other antigens such as infectious bronchitis virus, IBDV, egg drop syndrome virus and reovirus can be incorporated into emulsion with NDV making it bivalent or polyvalent. The advantages of inactivated vaccines are the very low level of adverse reactions in vaccinated birds; the ability to use them in situations unsuited for live vaccines, especially if complicating pathogens are present; and the extremely high levels of protective antibodies of long duration that can be achieved (Alexander, 2003). Inactivated vaccines are, however, expensive to produce and apply since they have to be applied to each bird individually by injection.

2.8.2. Newcastle disease control in village indigenous chickens

Much has been written about ND and its control in the commercial poultry sector. Comparatively little literature is available on ND and its control in the indigenous poultry sector although most authors agree that it is a major constraint to village chicken production (Sonaiya, 2000; Spradbrow, 1993). While the basic characteristics of the ND virus encountered in the commercial and family sectors are similar, the production systems used to raise village chickens and the socio – economic status of their owners are different, making ND control in the village indigenous chicken sector a very complex issue. The control of ND in the village indigenous chicken, as in the commercial sector, requires a multifaceted approach (Alexander, 1997).

Australian V4 vaccine, selected for thermostability and immunogenicity, specifically developed for use in village flocks in tropical countries is administered in coated, pelleted feed (Spradbrow, 1993). Its efficacy depends on the feed vehicle used (Nasser *et al.,* 2000; Spradbrow, 1992). It is, however, more efficacious and offers optimal protection when given by eye – drop (Bell, *et al.,* 1995; Foster *et al.,* 1999). Vaccinated chickens with HI antibody titres lower than 2^3 cannot be presumed to be susceptible to challenge, while absolute protection cannot be attained when ND vaccine is used under village conditions, protection rates in excess of 60% are obtainable and acceptable (Spradbrow, 1993). The possibility of natural transmission of V4 vaccine strains from vaccinated to non – vaccinated chicken, in addition to being heat resistant, makes it suitable for use under village condition (Ahlers *et al.*, 1999). However, work done in Tanzania and

elsewhere in Africa on the use of V4 has not indicated much success with feed administration (Foster *et al.*, 1999).

Australian Centre for international Agricultural Development (ACIAR), has also produced strain I_2 that was chosen for antigenicity and thermostability but with similar immunogenic properties of V4. Strain I_2 is produced by allantoic cavity inoculation. The allantoic fluid is then harvested, and either freeze-dried or stored at 4^0C and dispatched in quantities determined by the estimated titre and the number of chickens to be vaccinated. Refrigeration is not required for transport or for short term storage in a village (Bensink and Spadbrow, 1999).

2.8.3. Biosecurity and other tailored measures in control of Newcastle disease

Several studies have highlighted the critical role of biosecurity in disease prevention (Garner and Beckett, 2005; East *et al.*, 2006) and the chicken industries have recognized this by the development of their National Biosecurity Manuals (Grimes and Jackson, 2001; Anonymous, 2003).

Some of the control measures that are recommended especially during Newcastle disease outbreaks includes; isolating all sick chickens; slaughtering chickens that are very ill; avoid transporting of chickens that are ill or dead to other areas that are free of the disease; burying or burning all dead chickens and if, for any reason it is not possible to do this, any part of the chicken that has not been used should be buried or burned, avoid vaccinating chickens that are showing signs of illness; once an ND outbreak has

commenced in a village, vaccination should not be carried out as it is impossible to identify birds that are incubating the disease but not yet showing signs of illness afterall farmers will often associate the vaccine with the death of chickens that are vaccinated in the face of an outbreak, advising farmers to wait for at least one month after the last mortality before re-stocking in addition to contacting the veterinary services officer, extension officer, extension Worker or community livestock worker in their area when they notice any signs of illness (Alders and Spradbrow, 2001).

Other control strategies include; (i)Avoid the introduction of new birds to flocks during the periods of the year when ND occurs more frequently, (ii) Do not return from market with chickens that have failed to sell. Instead, arrange to keep them in another place, (iii) Avoid contact with people, cars and animals that have been in contact with the virus and other parts of infected chickens (e.g. eggs, feathers, etc.). Dogs and cats can also transmit the virus if they have access to chickens killed by ND, (iv) Minimise contact between chickens and other poultry, such as ducks, pigeons, turkeys and guinea fowl, (v) Good housing can reduce disease transmission. An elevated chicken house that is well ventilated allows faeces to fall through to the ground and so minimises contact with various infectious agents (Figure 8). Keep chickens and chicks away from the base of the chicken house where the faeces have accumulated or clean the area regularly. Encourage the use of local remedies to control ectoparasites (e.g. fleas and mites) in the houses when commercial insecticides are not available (and Spradbrow, 2001a),
(vi) House hens with young chicks in a clean, safe chicken house, (vii) Provide some supplementary feed, such as maize bran, ground grains, green leaves, ground sea shells,

insects, insect larvae and worms. Good nutrition will give chickens a better chance of combating infections. Supplementary feeding is especially important for chicks, and a creep feeder can be made from local materials to ensure that chicks are able to receive food without greatly increasing the amount of food given to the household, A creep feeder also provides chicks with shelter from airborne predators, (viii) Always provide water; fresh, clean water is best when available ((Alders and Spradbrow, 2001).

Based on the above literature review, it is clear that ND is a disease of economic importance and the factors leading to the maintenance of the carrier status in free – range indigenous chickens are not well understood and will be investigated in this study.

CHAPTER 3

3.0. Experiment 1: A retrospective study of factors associated with Newcastle disease outbreaks in village indigenous chickens in five agro – ecological zones in Mbeere and Embu districts, Kenya

3.1. Introduction

Seasons have been shown to influence the ND outbreaks in various parts of the world (Sharma *et al.*, 1986; Mukiibi, 1992; Otim *et al.*, 2007). In addition, age and sex have been shown to affect the survival rate following ND outbreaks (Beard and Hanson, 1984; Kutubuddin, 1973; Huchzermeyer, 1993). The status of the influence of age, sex, temperature changes, feed supplementation, confinement altitude and humidity in the Kenyan village chicken on ND prevalence is unknown and needs to be evaluated. In this experiment, the study areas were purposefully chosen in order to capture the diverse climatical changes. Embu and Mbeere districts were selected because Embu district is wet, humid and relatively cold while Mbeere is hot and dry. It was hypothesized that climatic and weather changes have no effect on ND outbreaks.The climatic and ecological study was used to test the effect of climatic changes on disease occurrence in the five ecological zones. This type of study has not been undertaken before. Thus a study was designed to investigate the risk factors that may be associated with outbreaks of ND in free – range indigenous chicken in a village setting with a view to testing the hypothesis stated above.

3.2. Materials and Methods

3.2.1. Study area

Since the study entailed investigation of the various associated factors such as: seasons, variations in temperature and farming practices, with ND outbreak, there was need to chose an area that represents them very well.Two districts, Embu and Mbeere, that were adjacent and that provide the required ecologies were chosen. Embu is cold and Mbeere has a hot climate.

Mbeere district lies between latitudes $0^0$20′and $0^0$50′ South and longitude 37^0 16′ and 37^0 56′ East. It has two rainy seasons with the long rains falling between mid March and June while the short rains are experienced from October to December. The extensive altitudinal range (500 to 1200 metres above sea level) of the district influences the temperature, which ranges from 20^0C to 32^0C. July is usually the coldest month with average monthly minimum temperature of 15^0C. March is the warmest month with average monthly, maximum temperature rising to 30^0C. The rainfall is however not very reliable and it ranges between 640 – 1100mm per year. The dry seasons are between January and early March; and between August and September. The livestock population in Mbeere district is as follows; 50,960, 108,052, 55,052, and 165,090 for cattle, goats, sheep and poultry, respectively (Personal communication, Nairobi Provincial Livestock Officer, 2007). The indigenous chickens were kept on free – range system.

Embu district lies between latitudes $0^0$8′ and $0^0$35′ South and longitudes $37^0$19′ and $37^0$42′ East. Rainfall is bimodal with two distinct rainy seasons. The long rains fall between March to June while the short rains come in October to December. The amount received varies with altitude

but average 1,495mm per year. However, areas above 1,700m, display a different pattern. The pattern changes with altitude to a tri – modal pattern, which has a peak in July and August. The temperature ranges from a minimum of 12^0C in July to a maximum of 27.1^0C in March, with a mean of 20.7^0C. The dry seasons are between January and February; and between July and September. The livestock population in Embu district is as follows; 42,740, 18,606, 9,442, and 83,582 for cattle, goats, sheep and poultry respectively (Personal communication, Nairobi Provincial Livestock Officer, 2007). The indigenous chickens were kept on free – range system.

Five agro-ecological zones (AEZ) were chosen, three of them being in Embu district, namely: Lower highland 1 (LH1), Upper midland 2 (UM2), and Upper midland 3 (UM3) in Manyatta, Runyenjes, and Central divisions respectively. Two AEZ were in Mbeere districts, thus Lower midland 3 (LM3) and Lower midland 5 (LM5), located in Gachoka division (**Table 3.1**). The distribution of these zones is shown in **Figure 3.1** (Jaetzold and Schmidt, 1983).

Table 3.1. Description of the five agro – ecological zones in respect to location

Agro – ecological zones	
Lower highland 1 (LH1)	
Upper midland 2 (UM2)	
Upper midland 3 (UM3)	
Lower midland 3 (LM3)	Gachoka division
Lower midland 5 (LM5)	Gachoka division

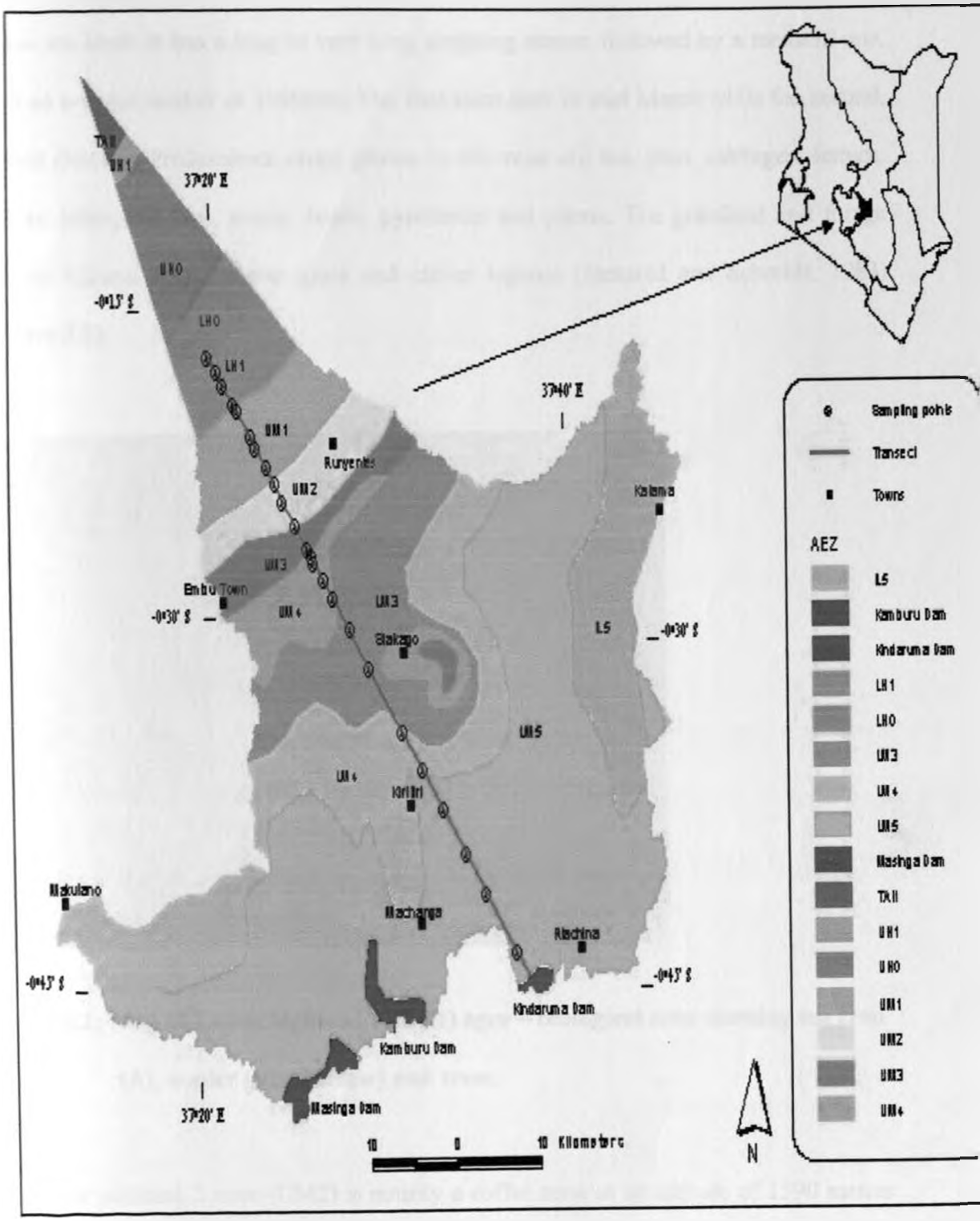

Figure 3.1: Map of the Embu-Mbeere agro-ecological zones (Source: Jaetzold and Schmidt, 1983)

The lower highland zone 1 (LH1) is a tea – dairy cattle zone at an altitude of 2070 metres above sea level. It has a long to very long cropping season followed by a medium one, with an average rainfall of 1080mm. The first rains start in mid March while the second, in mid October. Predominant crops grown in this zone are tea, peas, cabbages, lettuce, carrots, kales, potatoes, maize, beans, pyrethrum and plums. The grassland and forage include Kikuyu grass, napier grass and clover legume (Jaetzold and Schmidt, 1983) (**Figure 3.2**).

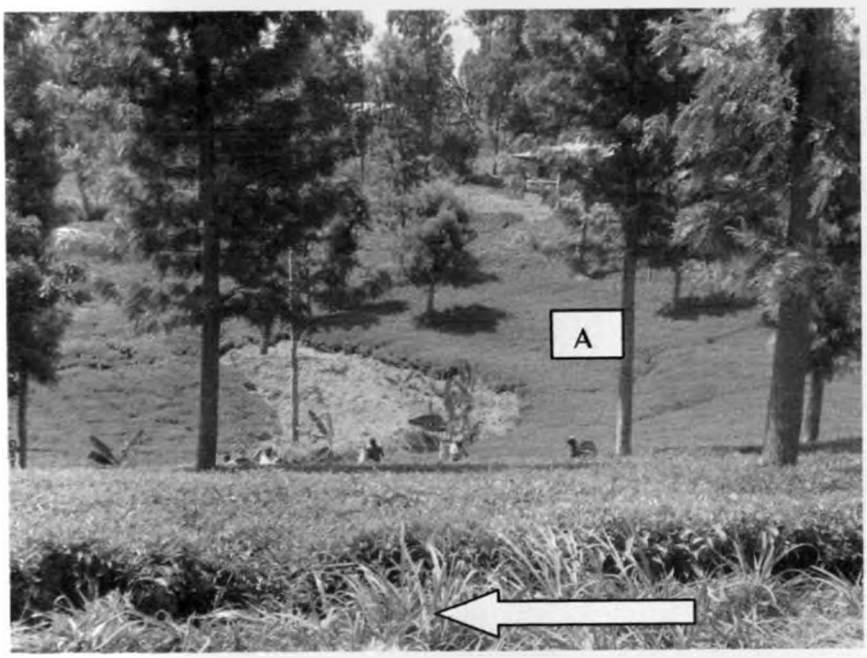

Figure 3.2: Map of Lower highland 1 (LH1) agro – ecological zone showing tea crop (A), napier grass (arrow) and trees.

The upper midland 2 zone (UM2) is mainly a coffee zone at an altitude of 1590 metres above sea level. It has a short - to - medium cropping season and an average rainfall of 615mm per year. The first rains start in mid March while the second, mid October. The

following crops are grown: coffee, sweet potatoes, sunflower, beans, cabbages, kales, tomatoes, onions, Meru foxtail millet, sorghum, bananas, cassava, maize and sugar cane. The grassland and forage include: star grass, napier and Bana grass (Jaetzold and Schmidt, 1983).

The upper midland 3 zone (UM3) is a marginal coffee zone at an altitude of 1280 metres above sea level. It has a medium to short cropping season with an average rainfall of 460mm per year. The first rains start at end of March while the second, mid October. The crops grown include: Katumani maize, sorghum, sunflower, onions, cabbages, Meru foxtail millet, pineapples, bananas, pawpaws, avocadoes, citrus, cassava and coffee. The grassland and forage include: zebra grass, napier and bana grass (Jaetzold and Schmidt, 1983).

In Mbeere district, the lower midland 3 zone (LM3), which is also referred to as a cotton zone, is at an altitude of 1070 metres above sea level; has an average rainfall of 300mm per year. It has two short cropping seasons. The first rains start at end of March and the second, mid of October. The following crops are grown: cotton, sorghum, Katumani maize, millet, green grams, cowpeas, pigeon peas, dwarf sunflower, sisal, cassava, pineapples, mangoes, macadamia nuts, tobacco, and sweet potatoes. The following vegetation and forage is predominant in this zone: high grass savanna with zebra grass, bana grass, Siratro and horse tamarind (Jaetzold and Schmidt, 1983).

The lower midland 5 zone (LM5), also refered to as livestock – millet zone, covers the central belt of Mbeere district extending to Mwea plains on the South West, at an altitude of 760 metres above sea level; has an average rainfall of 180mm per year. It has two short cropping seasons. The first rains start at end of March while the second, end of October. The following crops are grown: bulrush and foxtail millet, green grams, cowpeas, chickpeas, bambarra groundnuts, dwarf sunflower, moth beans and maize. The vegetation in this zone includes mixed short grass savannah with buffel grass, horsetail grass and saltbush (Jaetzold and Schmidt, 1983) (**Figure 3.3**).

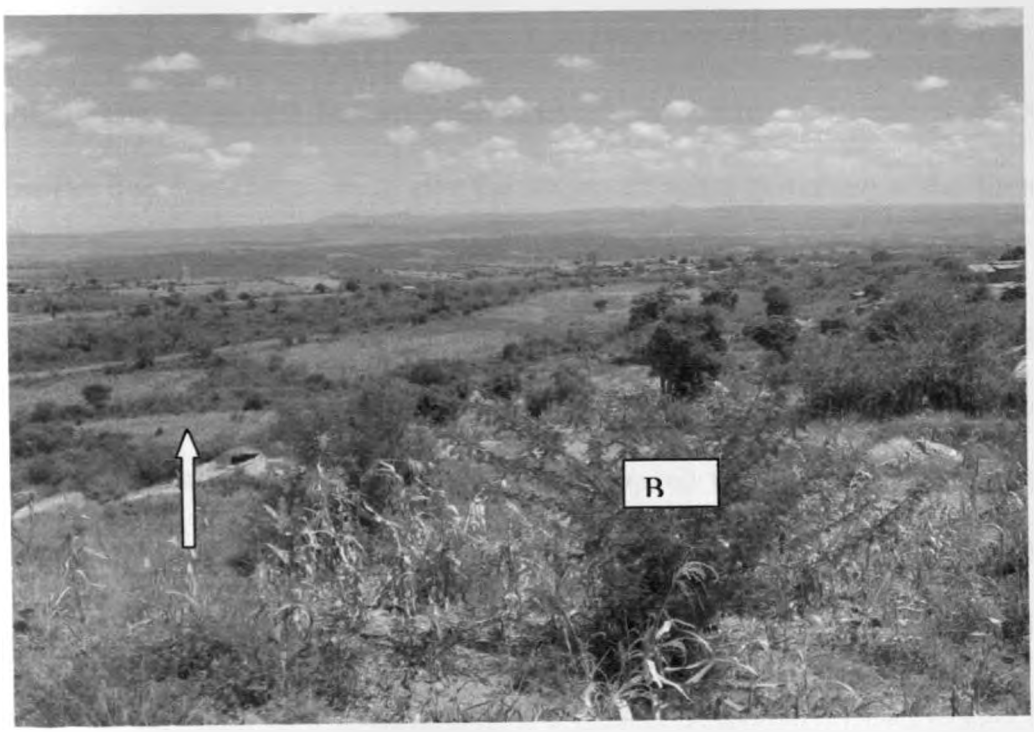

Figure 3.3: Map of Lower midland 5 agro – ecological zone demonstrating mixed short grass savanna (arrow) and saltbushes (B)

3.2.2. Experimental design

Seventy five farmers were randomly selected, 15 from each agro - ecological zone described above. The households' inclusion criterion for the study was: possession of a flock size of at least five chickens with no history of flock vaccination. Open and closed questions and structured interviews were used to collect data. The researcher visited each household and asked the farmer all the questions as they appeared in the questionnaire (**Appendix 1**). The answers were carefully recorded before proceeding to the next household.

During interviews, the farmers were assessed on whether they understood Newcastle disease; they were requested to give the vernacular name (*Kivuruto*) of the disease and to describe the respective signs of the disease. The data collected included: farmers' perception on the occurrence Newcastle disease; household set - up; poultry management systems used; types of poultry kept by the household and major constraints to poultry production. The farmers were also asked to respond to questions on factors that were thought to be linked with ND outbreaks. These were: (a) confinement of birds, (b) seasons, (c) lack of feed supplementation, (d) age susceptibility, (e) survival rate, (f) management and handling of sick birds, (g) disposal of poultry faecal matter, (h) transportation of birds, (i) temperatures, (j) new birds introduced to the flock, (k) other domestic birds in the flock, and (l) vegetation including flowering crops. The farmers were also asked to name other major diseases that caused chicken losses.

3.2.3. Statistical analysis

Data was cleared and then analyzed using the analysis of variance procedures and chi – square test (χ^2) of the statistical analysis systems (SAS Institute Inc., Cary, NC, USA, 2002 -2003).

3.3. Results

3.3.1. Farmers perception of Newcastle disease

All the farmers interviewed showed that they understood Newcastle disease by describing it with the right vernacular name '*Kivuruto*'. They also described the following clinical signs, which are usually attributed to the disease very well, namely: depression, anorexia, greenish diarrhea, coughing, sleepiness, high mortality and morbidity. In their description, the farmers indicated that the disease killed all the birds in the village once it occurred and that it caused the birds to sleep and die quickly.

3.3.2. Major poultry diseases

The major diseases documented by the farmers in village indigenous chicken were Newcastle disease, worm infestation, and fowl pox. Newcastle disease was ranked highest in all the agro-ecological zones as follows: LH1 (50%), UM2 (61.1%), UM3 (80%), LM3 (71.5%) and LM5 (93.8%) (**Figure 3.4**).

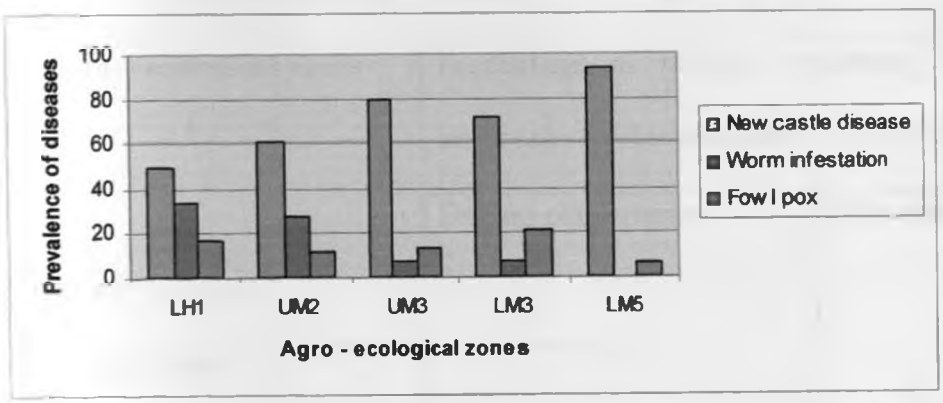

Figure 3.4: Prevalence of major diseases reported in village indigenous chickens in different agro - ecological zones

3.3.3. Factors associated with Newcastle disease occurrence in village indigenous chickens

3.3.3.1. Influence of confining chicken on Newcastle disease outbreaks

Ninety-five (95%) percent of all the households confined their chickens at some particular period of the year, mainly during the rainy seasons (April to June and October to December). Newcastle disease outbreaks were common in all the agro - ecological zones during confinement (66.7%, 73.3%, 80%, 66.7%, and 7.7% for and LH1, UM2, UM3, LM3, and LM5, respectively) than other periods, except in the Lower Midland 5 where it occurred in unconfined period as shown in **Table 3.2**. The difference in occurrence of ND with and without confinement and at different confinement times was statistically significant ($P<0.05$).

Table 3.2: Percentage of farmers reporting Newcastle disease outbreaks in five agro - ecological zones during chicken confinement

Agro – ecological zones	Percentage of farmers reporting Newcastle disease outbreaks in chickens with or without confinement	
	During confinement	Not confined
Lower highland 1	66.7	33.3
Upper midland 2	73.3	26.7
Upper midland 3	80	20
Lower midland 3	66.7	33.3
Lower midland 5	7.7	92.3

3.3.3.2. Influence of seasonal variation on the occurrence of Newcastle disease

Newcastle disease outbreaks were commonest during the wet than the dry season in all agro – ecological zones with exception of LM 5 and LM3 where it was commonest in the dry season. In the cooler zones, LH1, UM2 and UM3, ND outbreaks occurred during the rainy season, and very few cases during the dry season. The warmer (LM3 and LM5) zones had most cases in the dry season and just before the rains. There were 4 peaks, with respect to ND outbreaks as follows: late in the rainy season, occurring in UM2 only; middle of the rainy season, recorded in UM3 and LH1; just at the start of the rain, in LM3; and the middle of the dry season, in LM5 **(Figure 3.5)**. The difference in percentage occurrence in various agro - ecological zones was statistically significant ($P<0.05$).

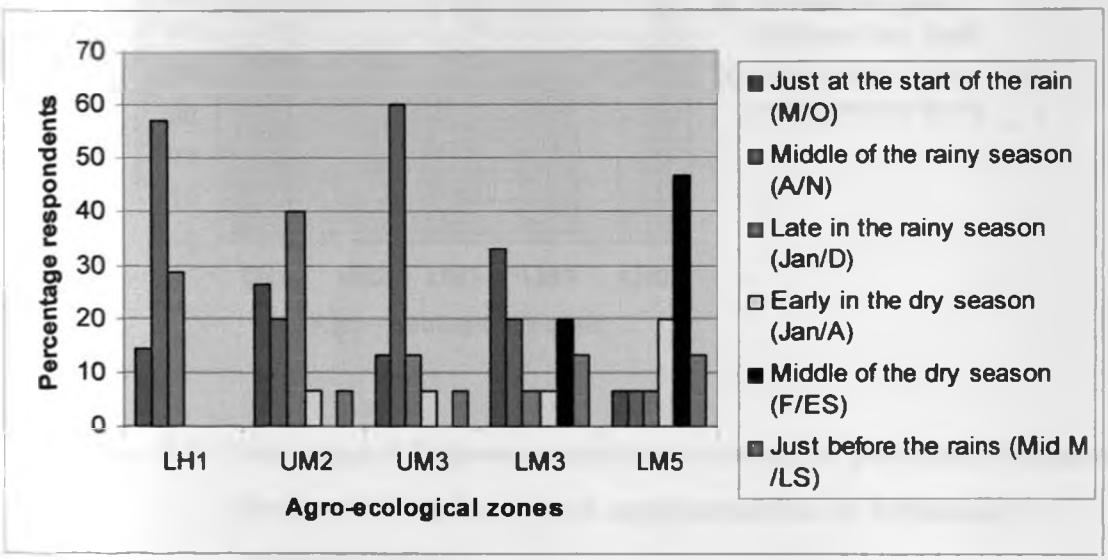

Figure 3.5: Percentage of farmers indicating the seasonal (wet and dry) occurrence of Newcastle disease in various agro - ecological zones

Legends:

M/O: March and October; **A/N:** April and November; **Jan/D:** January and December; **Jan/A:** January and August; **F/ES:** February and early September; **Mid M/LS:** Mid March and late September

3.3.3.3. Effect of feed supplementation on Newcastle disease outbreaks

Newcastle disease was reported to be more prevalent in chicken flocks that were not provided with supplementary feeding [UM3 (63.6%), LM5 (80%), and LH1 (53.3 %)] as compared to the ones that were supplemented [UM3 (36.4%), LM5 (20%), and LH1 (46.7%)], with the exception of zones UM2 and LM3, where there were no differences (**Figure 3.6**). The difference in the percentage of farmers who were supplementing their chickens and those that were not was not statistically significant, across agro – ecological zones ($P>0.05$).

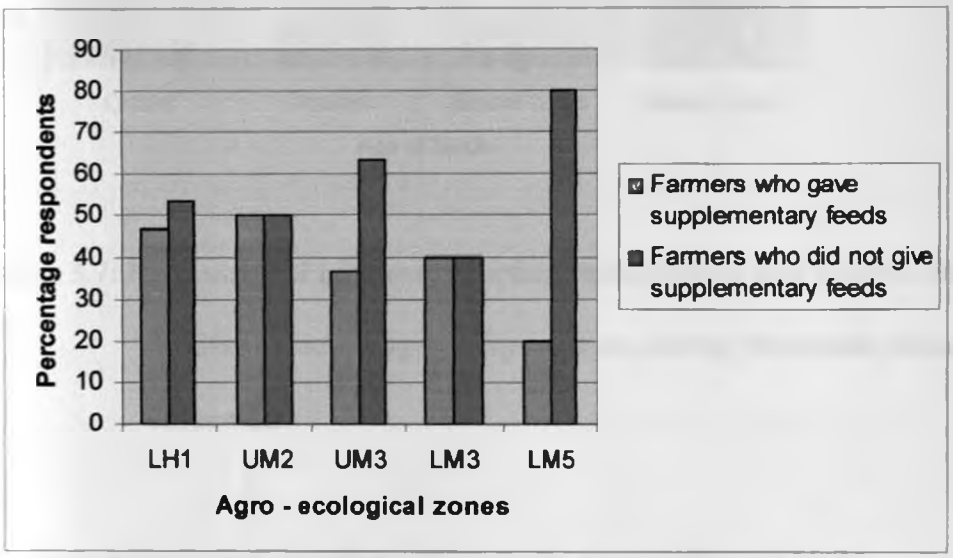

Figure 3.6: Percentage of farmers reporting occurrence of Newcastle disease in flocks with or without feed supplementation in various agro – ecological zones

3.3.3.4. Age influence and survival rate during Newcastle disease outbreaks

Chicks were reported to be the most susceptible age group (58.2%), followed by hens (19.8%). However, following ND outbreak, hens had the highest survival rate

(47.6%), followed by cocks (30.2%), growers (15.9%), and chicks (6.3%), respectively (**Figure 3.7**). Age seems to affect the survival rate and susceptibility of village chickens to ND ($P<0.05$).

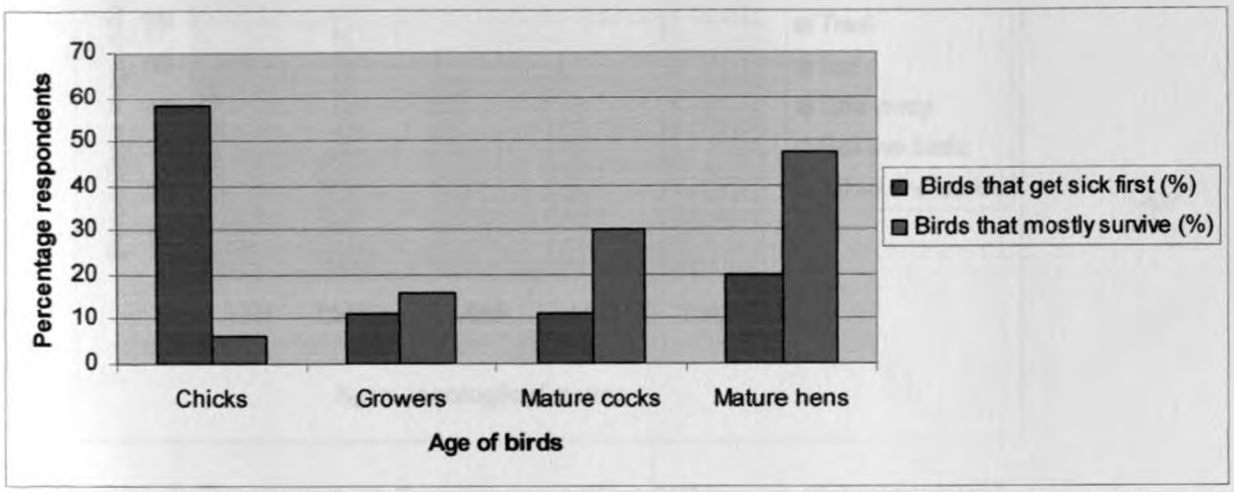

Figure 3.7: Percentage of farmers reporting susceptibility and survival rate of different chicken age groups and sex during Newcastle disease outbreaks

3.3.3.5. Management and handling of Newcastle disease infected birds

Farmers in all the agro - ecological zones, reported that some of the chickens that manifested clinical signs of Newcastle disease were consumed at home. Some farmers attempted treating birds using both conventional drugs and herbs, trying to salvage them. Other farmers in the LH1 (30%) and UM2 (7.1%) gave away the sick birds to relatives and neighbors. A few farmers (13.3%) in LM5 sold their sick live birds, while 7.1% of the farmers in LM3 disposed their sick birds through slaughter and burial. In UM2 most

farmers ate the sick birds (**Figure 3.8**). The mode of management and handling of infected chickens differed across agro – ecological zones ($P<0.05$).

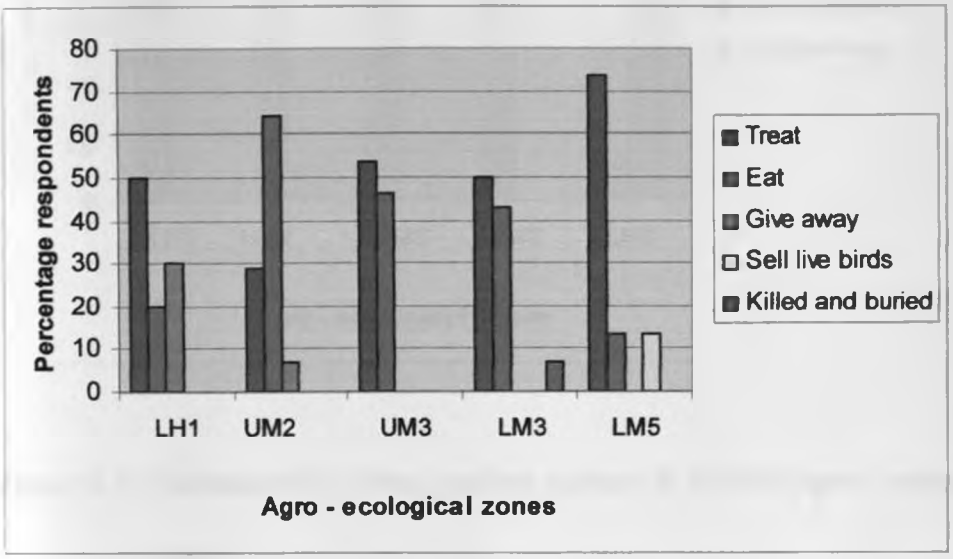

Figure 3.8: Percentage of farmers reporting different management and handling methods of Newcastle disease infected birds in various agro- ecological zones

3.3.3.6. Disposal of poultry fecal matter in different agro –ecological zones

The frequency of cleaning of the poultry houses per week across the agro - ecological zones were as follows: 35.4%, 20.0%, 9.2%, 26.1% and 9.3% for once, twice, thrice, more than 4 times and not cleaned regularly, respectively. The farmers in UM3, LM3, and LH1 zones exclusively used the poultry manure in their farms as fertilizers. Most of the farmers (80%) in LM5 were throwing the manure away as rubbish while a few farmers were using it as fertilizer in the farms. In UM2, most farmers used the manure in their farms and a few threw it away (**Figure 3. 9**). The disposal of poultry fecal matter was different between agro – climatic zones ($P<0.05$).

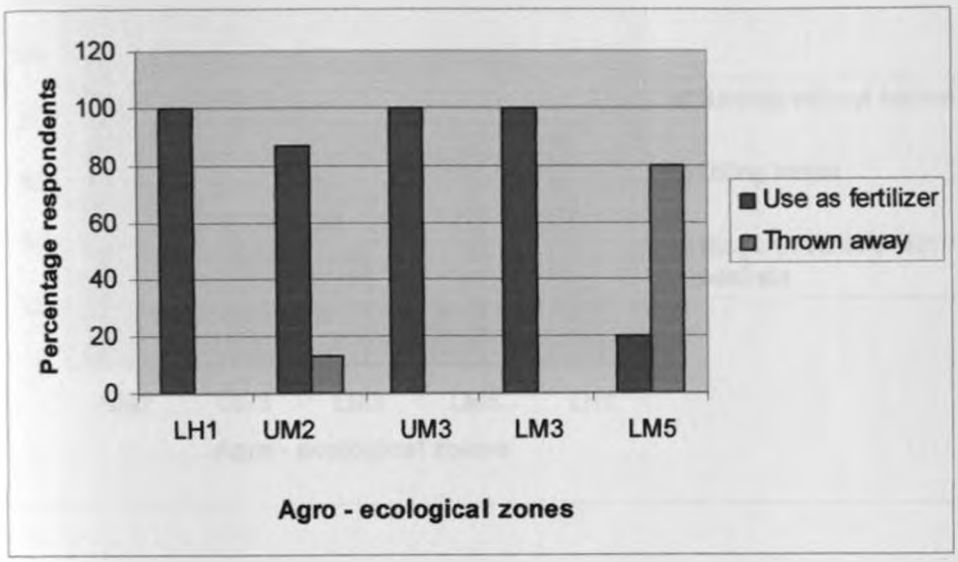

Figure 3.9: Methods of handling poultry manure in the five agro – ecological zones

3.3.3.7. Methods of transportation of birds to markets

The mode of transportation of birds to the market was also assessed since this may influence disease transmission. It was observed that varying percentage of farmers in the UM2 (100%), LM3 (60%) and LH1 (75%) zones transported their birds to the market, using bicycles without baskets. Majority of the farmers in the LM5 carried chickens by hands to transport their birds while in UM3 bicycles or vehicles, and hands were used equally (**Figure 3.10**).

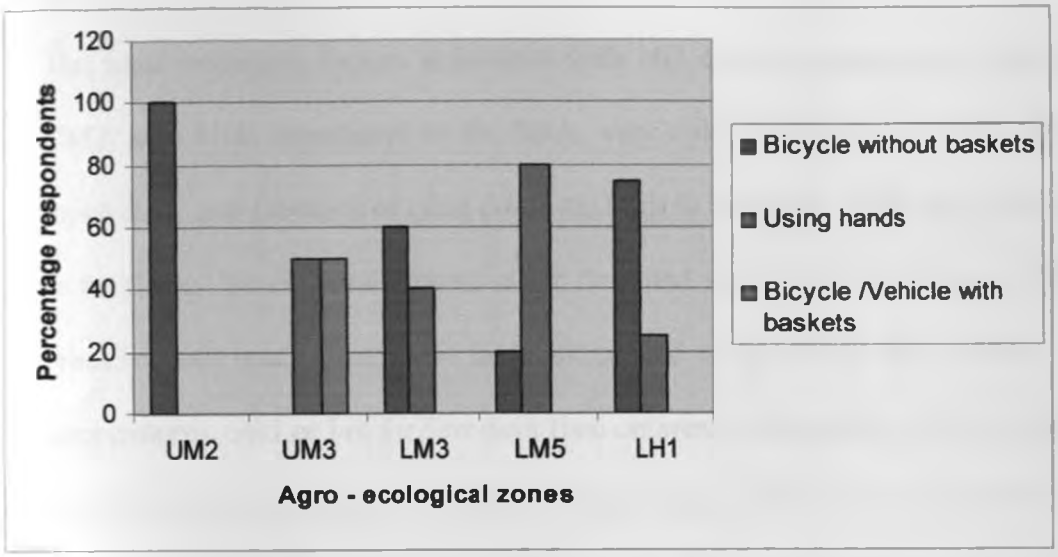

Figure 3.10: Methods of transportation of birds to the market in different agro – ecological zones

3.3.4. Ecological and climatic factors associated with Newcastle disease outbreaks

Various other factors were reported to be associated with ND outbreaks in different agro - ecological zones. Those associated in zone UM3 were strong wind and very cold temperatures: in LM3 and LH1were: strong wind without much dust: whereas very cold temperatures were associated with disease in UM2 and LH1. In the LM5, strong wind without much dust and very hot temperatures had equal weight, each accounting for 30.6%. Based on farmers' responses, strong wind without much dust, introduction of new birds to the flock and very cold temperatures were most important factors associated with ND outbreaks (**Table 3.3**).

The most important factors associated with ND outbreaks per zone were as follows: UM2: new birds introduced to the flock; very cold temperatures; strong wind without much dust, and presence of other domestic birds in the flock; UM3: new birds introduced to the flock; , strong wind without much dust, and very cold temperatures; LM3: , strong wind without much dust,; new birds introduced to the flock; dust storms; very cold temperatures, cold or hot for few days then outbreak and presence of kales and greens in the field; LM5: , strong wind without dust storms,; very hot temperatures; new birds introduced to the flock and dust storms and in LH1: new birds introduced to the flock; strong wind without dust storms and very cold temperatures (**Table 3.3**).

Table 3.3: Ecological and climatic factors associated with Newcastle disease outbreaks in different agro – ecological zones

Agro - ecological zones	Other factors observed during Newcastle disease outbreaks (% responses)										
	a	b	c	d	e	f	g	h	i	j	k
LH1	31.3	37.3	18.8	6.3	0.0	0.0	0.0	0.0	0.0	6.3	0.0
UM2	18.5	33.4	22.2	0.0	7.4	11.1	7.4	0.0	0.0	0.0	0.0
UM3	35.3	32.5	17.6	0.0	0.0	8.8	2.9	0.0	0.0	0.0	2.9
LM3	32.1	14.3	10.7	3.6	14.3	0.0	10.7	3.6	10.7	0.0	0.0
LM5	30.6	16.6	0.0	30.6	11.1	2.8	0.0	8.3	0.0	0.0	0.0
Overall total responses	**147.8**	**134.1**	**69.3**	**40.5**	**32.8**	**22.7**	**21.0**	**11.9**	**10.7**	**6.3**	**2.9**

Legend:

a: Strong wind without dust storm; **b:** New birds introduced to the flock from the market; **c:** Very cold temperatures; **d:** Very hot temperatures; **e:** Dust storms; **f:** When there are other domestic birds in the flock; **g:** Kales and greens in the field; **h:** New birds introduced to the flock as gift from friends; **i:** Cold and hot for few days then outbreak; **j:** Flowering of fruit-trees; **k:** Flowering beans

3.4. Discussion

This study has shown that many constraints prevent the development of village indigenous chicken production in Kenya, the major ones being; diseases, predation, feed problems, accidents and lack of medication. Of these, poultry diseases were found to be the outstanding constraints that reduced total number of birds and impaired productivity (Dessie and Ogle, 1996). The interviewed chicken flock owners considered ND as the most important poultry disease affecting productivity in chickens, as is the case in other countries (Awan *et al.*, 1994; Zeleke *et al.* 2005). The disease was reported to occur in different sexes, ages of birds and agro – ecological zones; this supports Musiime's (1992) observation that ND virus may be distributed all over the country, although he had no data on Eastern Kenya. However, Musiime's studies had not compared the ND outbreaks in different sexes and ages of birds and in different agro – ecological zones as illustrated in this study.

Environmental and social stresses are known to increase the level of circulating corticosteroids, which in turn affect the immune system in pigs and other mammals (Harbuz and Lightman, 1992; Morrow-Tesch *et al.*, 1994). In this study, features that simulate stress factors such as confinement, lack of feed supplementation and seasonal weather effects were assessed to determine whether they were associated with ND outbreaks in different agro – ecological zones. In this regard, most farmers associated ND outbreaks with confinement, seasonal weather patterns, lack of feed supplementation, and introduction of new birds to the flock and presence of other domestic birds in the flock. However, other factors such as disposal of poultry fecal matter, management and

handling of sick birds and mode of transportation of birds to the markets were not directly associated with ND outbreaks though they varied across the agro – ecological zones.

Most ND outbreaks occurred during the wet seasons (March to June; October to December) in all zones except the lower midland 5 (LM5). Very cold (July) or very hot temperatures (February) were associated with ND outbreaks in this study, supporting what was reported by Musiime, (1992) that ND infections were more common during cold and dry periods. In addition, windy conditions with or without obvious dust storms were associated with ND occurrence in the study area. It is also reported that ND occurs year – round in most village chicken populations, but was common and severe at times of climatic stress (Martin, 1992). In Thailand, outbreaks were associated with change of season, particularly the start of the wet season (Thitisak *et al.* 1988). In this study, most outbreaks were reported to occur in the middle of cold or hot season depending on the agro-ecological zone. Cold weather has been cited as a contributory factor in ND outbreaks in Kenya and Vietnam (Nyaga *et al.,* 1985; Dao and Pham, 1985), as well as hot weather in Mauritania (Bell *et al.* 1990). A higher seasonal incidence of ND has been reported in dry season in Zambia (Sharma *et al.*, 1986), and Uganda (Mukiibi, 1992); and in winter in Bangladesh (Asadullah, 1992) and Vietnam (Nguyen, 1992). These climatic changes may be associated with inducing stress in the village flocks and perhaps stress is the common variable applying in all these situations. Interestingly, another study carried out by Otim *et al.* (2005) showed no significant difference in incidence of ND in dry or rainy seasons in Uganda, leading to a conclusion that seasons per se might not be risk

factors, which is at variance with reports of other studies quoted. The ecological zone LM5 had ND occurrence similar to that of Zambia, Mauritania and Uganda, unlike the other zones, which behaved as in Thailand, Vietnam and Bangladesh. The agro – ecological zones appeared to influence the time of the outbreak in this study.

Confinement in most zones was found to be a factor for ND outbreaks. This may be due to confinement stresses, close interaction of the birds and insufficient feeding. Virulent NDV has been isolated frequently from captive caged birds (Senne *et al.*, 1983; Alexander, 2000) and some of these birds came down with clinical ND (Kaleta and Baldauf, 1988; Carter, 2005). The same may be occurring in our study area where confinement as a stress inducer may be playing a role in the release of virulent NDV in carrier birds and consequently leading to manifestation of clinical disease and major ND outbreaks. The contribution of stress to NDV endemicity was further investigated and reported elsewhere in this thesis.

Newcastle disease outbreaks were more prevalent in flocks that were not supplemented with feed. Feed deprivation to birds, especially during confinement, is a major source of stress. This may further lead to depression of immune system due to lack of vital nutrients such as valine, as reported in commercial birds (Bhargava *et al.*, 1970), reduced amino acids: lysine, cystine and methionine (Chaiyapoom *et al.*, 2005) thus increasing the susceptibility of the birds to disease. In addition, vitamins A, E and B complex significantly affect the immune response to ND vaccine (Haq *et al.*, 1996). The fact that majority of the farmers (80%) in LM5 were not supplementing their flocks may have

contributed to the high incidence of ND outbreaks in this particular agro - ecological zone as compared to the others.

In this study, kales and other green plants (including flowering fruit plants) were significantly associated with the ND outbreaks in some agro – ecological zones (UM2, LM3 and LH1). This has not been reported before. Since some of these plants provide food and shelter during the hottest time of the day to indigenous chickens and wild birds, the mutual sheltering of NDV carrier birds may foster NDV's easy spread in such clustered circumstances.

This study revealed that chicks are more susceptible to Newcastle disease than any other age group. This finding is in agreement with the findings of Beard and Hanson (1984) who found that chickens become increasingly resistant to ND with age. However, it is unlike in Nigeria (Ezeokoli *et al.*, 1984) where mortality due to ND in unvaccinated growers was significantly higher than in unvaccinated adults and chicks. Velogenic NDV strains will cause disease in healthy non – immune adults, but some birds will survive (Martin, 1992). Hens had the highest survival rate followed by cocks in this study, which is similar to that reported in Bangladesh (Kutubuddin, 1973). In addition, Huchzemeryer (1993) noted that brooding hens and hens with clutches of chicks that were kept segregated could also escape infection. This could be occurring in the study area.

Transportation of poultry is a complicated, multifactorial, stressful and traumatic event (Elrom, 2000). It is reported that latent ND virus in vaccinated or non – vaccinated birds

may be shed by birds subjected to stresses, such as transport or concurrent disease (Heuschele and Easterday, 1970). In this study, hand carrying of birds to the markets was prevalent in agro-ecological zones with highest reports of ND outbreaks. Hand transportation is stressful as some birds are transported upside down and take too long to reach the market. This has not been reported before. Furthermore, contaminated carrier boxes have been associated with the spread of ND infection in California in 1972 (Utterbuck and Schwartz, 1973).

With respect to transmission and spread of the disease in village chickens, factors such as mode of disposal of manure and carcasses of ND infected birds, management of sick birds and the source of restocking birds were evaluated. Consumption of the sick birds by the farmers was the most common method of disposal of infected birds in all the agro - ecological zones. Disposal of the offal and feathers from such infected birds that are consumed at home can easily spread disease to clean birds in the home and those of the neighbours. The study revealed that farmers in all agro - ecological zones attempted to treat their sick birds using herbs (especially LM5) but their efficacy has not been tested. In that case, infected birds linger more in the homes, giving more opportunity for disease spread. Farmers from LM5 consumed the sick birds and sold off the birds that appeared healthy in the flock in order to reduce losses due to anticipated deaths of birds. Interestingly, this zone had the highest ND prevalence rate (93.8%), which means that an outbreak starting at one focus could have spread rapidly as the farmers moved the sick birds from their farms to the markets by hands. In this way, birds act as a source of infection to the flocks of the farmers who purchase the sick birds and take them home.

Indeed, many farmers in all the agro ecological zones linked ND outbreaks with introduction of new birds bought from the market. This observation concurs with the findings of Alexander, (1988b) and Spradbrow (1993) who reported that live bird markets are often involved in the spread of the Newcastle disease. Poultry manure was disposed in two ways, as fertilizer on the farms or thrown away as rubbish. Many farmers in the zone that reported the highest ND prevalence rate (LM5) were throwing the fecal matter away as rubbish near the homesteads, where it was easily accessable to the birds. Feacal material used as farm fertilizer is spread out away from the chickens and NDV in them would be more exposed to destruction by heat from the sun and dessication. Newcastle disease virus in fecal heaps is easily spread by aerosol and contaminated fomites and can be ingested by the birds. In addition, shedding of the virus commences before clinical signs of ND occur and recovered birds or those exposed to virulent virus continue to shed NDV (Spradbrow, 1999). Newcastle disease virus can survive in fecal matter for more than 8 weeks in hot dry tropical areas at a temperature of 40^0C (Warner, 1989) and up to 3 months at temperatures of $20 - 30^0$C; and longer at cooler temperatures (Alexander, 1988b). Furthermore, it was observed that ND virus was found in manure 16 days post depopulation of infected chickens (Kinde *et al.* 2004). Thus, there is a possibility of infected manure from village flocks maintaining the infection on the farm and mechanically spreading NDV to the neighbouring farms.

Rearing of other domestic birds such as ducks, turkeys, doves, geese and guinea fowls together with chickens increases the risk of ND outbreaks in indigenous chickens (Spradbrow, 1999). Only six of the seventy-five households in this study reared other

birds (ducks, guinea fowls, turkeys, doves) together with chickens, and some of these farmers associated the ND outbreaks with these birds. Geese, turkeys, doves, and guinea fowls can become infected with NDV, shed the virus, and act as a source of infection for chickens, even if they do not develop clinical signs (Martin, 1992). Newcastle disease has been diagnosed in guinea fowls by various workers (Durojaiye and Adene, 1988; Okaeme *et al.* 1988; Haruna *et al.* 1993). Similarly ducks become infected with NDV and show few clinical signs and can play a role in the spread of the virus (Spradbrow, 1999; Alexander, 2001b).

In conclusion, this study has demonstrated that several factors namely: confinement, lack of feed supplementation, cold temperatures, winds, introduction of market birds, disposal of manure and sick birds are major risk factors to the occurrence of ND in village indigenous chickens. However, seasonal outbreaks depend on the agro-ecological zone in which the birds are reared. It is recommended that ND vaccination be encouraged since the farmers were not vaccinating against the disease, flock owners be educated on disease transmission and prevention, and be discouraged from restocking their farms with chickens bought from the markets.

CHAPTER 4

4.0. Experiment 2: Study of the Newcastle disease virus prevalence in healthy village indigenous chickens in two climatic and ecological areas

4.1. Introduction

Many reports and studies (Bell *et al.*, 1990; McBride *et al.*, 1991) suggest a continuous presence of NDV in village poultry populations. Some of the risk factors that have been associated with the maintenance of ND include: carrier chickens, village poultry population dynamics, other poultry species, wild birds and heterogeneity of NDV (Awan *et al.*, 1994). Although clinically diseased chickens are the most important hosts for Newcastle disease virus, latently infected birds and survivors of natural infection, which still harbour the agent, may also act as reservoirs. Village chickens may be exposed naturally to virulent virus shed from recovered birds, vaccinated birds having various levels of antibodies in their blood, non – susceptible species carrying virulent virus or susceptible birds yielding virulent virus, which may have evolved from passages in birds of mesogenic viruses (Westbury *et al.*, 1984; De Leeuw *et al.*, 2003).

Newcastle disease was shown to be present in all agro – ecological zones as per farmers' reports in the previous experiment on risk factors associated with Newcastle disease occurrence in village indigenous chickens in Embu and Mbeere districts. It was

hypothesized that, chickens with high antibody levels of Newcastle disease virus are not involved in maintaining the virus in the village poultry flocks.

Thus, it was found necessary to carry out laboratory isolation and serological tests for NDV from two extreme agro – climatic zones namely, Lower highland 1 and Lower midland 5. The aim of this study was therefore to determine the prevalence of NDV and NDV antibodies in naturally exposed non – vaccinated village indigenous chickens, in varying climatic and ecological zones in Kenya.

4.2. Materials and methods

4.2.1. Sources of chickens

The farms from which the chickens were purchased were located in two varied zones in terms of climate and ecology namely: lower midland 5 (LM5) in Mbeere district and lower highland 1(LH1) in Embu district (as described in chapter 3). The flock size was variable and all the birds were on free – range system in both agro – ecological zones.

4.2.2. Experimental design

One hundred and forty four apparently healthy chickens (71 from LH1 and 73 from LM5), consisting 59 growers (31 females, 28 males); 35 chicks (15 females, 20 males; and 50 adults (26 females, 24 males) were randomly sampled. There were equal numbers of females and males (72 each). The chicks were less than 2 months old; growers were between 2 to 8 months old; and adults, above 8 months of age. All the birds were transported in cages to Kabete university campus for sampling. Cloacal and

oropharyngeal swabs were processed for ND viral isolation as described below while the serum samples were tested for ND specific antibody by haemagglutination inhibition (HI) test. The isolated viruses were then tested for pathogenicity using mean death time and intracerebral pathogenicity index.

4.2.3. Collection and processing of swabs and blood

Swabs were taken from oro–pharynx and cloaca using sterile cotton – tipped applicator swabs. The swabs were placed in 2 ml viral transport medium comprising minimum essential medium, penicillin, streptomycin at a concentration of 5000 units / ml and 2.5 mg/ml amphotericin B and transported in a cool box to the laboratory. The swabs were expressed, centrifuged at 3500 rpm for 10 minutes and the supernatant transferred to a sterile bijoux bottle. All the samples were stored at -20^{0}C until virus isolation was done.

In addition, blood from the brachial vein was collected into universal bottles, without anticoagulant. Serum samples were separated from respective clotted blood samples by centrifugation at 500 rpm for 15 minutes, and then heated at 56^{0}C for 30 minutes to inactivate nonspecific hemagglutination inhibitors. The serum samples were then decanted, aliquoted into screw capped vials and kept frozen at -20^{0}C until HI tests were performed.

4.2.4. Embryonated eggs

Specific pathogen free embryonated eggs were obtained from Kenya Agricultural Research Institute (KARI), incubated at 37^{0}C in a humidified incubator and candled on

day 9 post incubation. Any infertile eggs and those with dead embryos were discarded, while those with live embryos were used for sample inoculation to recover virus, and for evaluation of the mean embryo death time (MDT) or for preparing primary chicken embryo fibroblasts.

4.2.5. Preparation of chicken embryo fibroblasts

Primary chicken embryo fibroblast (CEF) cultures were prepared using 9 to 11 – day – old embryonated chicken eggs, as per standard procedures (Kumanan and Venkatesan, 1994). Briefly, the shells of the embryonated chicken eggs (ECE) were disinfected by wiping with 70% alcohol soaked swabs. The eggshell above the air cell margin was removed and the shell membranes cut. The live embryos were lifted from the eggs with sterile forceps into a sterile petri dish containing phosphate buffered saline (PBS) with 500 units of penicillin and 500µg of streptomycin. The head, forelimbs, and hindlimbs of the embryo were cut, and a mid-ventral incision was made on the embryo to remove all the internal organs. The torso was washed in two changes of sterile medium and cut into small cubes. Trypsinization of the embryo was done by adding trypsin to a final concentration of 0.25% of the minimum essential medium (MEM). The cell suspension containing the trypsinized cells was decanted into fresh centrifuge tubes and centrifuged at 1200 rpm for 15 minutes to pellet the cells. The cells were resuspended in MEM containing 5% fetal bovine serum (FBS), counted using Neuber chamber and seeded at a concentration of $3x\ 10^{6}$ cells / ml in flat – bottomed – 96 well tissue culture plates (Costar, Cambridge, MA). They were maintained at $37^{0}C$, 5 % carbon dioxide for 2 to 4 days so that the monolayer could develop (Kumanan and Venkatesan, 1994).

4.2.6. Propagation and harvesting of Newcastle disease virus

Confluent CEF cultures were inoculated with 1 ml of specimen material and incubated for 1 hour at 37^0C to allow virus adsorption. The nonadsorbed virus was removed by addition of 1 ml of fresh medium to the monolayers which were tilted from side to side and the medium aspirated out. Fresh medium (1 ml) was added and the cells incubated for 4 to 5 days at 37^0C. Regular microscopic examination was carried out to look for the development of characteristic cytopathic effects (CPE). The virus was harvested at the height of CPE by subjecting the cultures to three cycles of alternate freezing and thawing (Kumanan and Venkatesan, 1994). The harvested virus was tested for hemagglutination activity.

4.2.7. Preparation of red blood cells

Five millilitres of blood was collected from chicken in Alsevers solution and centrifuged at 500 rpm for 5 minutes. The supernatant and buffy coat was removed and the red blood cells (RBCs) washed three times in phosphate buffered saline (PBS) pH 7.2. The RBC were then resuspended in PBS to a final concentration of 0.5% v/v, (Hsiung, 1973) stored at 4^0C and used within 3 – 4 days.

4.2.8. Virus detection using haemagglutination test

The hemagglutination test was carried out after the method of OIE (2000). Briefly, a volume of 0.025 ml of PBS was dispensed into each well of a plastic U- bottomed – 96 well microtitre plate (Costar, Cambridge, MA) and 0.025 ml of the harvested virus growth in CEF was placed in the first well. Two fold dilutions were made using 0.025 ml

volumes transferred from well to well across the plate. A volume of 0.025 ml of 1% (v/v) chicken red blood cells was dispensed in each well including the RBC control wells and the plate mixed by tapping the plate gently. The RBCs were allowed to settle for about 40 minutes at room temperature, when the control RBCs had settled to a distinct button. Haemagglutination end point was determined by tilting the plate and observing the presence or absence of tear – shaped streaming of the RBCs. The titre was taken as the well with the highest dilution giving complete haemagglutination and no tearing (OIE, 2000).

4.2.9. Haemagglutination inhibition test

The hemagglutination inhibition test was carried out as described in OIE (2000). Briefly, a volume of 0.025 ml of PBS was dispensed into each well of a plastic U- bottomed – 96 well microtitre plate (Costar, Cambridge, MA) and 0.025 ml of test serum was placed into the first well of the plate. Two fold dilutions were made by transferring 0.025 ml volumes of the diluted serum to next well and discarding 0.025 ml after mixing in the last well of the plate. Four haemagglutination units (4 HAU) of the virus antigen in 0.025 ml volumes were added to each well and in the first two wells of an additional row of wells. Two fold dilutions of the virus antigen were made from the second upto the sixth well. The plate was left to incubate for 30 minutes at room temperature. Then 0.025 ml of 1% (v/v) chicken RBCs were added to all wells including the antigen control wells and RBCs control wells containing only PBS. The plate was incubated for about 40 minutes at room temperature. The plates were read when the RBCs in control wells had settled to a button. Wells showing teardrop were positive for hemagglutination

inhibition. The antigen control wells were read to confirm 4 HAU by tilting the plate to observe the tear drop formation. The HI titre was taken as the highest dilution of serum in the well showing complete inhibition of hemagglutination. The validity of the results was assessed against a negative control serum and a positive control serum included in the test (OIE, 2000).

4.2.10. Pathogenicity tests

4.2.10.1. Mean death time

The virulence of some (12 out of 21) of the recovered NDV isolates was determined by the mean death time (MDT) in embryonated chicken eggs (OIE, 2000). Harvested CEF fluid was diluted in sterile PBS to give a ten -fold dilution series between 10^{-6} and 10^{-9}. For each dilution, 0.1 ml was inoculated into the allantoic cavity of each of five 9-day – old embryonated chicken eggs, which were then incubated at 37^0C. The remaining virus dilutions were retained at 4^0C and another five eggs were inoculated with 0.1 ml of each dilution 8 hours later and left at 37^0C. Each egg was examined twice daily for 7 days and the time of any embryo deaths were recorded. The highest dilution at which all embryos died was considered the minimum lethal dose. The MDT was recorded as the mean death time in hours for the minimum lethal dose to kill the embryos.

4.2.10.2. Intracerebral pathogenicity index

Virulence of 12 out of 21 of the recovered NDV isolates was also determined using the intracerebral pathogenicity index (ICPI) in day old chicks as described below. Briefly, fresh infective allantoic fluid with a HA titre $>2^4$ (>1/16) was diluted 1/10 in sterile

isotonic saline. Each of the 10, one day old chick hatched from specific pathogen free (SPF) eggs was injected intracerebrally with 0.05 ml of the diluted virus (OIE, 2000). A total of 12 randomly selected isolates were tested. The chicks were examined every 24 hours for 8 days. At each observation, the birds were scored: 0 if normal, 1 if sick, and 2 if dead. The intracerebral pathogenicity index (ICPI) was then calculated using standard procedures (OIE, 2000).

4.2.11. Statistical analysis

Data on HI titres and virus recovery were log transformed before being analysed using SAS software (SAS Institute Inc., Cary, NC, USA, 2002 -2003). The Waller – Duncan K – ratio t test and Ryan – Einot – Gabriel – Welsch multiple range test (Steel and Torrie, 1980) were used to analyse the antibody responses. The titres were compared across the various age groups, between sexes and agro -ecological zones.

4.3. Results

4.3.1. Prevalence of Newcastle disease antibodies and virus in the Lower highland 1 (wet - humid) and Lower midland 5 (dry - hot) agro – ecological zones

Prevalence of Newcastle disease virus was higher in the Lower midland 5 (17.8%) as compared to Lower highland 1 (LH1), which had a prevalence of 9.9 % ($P < 0.05$). Lower midland 5 (LM5) had a NDV seroprevalence of 8.2% while LH1 had 4.2% (**Figure 4.1**).

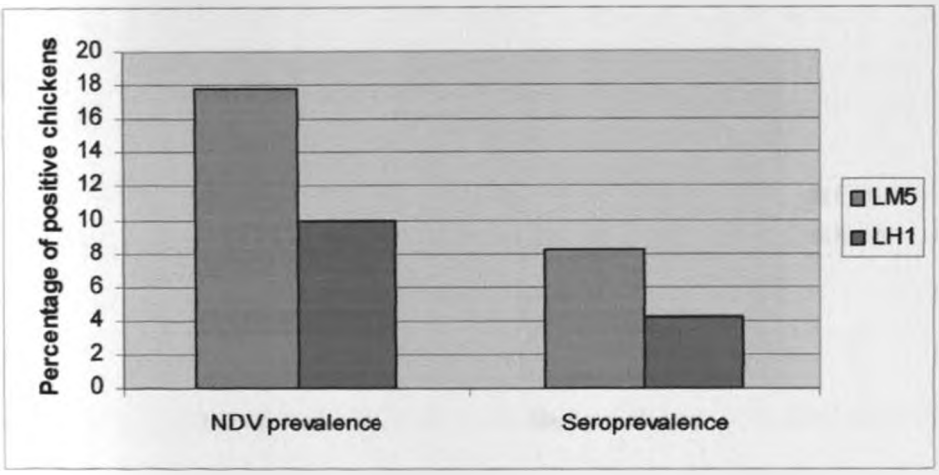

Figure 4.1: Prevalence of Newcastle disease virus and antibodies in village indigenous chickens in the Lower highland 1 and Lower midland 5 agro - ecological zones

Legend: NDV = Newcastle disease virus; **LH1:** Lower highland 1;
LM5: Lower midland 5

4.3.2. Prevalence of Newcastle disease antibodies and virus in different age groups and sexes of village indigenous chickens

Prevalence of Newcastle disease virus was highest in female birds (22.2%) of all the age groups, while males had a prevalence rate of 5.6%. Similarly, the seroprevalence was higher in females than males of the same age groups: 6.9% and 5.6% for females and males, respectively **(Figure 4.2).** In this study, the difference in NDV prevalence between the males and females was statistically significant ($P < 0.05$) while the seroprevalence was not significant ($P > 0.05$).

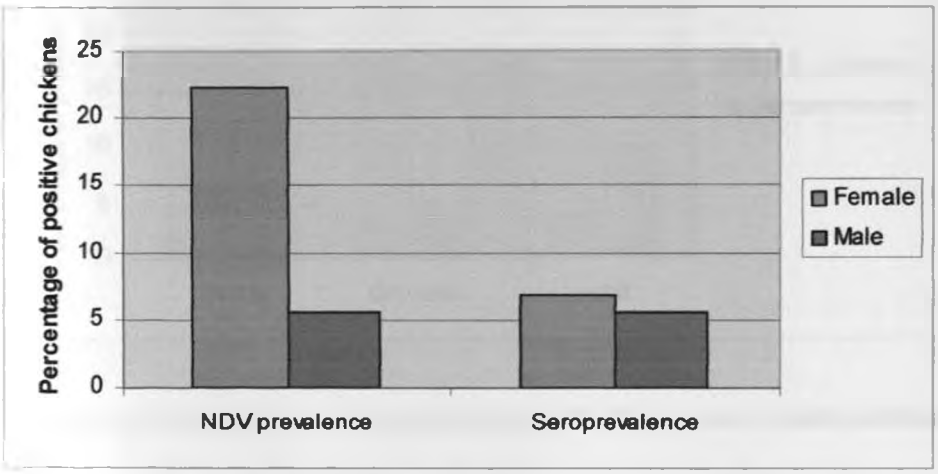

Figure 4.2: Overall prevalence of Newcastle disease virus and antibodies in different sexes of village indigenous chickens

Legend: NDV = Newcastle disease virus

Among the three age groups, the growers had the highest NDV prevalence of 25.4%, while chicks and adults had an NDV prevalence of 8.6% and 4%, respectively. In respect to antibody prevalence, adult birds had the highest NDV seroprevalence of 10% followed by growers (5.1%) and lastly chicks with 2.9% (**Figure 4.3**). The Newcastle disease virus prevalence between the three age groups was statistically significant ($P < 0.05$).

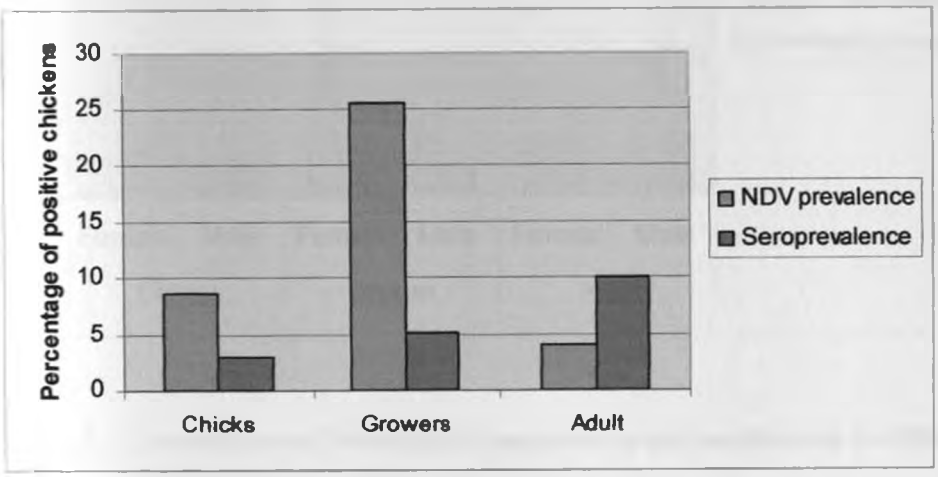

Figure 4.3: Overall prevalence of Newcastle disease virus and antibodies in different age groups of village indigenous chickens

Legend: NDV = Newcastle disease virus

Grower females had higher NDV prevalence of 38.7% compared to that of their male counterparts (10.7%). Female chicks had an NDV prevalence of 13.3% while males had 5%, and adult females had 7.7% while no virus was isolated from adult males. In respect to antibody prevalence, adult females had the highest NDV seroprevalence of 11.5% and

their male counterparts 8.3%. Grower females and males had a seroprevalence of 6.5% and 3.6% respectively. Lastly, male chicks had a seroprevalence of 5% while all female chicks were seronegative **(Figure 4.4)**.

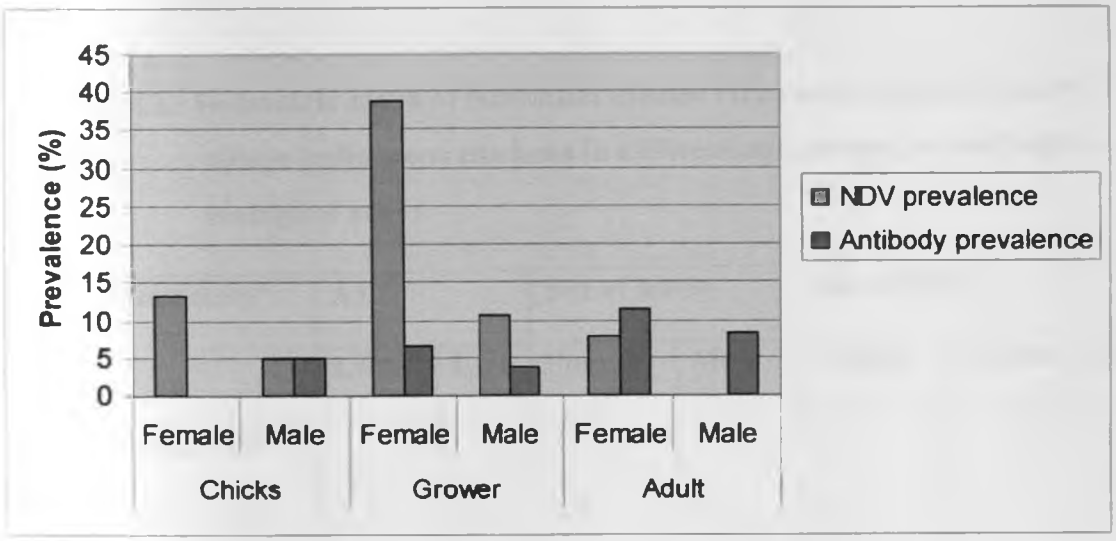

Figure 4.4: Prevalence of Newcastle disease virus and antibodies in different sexes and age groups of village indigenous chickens

Legend: %: Percentage; **NDV:** Newcastle disease virus

4.3.3. Effect of age, sex and climate on Newcastle disease virus titers in village indigenous chickens

Chickens from lower highland 1 had higher mean titres of antibodies as compared to those from Lower midland 5. Female birds had the highest geometric mean titres of both NDV and antibodies in the two agro - ecological zones. The NDV titres were highest in

adults, followed by growers and lowest in chicks while antibody levels were highest in the growers, followed by chicks and lowest in adults **(Table 4.1)**. Age of the birds significantly affected the mean titers of NDV and antibody in various chicken age groups ($P < 0.05$), while sex did not affect the two parameters ($P > 0.05$).

Table 4.1.: Geometric mean of Newcastle disease virus and antibody titres of village indigenous chickens in different age groups, sex and agro – ecological zones

Parameters*	**AEZ**		**Sex of birds**		**Age of birds**		
	LM5	**LH1**	**Female**	**Male**	**Chicks**	**Grower**	**Adult**
G.mean ± SE (NDV – HA)	2.1	2.1	2.2	2.0	2.0	2.1	2.4
G. mean ± SE (Antib– HI)	3.5	4.3	4.0	3.5	4.0	4.2	3.5

Legend:

NDV - HA: Newcastle disease virus hemagglutination titers; **Antib –HI:** Antibody hemagglutination inhibition titers; **AEZ:** Agro - ecological zone; **LM5:** Lower midland 5; **LH1:** Lower highland 1; **G. mean ± SE:** Geometric mean plus or minus standard error; ***:** Titers $\log_2$

4.3.4. Pathogenicity of recovered Newcastle disease virus isolates

The mean death time (MDT) of 12 isolates tested ranged between 48 and 56 hours. The lowest intracerebral pathogenicity index (ICPI) was 1.5 while the highest was 1.8. Majority of the isolates had an ICPI of 1.7. All the 12 isolates tested were from female birds only **(Table 4.2)**. All were velogenic strains.

Table 4.2: Mean death time and intracerebral pathogenicity indices for Newcastle disease virus isolates from field birds

Isolate code	Sex	Age	AEZ	MDT (in hours)	ICPI	Pathotype	Antibody level (log_2 10)
MB 5	F	G	LM 5	48	1.7	Velogenic	0
MB 9	F	G	LM 5	48	1.5	Velogenic	0
MB 14	F	G	LM 5	48	1.7	Velogenic	0
MB 27	F	G	LM 5	56	1.8	Velogenic	4
MB 35	F	G	LM 5	48	1.7	Velogenic	0
MB 37	F	G	LM 5	56	1.7	Velogenic	0
EM 41	F	A	LH 1	56	1.7	Velogenic	0
EM 47	F	A	LH 1	56	1.6	Velogenic	0
EM 61	F	A	LH 1	48	1.6	Velogenic	0
EM 66	F	A	LH 1	48	1.8	Velogenic	0
EM 88	F	G	LH 1	56	1.7	Velogenic	0
EM 108	F	G	LH 1	56	1.7	Velogenic	0

Legend:

MDT: Mean death time; **ICPI:** Intracerebral pathogenicity index; **MB:** Mbeere; **EM:** Embu; **AEZ:** Agro – ecological zones; **F:** Female; **A:** Adult; **G:** Grower

4.4. Discussion

The prevalence of Newcastle disease virus was higher in LM5 than the LH1 and the difference was statistically significant. The fact that NDV prevalence rate was higher in LM5 than LH1 may be attributed to the diversity in the management practices (this include confinement and mode of disposal of poultry waste and carcasses) favouring the maintenance of the virus in the village poultry populations as reported in experiment 1 of this thesis. These findings agree with farmers' responses on the occurrence of ND in the two agro – ecological zones who reported LM5 to have more outbreaks of the disease than LH1. The two zones have varied climate, farming and chicken management systems. Such a comparison has not been undertaken in Kenya before. Previous studies have associated ND with change of seasons, particularly the start of the wet season (Thitisak *et al.* 1988; Jintana, 1987). Cold weather has been associated with ND outbreaks in some countries (Dao and Pham 1985), while in others it is hot weather (Bell *et al.*, 1990).

The seroprevalence differed across the two agro - ecological zones, with LM5 having a higher seroprevalence than the LH1 although the difference was not statistically significant ($P > 0.05$). Yongolo (1996) reported a sero prevalence varying from 25 % to 81.5% in Tanzania, which also had variation in different months and localities, but not in specific agro – ecological zones. The many risk factors identified in this study and the free-range management system may be responsible in creating uninterrupted cycles of infections whereby the virus passes from one chicken to another throughout the year.

Female chickens had higher mean NDV titres compared to the male birds. Hens and pullets may therefore be playing a significant role in the carriage and maintenance of the virus in these rural poultry populations. Previous studies by Kutubuddin, (1973) indicated that male birds were more affected by NDV than female birds. However, in this study the females had higher viral load than male birds in all age groups. Huchzemeryer (1993) noted that brooding hens and hens with chicks that were kept segregated could also escape infection. Chickens that may have survived previous ND outbreaks produce chicks, which become susceptible to ND after the maternal antibodies have waned (Huchzemeryer, 1993). The actual cause of this apparent sex related differences in NDV carriage is not yet understood.

All the NDV isolates were velogenic. It is not clear why apparently healthy birds, which had no antibodies, would harbour velogenic NDV strains. Perhaps they were protected through other non – antibody mediated mechanisms, e.g. cell mediated immunity from prior exposure to virus that is assuming that antibodies had declined to zero levels as shown in these birds. On the other hand, the birds could have been incubating the disease and would have shown clinical disease if they were sampled several days later. However, the two situations may not have been the explanation and therefore, this phenomenon requires further investigation. Although virulent NDV have been recovered from healthy looking birds previously it was not recorded whether these birds had high antibody levels (Awan *et al.*, 1994). For the one isolate in our study that was recovered from a healthy looking bird with high antibody titer, one can explain why the bird still looked healthy. In this case, though infected, the bird can be taken to have been protected from clinical

disease by the antibodies. This is what normally occurs when vaccinated birds are challenged by virulent virus in an epidemic. The birds with protective levels of antibodies get infected but they overcome this infection by neutralizing the virus and infection does not progress into full disease (Alexander, 1997).

In conclusion, the present study indicates that NDV occurrence in Kenya is higher in village chickens that are kept in warm (LM5) than cold climates (LH1). Further, the study demonstrates for the first time that in Kenya, factors such as climate, ecology, age and sex of birds influence the viral carriage in the village birds. It is still not clear why healthy looking birds yield virulent NDV and this requires further investigation.

CHAPTER 5

5.0. Experiment 3: Determination of sera and egg yolk antibodies as indicators of Newcastle disease virus carrier status in naturally exposed healthy village indigenous hens

5.1. Introduction

The village poultry flocks are believed to keep the virus in circulation and act as reservoirs to exotic breeds in commercial farms (Spradbrow, 1993). The circumstances in which the village birds sustain NDV in the flocks have not been fully explained. The resource derivable from chickens cannot, therefore, be fully utilized unless the disease is controlled (Adu *et al.*, 1986; Nwosu and Okeke, 1989; Olabode *et al.*, 1992).

Commonly used vaccines such as Hitchner B1 and La Sota induce high levels of IgA, IgY, and IgM antibodies (Russel and Koch, 1993). The antibody titre of log $2^{5.2}$ has been reported to provide 100% protection against challenge and log 2^{3} protective against infection (Allan *et al.*, 1978; Allan and Gough, 1974). Booster vaccination or reinfection after some weeks produces secondary immune response (Allan *et al.*, 1978).

Antibodies are detectable in the upper respiratory and intestinal tracts about the time humoral antibodies are first detected. The immunoglobulins (Ig) produced in the upper respiratory tract appear to be predominantly IgA with some IgG. Similar secretions occur

in the Harderian glands following ocular inoculation (Parry and Aitken, 1977). It has been demonstrated that viral replication in the Harderian gland can result in the production of lachrymal IgG, IgA, and IgM. Immunoglobulin M (IgM) has been found to be responsible for the clearance of virus in intraocular infections (Russel, 1993; Russel and Koch, 1993; Russel and Ezeifeka, 1995).

The main type of Ig isolated from egg yolk is generally referred to as "IgY"; other Ig classes are present, but only in negligible amounts (Schade *et al.,* 1996). The antibody levels in the yolk have been reported to be predictive of the hen's titer (Piela *et al.*, 1985; Silim and Venne, 1989). The serum antibody level must reach a specific level before specific antibody appears in the egg yolk, and to a certain extent, the egg yolk reflects the serum concentration of IgG over 6 to 7 days (Bollen and Hau, 1997). Following immunization of chickens at the beginning of the egg – laying period, it was found that the older chickens had consistently higher yolk titers than the younger chickens, which means that the NDV would probably survive longer in older birds (Bollen and Hau, 1999). The relationship between virus persistence and serum - egg yolk antibody levels is not clearly understood. Thus, it was hypothesized that there is no association between Newcastle disease virus antibody levels in sera and eggs of indigenous hens and Newcastle disease endemicity.

Previous studies (Yeo *et al.*, 2003; Msoffe *et al.*, 2006) which utilized vaccinated commercial hens reported positive association of NDV serum titers to the egg yolk titers. However, none of these studies linked the levels of antibodies in the matched eggs and

sera of carrier village indigenous hens or hens that had previous exposure to natural infection of ND. Thus, the objective of this study was to establish the role of serum and egg yolk antibodies as indicators of NDV carrier status in indigenous hens.

5.2. Materials and methods

5.2.1. Source of laying hens and eggs

The laying hens with no history of vaccination were bought from farmers distributed in 5 agro –ecological zones (LH1, UM2, UM3, LM3, and LM5). The location of the AEZ is described in section 3.2.1.

Experimental design

One hundred and thirty three laying hens were sampled as follows: 27 in LH1, 25 in UM2, 25 in UM3, 27 in LM3 and 29 in LM5. Cloacal and oropharyngeal swabs were processed for ND viral isolation using chicken embryo fibroblasts while the serum samples, eggs and ovules were tested for NDV specific antibody by the hemagglutination inhibition test as described in section 4.2.9.

5.2.3. Selection of study birds

Hens that were in lay were sampled from 67 households, with at most two hens being sampled from each household. The hens were ascertained to be in lay by measuring, using fingers, the spaces between the pubic bones and between the pubic and the keel

bones (Moreng and Avens, 1985; Bebora *et al.*, 2005). Any bird that had at least three fingers spread was considered to be in lay.

5.2.4. Collection and processing of samples

Swabs were taken from oro–pharynx and cloaca using sterile cotton – tipped applicator swabs and the samples were processed as described in section 4.2.3.

Yolk material was carefully prepared by breaking each egg into a separate sterile petri dish. The albumen was removed and the egg yolk membrane or the membranes covering the ovule was incised. 2.5 ml of yolk material, were aspirated and dispensed into a volume of 2.5 ml phosphate – buffered saline (PBS) to achieve a 1: 2 dilution.

Antibodies in the egg yolks were then extracted using phosphate buffered saline method as follows: a volume of 0.5 ml egg yolk (diluted earlier at 1:2) was mixed with 0.5 ml PBS, resulting in 1:4 dilution of egg yolk. All treated egg yolk samples were then incubated at room temperature for one hour. The mixture was centrifuged at 1000 x g for 10 minutes and the supernatant was transferred to a sterile bijoux bottle and kept at -20^{0}C in aliquots until used.

5.2.5. Preparation of chicken embryo fibroblasts

Primary chicken embryo fibroblast cultures were prepared as described by Kumanan and Venkatesan (1994). The procedure has also been fully described in section 4.2.5.

5.2.6. Propagation and harvesting of virus

Newcastle disease virus isolation was carried out on confluent CEF cultures following the method described by Kumanan and Venkatesan (1994) briefly described in section 4.2.6

5.2.7. Preparation of red blood cells

The RBCs were prepared as described by Hsiung (1973) and briefly described in section 4.2.7.

5.2.8. Virus detection using haemagglutination test

The protocol for the hemagglutination test is as described by OIE (2000) and briefly described in section 4.2.8.

5.2.9. Haemagglutination inhibition test

The hemagglutination inhibition test is as described by OIE (2000) and briefly described in section 4.2.9.

5.2.10. Data analysis

Data on HI titres for sera and egg yolk were analyzed using SAS software (SAS Institute Inc., Cary, NC, USA, 2002 -2003). Pearson correlation coefficients and comparisons of means (LSD) were used to compute the correlation between serum antibody titres and egg yolk antibody titres (Steel and Torrie, 1980). The correlation between yolk antibodies in mature and immature eggs (ovules) was also computed. Five percent (5%) critical level was used to determine the significance of the differences between comparisons.

5.3. Results

5.3.1. Prevalence of Newcastle disease antibodies in egg yolk in different agro – ecological zones

Lower highland 1 had the highest percentage of positive chickens with respect to egg – yolk antibodies (96.3%) followed by UM2, LM5, LM3 and UM3 with 96.0%, 93.1%, 88.9% and 84.0%, respectively. The highest HI antibody titer of 4096 was recorded in upper midland 2 only. The antibody titres ranged from $\log_2$ 4 to 9 for LM5 and $\log_2$ 4 to 10 for LH1 (**Table 5.1**). However, the difference across the various agro-ecological zones was not statistically significant (P>0.05).

Table 5.1: Number of hens per agro – ecological zones with Newcastle disease virus antibodies in the egg yolk

Range of Newcastle disease virus antibody titers in the egg yolk ($\log_2$ 10)	**No. of yolk positive for Newcastle disease virus antibodies**				
	LH1	UM2	UM3	LM3	LM5
3 - 4	3	5	10	2	7
5 – 6	5	3	5	9	13
7 – 8	14	6	3	7	6
9 – 10	4	9	3	5	1
11	0	0	0	1	0
12	0	1	0	0	0
Total no. Positive	26	24	21	24	27
Percentage positive	96.3	96	84	88.9	93.1

Legends

AEZ: Agro –ecological zone; **UM2:** Upper midland 2; **UM3:** Upper midland 3; **LH1:** Lower highland 1; **LM3:** Lower midland 3; **LM5:** Lower midland 5; **No:** Total number of yolk samples

5.3.2. Antibody titers in carrier village hen's sera, mature eggs and ovules

The geometric mean HI titres were significantly higher in egg yolk than sera of the same hens ($P < 0.05$). Geometric mean titers for serum antibodies were 5.0, 6.0, 3.0, and 4.0 for LH1, UM3, LM3 and LM5, respectively. On seroprevalence, birds with titre were 11.1% for LH1, 4.0% for UM3, 7.4% for LM3 and 3.4% for LM5. All the hens from UM2 were seronegative and in addition did not yield NDV. The percentage of hens that yielded the ND virus in different agro – ecological zones were: LH1 = 3.7%, UM2 = 0.0%, UM3 = 8.0%, LM3 = 0.0% and LM5 =3.4% (**Figure 5.1**). All the viral isolates were velogenic NDV. All hens that were positive for NDV had no antibody in their sera.

The geometric mean NDV antibodies titers in ovules were as follows: LH1= 5.6, UM2 = 6.3, UM3 =4.2, LM3 =5.7, and LM5 =5.6 while those in matched mature eggs were LH1 =7.5, UM2 =6.0, UM3 =4.0, LM3 = 6.6, and LM5 =5.2 (**Figure 5.2**). The geometric mean titres of matched mature egg yolk were higher than in ovules in the LH1 and LM3 agro-climatical zones. On the other hand, the geomeans were higher in the ovules than the mature egg yolks in UM2, UM3 and LM5. There was significant difference between geometric mean titres of mature eggs and ovules ($P<0.05$). The geometric mean titres of the mature egg yolks and ovules varied significantly across the five agro – ecological zones ($P<0.05$).

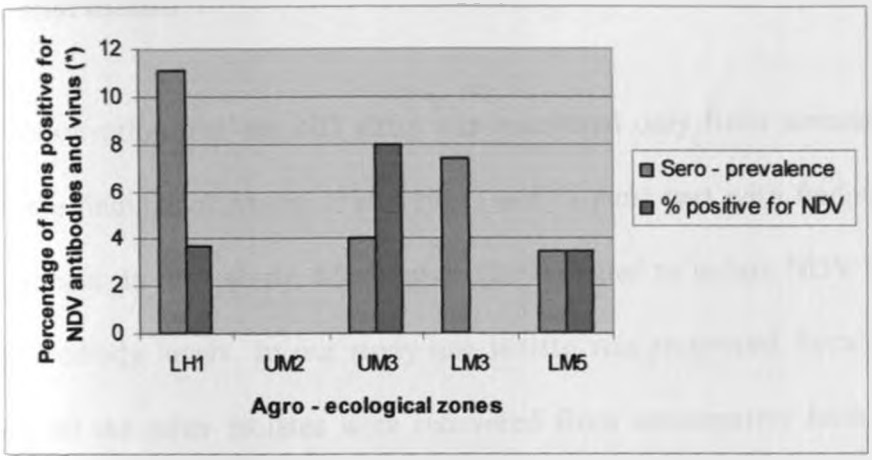

Figure 5.1: Prevalence of Newcastle disease virus and antibodies in laying hens from different agro – ecological zones

Legend: NDV: Newcastle disease virus; **%:** Percentage; **(*):** All hens that were positive for NDV had no antibody in their sera.

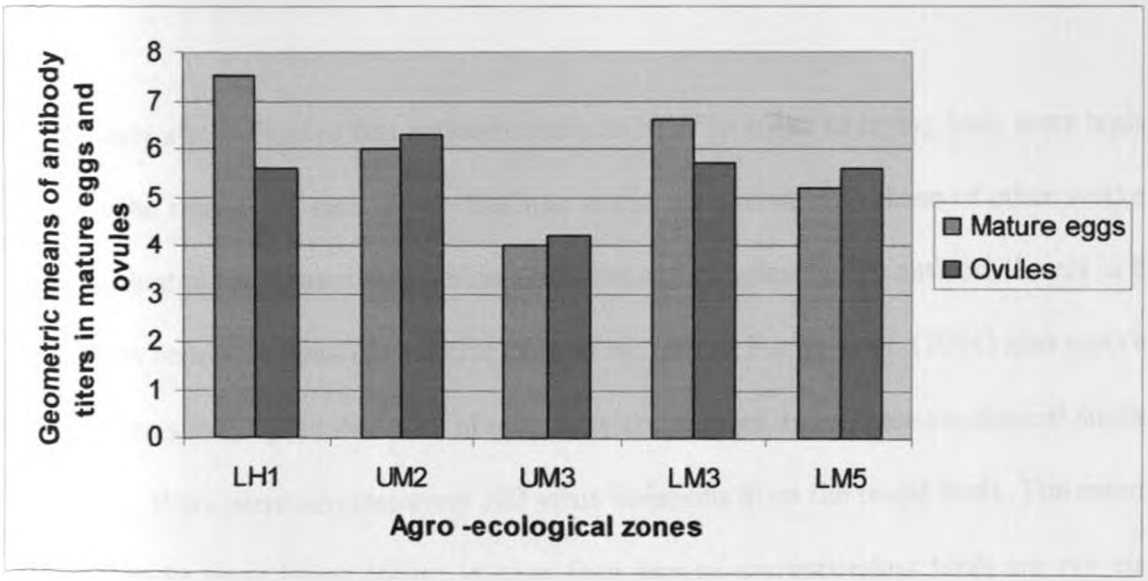

Figure 5.2: Geometric means of antibody titers in ovules and mature eggs of hens from different agro - ecological zones

5.4. Discussion

The observation that the ND virus was recovered only from seronegative hens agrees with the findings of Mushi *et al.* (2001) and in most part with findings of our previous experiment. In their study, Mushi *et al.* (2001) failed to isolate NDV from birds that had high antibody levels. In our study one isolate was recovered from a seropositive bird while all the other isolates were recovered from seronegative birds. Thus, hens were seronegative and infected; seropositive with antibodies in eggs or seronegative but with antibodies in eggs and ovules. Further more, Boven *et al.* (2008) reported that vaccinated birds with low or undetectable antibody titres may be protected against disease and mortality but that infection and transmission may still occur.

This study also indicated that antibody titers to NDV in yolks of laying hens were higher than in the respective sera. These findings are in agreement with those of other workers who evaluated vaccinated commercial chickens and reported higher antibody levels in the yolk than sera (Christian *et al.*, 2001; Yeo *et al.*, 2003). Farley *et al.* (2001) also reported higher titers in the yolk than sera of migratory cormorants. In all these serological studies, however, there were no concurrent ND virus isolations from the tested birds. The reasons for antibody titres being higher in eggs than sera of corresponding birds are not clear (Farley *et al.*, 2001). It has however, been reported that IgY in the egg follicles is passed selectively through receptors in large amounts into the yolk (Erhard and Schade, 2001). The concentration of IgY in the yolk is reported to be 1.23 times the one in the serum (Woolley and Landon, 1995). The amount of IgY transported to the yolk is believed to be

independent of egg size and known to be proportional to the maternal serum IgY concentration (Loeken and Roth, 1983). The selective transfer of Ig Y receptors can, therefore, partially explain the high concentration of antibodies to NDV observed in hen eggs in this study of healthy appearing, naturally exposed village indigenous hens.

The diversity and quantity of specific antibodies transmitted to offspring may reflect previous differences in the local disease environments experienced by female birds (Lundin *et al.*, 1999; Gasparini *et al.*, 2001). Other factors affecting maternal antibody transfer include: nutritional status, hen age and time of the season. Barua *et al.* (1998) have shown that young laying hens (180 days old) have over two-fold higher levels of IgG – containing cells in the ovary compared to either immature hens (50 days) or old laying hens (450 days old). Schade *et al.* (1991) describes the disparity in the concentration of antibodies in serum and yolk as due to the wide daily fluctuation of the amount of IgY in the yolk. However, both the content of IgY as a whole and the specific amounts of IgY in the serum and yolk, respectively, correlate positively if the time of passage into the yolk is taken into account (Erhard *et al.*, 1997).

The antibody titers to NDV in this study were higher in mature eggs than in the ovules from the same hen. There were also hens with antibodies found exclusively in yolks and not in sera. There is a possibility that the disparity is due to differences in period since start of lay and the last exposure to natural infection with NDV. This is the first report of Newcastle disease antibodies in matched mature eggs and ovules in naturally - exposed village chickens. Msoffe *et al.* (2006) reported higher HI titres in La Sota vaccinated

chicks than sera and eggs from their mother hens. They did not match each hen's sera with its egg and ovule after natural NDV exposure as in this study. Some workers (Msoffe *et al.*, 2006; Yeo *et al.*, 2003) have proposed that eggs could be used to assess the antibody levels in hens indirectly and this would not cause stress through bleeding to the flocks. Our study has reported higher antibody levels in eggs than hen sera and we suggest that the use of egg yolk alone might not correctly assess low levels of hen serum antibody.

In conclusion, the presence of NDV antibodies in the sera and yolks of non –vaccinated hens reported in this study indicated that the birds had previously been naturally exposed to NDV. This shows that NDV is prevalent in the area. A few of the hens in this study had protective levels of antibodies to NDV. In case of an infection with velogenic NDV, such birds would usually be expected to come down with clinical disease. In such a situation, the hens with high antibody levels though infected by NDV they would not die. However, those with low antibody levels or where antibody titers have fallen to zero due to natural waning off, would lead to disease outbreak. Detectable viral isolations from cloacal and oropharyngeal swabs were only made in seronegative hens. The virus strains recovered in this study were shown to be velogenic just like those recovered elsewhere from healthy appearing chicken (Awan *et al.*, 1994) and there is yet no explanation why the chickens were not sick. This scenario in our study therefore forms one component of a wider NDV endemicity model.

CHAPTER 6

6.0. Experiment 4: Effect of immunosuppression of ducks on Newcastle disease virus transmissibility to incontact chickens

6.1. Introduction

Sero – epidemiological and isolation studies have shown that velogenic NDV is endemic in rural chicken populations in some countries (Spradbrow, 1993; Otim *et al.*, 2007). The major mode of transmission appears to be by faecal – oral route, with respiratory route playing a role where close bird to bird associations exist (Alexander, 1988a). Besides domestic avian species, natural or experimental infection with NDV has been demonstrated in numerous other bird species. The disease resistant species appear to be aquatic birds while gregarious birds are the most susceptible (Kaleta and Baldauf, 1988). Psittacines are known to harbour and spread velogenic NDV (Olabode *et al.*, 1992).

Reports on susceptibility of ducks to Newcastle disease and the disease manifestation in this species has been conflicting. Nishizawa *et al.* (2006 and 2007) who worked on commercial breeds of ducks, mainly pekin, found that they did not show any signs of ND. Otim *et al.* (2006) also reported similar findings. However, Roy *et al.* (1992) reported that in unvaccinated commercial duck farm, the mortality due to ND was 10% and the affected birds showed anorexia and greenish - white diarrhoea. Thus, it seems that there are other factors, which play a role in the pathogenesis of Newcastle disease in ducks and the release of the virus to the environment. There is no information on the potential role

in dissemination of this virus to chicken and other food-producing poultry raised together or in close proximity to them.

In Kenya, ducks are the second largest in terms of domestic poultry population and are often reared together with chickens under village conditions (Nyaga *et al.*, 2002; Mbuthia *et al.*, 2003; MLD, 2005). Although there are several indications of the role of ducks in the transmission and maintenance of NDV in the rural poultry production system, no proper investigations into the dynamics of transmission of NDV from ducks to chickens have been conducted.

Under field conditions, immunosuppression may occur due to infection with other viruses such as infectious bursal disease virus (IBDV) and chicken infectious anemia virus (CIA) (Rosenberger and Gelb, 1978; Box *et al.*, 1988). Other sources of immunosuppression for the village chickens may include stress, mineral deficiencies, malnutrition, moulting, environmental factors such as too much heat or cold and reproduction cycles (Box *et al.*, 1988). It is not clear what role immunosuppression may play in the persistence of virulent virus in immune birds or carrier ducks. It was thus hypothesized, that immunosuppression has no effect on the transmissibility of Newcastle disease virus from carrier ducks to chickens.

In this study, the effect of immunosuppression on the ability of ducks to maintain and transmit velogenic NDV to in - contact chickens was investigated.

6.2. Materials and methods

6.2.1. Experimental birds

One-day-old indigenous ducklings and chicks were hatched from the poultry flock maintained at the University of Nairobi, Kabete campus premises. All the birds were reared in an isolation unit and then transferred to experimental units at the age of one year. They were wing tagged, tested and confirmed to be free of ND virus and antibodies.

6.2.2. Experimental design

Fifteen ducks and 15 indigenous chickens free from ND antibodies and NDV were used in this study. The ducks were subdivided into 3 groups as follows: (i) five immunosuppressed and inoculated with velogenic NDV; (ii) five non – immunosuppressed and inoculated with vNDV; and (iii) five negative control ducks. The 15 sentinel chickens were divided up into 3 subgroups as follows: (a) Five chickens mixed with immunosuppressed challenged ducks; (b) Five chickens mixed with non – immunosuppressed challenged ducks; (c) Five chickens kept in isolation as negative controls.

Immunosuppression, as described later, was carried out before inoculating the ducks with vNDV and also on days 13,14,15 and 16 post inoculation (p.i.) using the same dose rate for 4 days continuously in order to maintain ducks at a high level of immunosuppression. All the 10 ducks were inoculated intranasally with 0.2 ml of undiluted amnioallantoic fluids of vNDV with a titre of 1:1024, after 24 hours post inoculation with the last

injection of dexamethasone. The 5 chickens per group of ducks were introduced after 24 hours post inoculation of the ducks.

Cloacal and oropharyngeal swabs and blood samples for serum were taken on days 0, 1, 3, 6, 9, 12, 15, 18, 20, 23, 26 and 29 post inoculation (p.i.). The ducks and in – contact chickens were observed twice daily for any clinical signs of ND at the same time each day by the same person for all the 29 days of observation. All the surviving birds were sacrificed on day 29 p.i. and 6 tissues (liver, ceacal tonsils, kidneys, spleen, lungs and brain) were harvested from each bird for virus isolation. The serum samples were tested for ND specific antibody by the hemagglutination inhibition (HI) test.

6.2.3. Newcastle disease virus strain used

A velogenic Newcastle disease virus (strain L1) obtained from the repository maintained at the University of Nairobi was used in this study. It was a Kenyan field isolate characterized by standard methods (OIE, 2000). After initial characterization, the virus was purified three times using limit dilution in 9-day-old embryonated chicken eggs from a specific pathogen free flock (SPF). The mean embryo death time (MDT) was confirmed. The purified virus was aliquoted and maintained frozen at –20°C.

6.2.4. Immunosuppression of the ducks

Dexamethasone (Dexamethasone sodium phosphate and Sodium methyl hydroxybenzoate, Coophavet, St Herblon – BP 7- 44153 Ancenis Cedex – France), was used to stress ducks in this study. The respective groups of ducks were injected

intramuscularly with the dexamethasone at the rate of 2 mg per kilogram of body weight per day for 4 days continuously, then rested for 2 days and the injections resumed for 2 more days as done by Miller *et al.* (2003), with modification.

6.2.5. Collection and processing of samples

Swabs and blood samples were collected and processed as described on section 4.2.3. The tissues from each individual bird were homogenized to make a 10 % suspension in transport media (containing antibiotics (2000units/ml of penicillin; 2000 µg/ml of streptomycin and 2500 µg /ml of amphotericin B). The homogenate was then centrifuged at 1000 xg for 10 minutes and the supernatant stored at -20^{0}C until used.

6.2.6. Preparation of chicken embryo fibroblasts

Primary chicken embryo fibroblast cultures were prepared as described by Kumanan and Venkatesan (1994) and has been briefly described in section 4.2.5.

6.2.7. Virus propagation and harvesting

Newcastle disease virus isolation was carried out on confluent CEF cultures following the method described by Kumanan and Venkatesan (1994) and has been briefly described in in section 4.2.6.

6.2.8. Preparation of red blood cells

The RBCs were prepared as described by Hsiung (1973) and briefly described in section 4.2.7.

6.2.9. Virus detection using haemagglutination test

The protocol for the hemagglutination test is as described by OIE (2000) and briefly described in section 4.2.8.

6.2.10. Haemagglutination inhibition test

The hemagglutination inhibition test is as described by OIE (2000) and briefly described in section 4.2.9.

6.2.11. Data analysis

Data was cleared and then analyzed using SAS software (SAS Institute Inc., Cary, NC, USA, 2002 -2003). All the data were log transformed before analysis. Analysis of variance (ANOVA) of repeated measures and Chi-square was performed in SAS to determine the treatment main effects and the interaction between time (days) and treatment (Steel and Torrie, 1980).

6.3. Results

6.3.1. Clinical signs observed in ducks and chickens used in the cross transmission study

All the clinical signs observed for each bird per day were aggregated to form a total score. A total of 50 clinical signs were manifested by the indigenous chickens that were mixed with immunosuppressed (IS) NDV infected ducks (**Table 6.1**).These were: 18 depression, 9 ruffled feathers, 3 nervous tics, 5 sneezing, 6 greenish diarrhea and 4 sternal

recumbency (**Figure 6.1**). All the five chickens died between day 4 to 11 post mixing, while all the ducks survived.

Table 6.1: Type and total daily clinical signs observed in village indigenous chickens that were mixed with Newcastle disease virus infected immunosuppressed ducks

Type of clinical sign	Days post inoculation and number of birds with clinical signs											Total signs
	1	2	3	4	5	6	7	8	9	10	11	
Mortality	-	-	-	-	1	1	2	-	-	-	1	5
Depression	-	1	2	4	4	3	1	1	1	1	-	18
Ruffled feathers	-	-	-	4	2	3	-	-	-	-	-	9
Nervous tics	-	-	-	-	2	-	1	-	-	-	-	3
Sneezing	-	-	-	-	2	2	1	-		-	-	5
Greenish diarrhoea	-	-	-	-	-	1	2	1	1	1	-	6
Sternal recumbency	-	-	-	-	-	2	1	-	-	-	1	4
Total signs	**0**	**1**	**2**	**9**	**9**	**11**	**8**	**2**	**2**	**2**	**4**	**50**

Legend:

- : no clinical sign seen

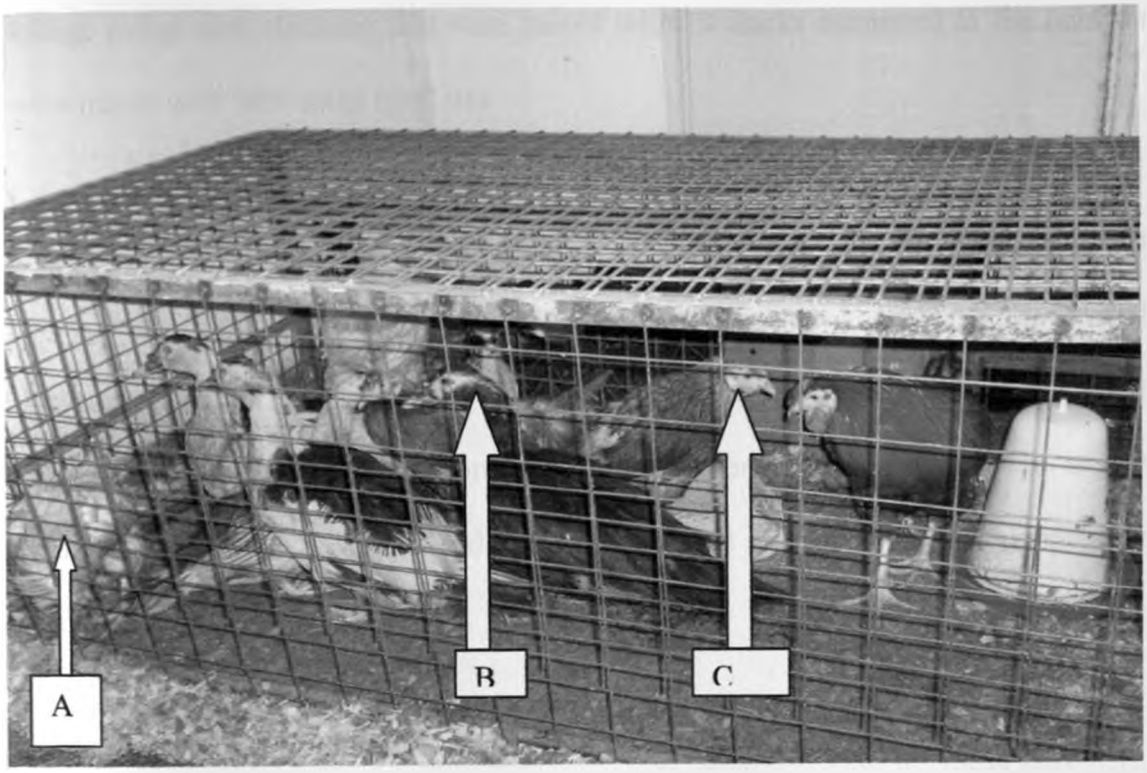

Figure 6.1: Mixed ducks (immunosuppressed) and chickens in cross – transmission study showing (A) moribund recumbent chicken, (B) a bright duck and (C) depressed chicken

A total of 28 clinical signs were manifested by the village indigenous chickens that were mixed with non – immunosuppressed (NIS) ducks exposed to NDV challenge (**Table 6.2**). The clinical signs included: 10 depression, 8 ruffled feathers, 3 sneezing and 3 greenish diarrhea. The first clinical signs were observed on day 4 p.i. and peaked on the fifth day. All the chickens died between day 6 to 10 post mixing with exception of one cock that survived up to the end of the experiment and was sacrificed on day 29 p.i. while all the ducks survived. The group mixed with NIS ducks, did not manifest nervous tics and sternal recumbency. **Figure 6.2** shows that more clinical signs were expressed by the

village indigenous chickens that were mixed with IS ducks compared to the ones that were mixed with NIS ducks ($p<0.05$).

Two out of the 5 IS ducks expressed clinical signs of ND, mainly mild depression from day 6 to day 8 p.i.. The depressed ducks clinically recovered after day 9 p.i.. The NIS ducks did not manifest any clinical signs of the ND and all the ducks survived up to the end of the experiment and therefore no mortality recorded for all ducks. The negative control birds (5 control ducks and 5 sentinel control chickens) did not manifest any clinical signs of Newcastle disease.

Table 6.2: Type and total daily clinical signs observed in village indigenous chickens that were mixed with Newcastle disease virus infected non – immunosuppressed ducks

Type of clinical sign	Days post inoculation and number of birds with clinical signs											Total signs
	1	2	3	4	5	6	7	8	9	10	11	
Mortality	-	-	-	-	1	3	-	-	-	-	-	4
Depression	-	-	-	2	3	1	1	1	1	1	-	10
Ruffled feathers	-	-	-	2	4	1	1	-	-	-	-	8
Nervous tics	-	-	-	-	-	-	-	-	-	-	-	0
Sneezing	-	-	-	-	-	2	1	-	-	-	-	3
Greenish diarrhoea	-	-	-	-	1	1	1	-	-	-	-	3
Sternal recumbency	-	-	-	-	-	-	-	-	-	-	-	0
Total signs	0	0	0	4	9	8	4	1	1	1	0	28

Legend:

- : no clinical sign seen

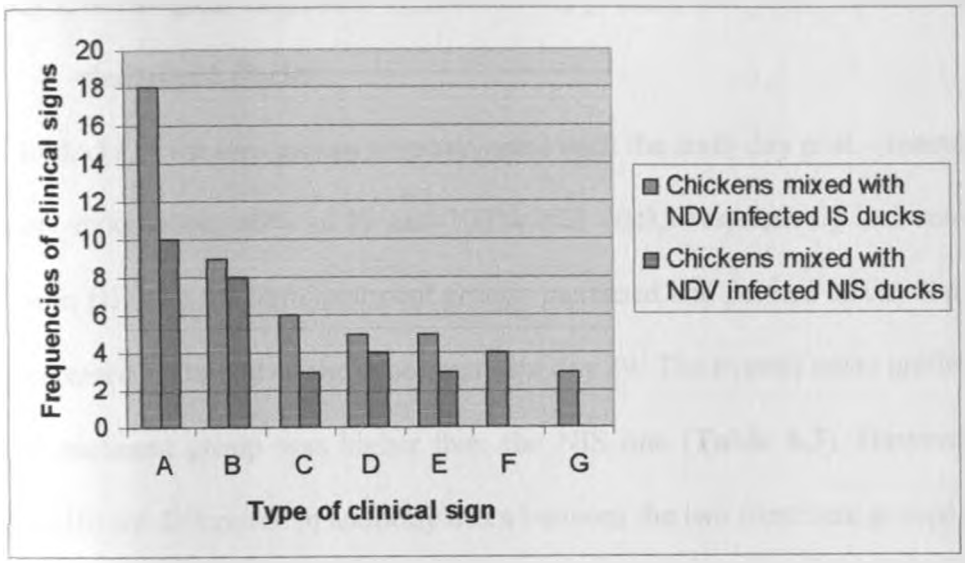

Figure 6.2: Effect of immunosuppression in infected ducks on clinical signs in in – contact chicken

Legend:

A: Depression; **B:** Ruffled feathers; **C:** Greenish diarrhea; **D:** Mortality; **E:** Sneezing; **F:** Sternal recumbency; **G:** Nervous tics; **IS:** Immunosuppressed; **NIS:** Non – immunosuppressed; **NDV:** Newcastle disease virus

6.3.2. Serological responses of immunosuppressed and non – immunosuppressed challenged ducks

No ducks in the two groups seroconverted until the sixth day post – inoculation. By day 6 post inoculation, 60% of IS and 100% NIS ducks respectively had seroconverted. The mean HI titers for both treatment groups increased and peaked on day 9 p.i. after which it decreased up to end of the experiment on day 29. The overall mean antibody titers for the IS treatment group was higher than the NIS one (**Table 6.3**). However, there was no significant difference in antibody titers between the two treatment groups ($p>0.05$).

All the chickens mixed with infected ducks, with exception of two birds (one survived upto day 9 p.i. and the other until the end of the experiment), died before they could raise antibodies to NDV. The two survivor chickens produced antibodies on day 9 p.i. The cock that survived to end of experiment had marked immune response that peaked on day 12 p.i. and then decreased gradually up to day 29 p.i.

Table 6.3: Mean hemagglutination inhibition titers of Newcastle disease virus challenged immunosuppressed and non - immunosuppressed ducks

Days post inoculation	NDV + IS		NDV + NIS	
	% of Ducks with Abs	Mean ± SE HI titre	% of Ducks with Abs	Mean ± SE HI titre
1	0.0	0.00	0.0	0.00
3	0.0	0.00	0.0	0.00
6	60	1.80 ±0.73	100	2.40 ± 0.40
9	100	6.40 ± 0.40	100	6.60 ± 0.40
12	100	6.00 ± 0.55	100	5.80 ± 0.49
15	100	5.00 ± 0.32	100	5.00 ± 0.32
18	100	4.60 ± 0.40	100	4.40 ± 0.51
20	100	3.60 ± 0.24	100	3.00 ± 0.32
23	100	3.60 ± 0.24	100	3.00 ± 0.32
26	100	3.00 ± 0.32	100	2.40 ± 0.24
29	100	2.60 ± 0.24	100	2.20 ± 0.20
Overall		**3.33 ± 0.30**		**3.16 ± 0.29**

Legend:

Abs: Antibodies; **SE:** Standard error; **NDV + IS** Ducks challenged with velogenic NDV (vNDV) after immunosuppression using dexamethasone; **NDV + NIS:** Non-immunosuppressed ducks and challenged with vNDV; **%:** Percentage; **HI:** Haemagglutination inhibition

6.3.3. Isolation of Newcastle disease virus from cloacal and oropharyngeal swabs of the experimental ducks

The ducks in the two treatment groups had detectable shedding of the ND virus from day 1 p.i. and stopped on day 15 p.i. There was no significant difference between the two treatments with respect to NDV shedding (P>0.05) (**Figure 6.3**).

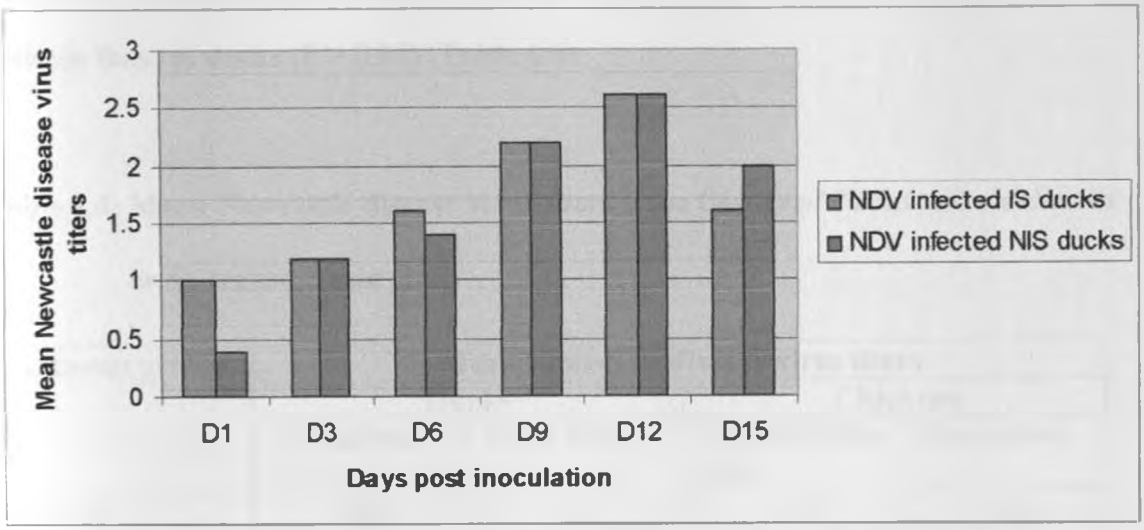

Figure 6.3: Mean Newcastle disease virus titers from cloacal and oropharyngeal swabs of immunosuppressed and non – immunosuppressed ducks

Legend:

NDV: Newcastle disease virus; **IS:** Immunosuppression; **NIS:** Non – immunosuppressed; **D1, D3, D6, D9, D12, 15:** Sampling days post inoculation

6.3.4. Recovery of Newcastle disease virus from tissues of chickens and experimentally infected ducks

The ND virus was recovered from pooled tissues of all the infected ducks on day 29 post inoculation. The mean NDV titers varied between the IS and NIS ducks and chickens mixed with each of the duck treatment group. However, there was no statistical significant difference in mean NDV titer between the two chicken treatment groups and between those of ducks ($P > 0.05$) (**Table 6.4**).

Table 6.4: Mean Newcastle disease virus titers from tissues of ducks and chicken in cross transmission study

Treatment groups	Mean Newcastle disease virus titers			
	Ducks		Chickens	
	% positive for NDV	Mean titers	% positive for NDV	Mean titers
IS +NDV	100	2.2	100	1.6
NIS +NDV	100	1.6	100	2.0
Control chickens	0	0	0	0
Non – infected ducks (control)	0	0	0	0

Legends:

IS+NDV: Ducks challenged with velogenic NDV (vNDV) after immunosuppression using dexamethasone; **NIS + NDV:** Non- immunosuppressed ducks, challenged with vNDV; **NDV:** Newcastle disease virus; **%:** Percentage

6.4. Discussion

In this study the in – contact chickens manifested varied clinical signs such as depression, ruffled feathers, diarrhoea, sneezing, nervous tics and sternal recumbency. However, only 2 immunosuppressed ducks showed depression and for only 3 days, while the rest did not show any clinical signs. This confirms what has been previously reported that ducks show very few or no signs of NDV even when infected with strains very virulent for chickens (Fagbohun *et al.*, 2000; Alexander, 2001b). Interestingly, Otim *et al.* (2006), using 3 week old chicks and 8 week old ducks reported neither clinical signs nor mortality in the in –contact chicks following inoculation of the ducks using a velogenic strain. However, the ducks in our experiment were immunosuppressed which may have predisposed them to the develop clinical signs of the disease unlike those non – immunosuppressed cohorts in our study and that of Otim *et al.* (2006).

Antibodies to NDV were detected in ducks 6 days post - inoculation. The positive HI titers obtained from the ducks indicated seroconversion and proved that the ducks were actually infected with NDV. The antibodies were also detected in two chickens that survived up to day 9 and 29 p.i. This means that in the event that both ducks and chickens are exposed to NDV, the ducks are likely to mount an immediate and strong immune response compared to chickens and this may partly explain the mechanism behind the resistance of these birds to NDV. Similarly, Otim *et al.* (2006) found that all the 3-week-old chicks and non – immunosuppressed grower ducks that were used in a preliminary cross- transmission study seroconverted seven days post inoculation. Although our study revealed that the immunosuppressed ducks had higher mean antibody titers than the non

– immunosuppressed ones, there was no statistical significant difference. This finding differs from earlier observations by Giambrone *et al.* (1985) and Otim *et al.* (2005) who reported that chicks fed aflatoxin, hence presumed immunosuppression produced lower antibodies than those not fed with aflatoxin.

This study showed that the mean NDV titers from cloacal and oropharyngeal swabs varied between IS and NIS infected ducks although the differences were not statistically significant and viral shedding was undetectable 15 days p.i. It is possible that the effect of immunosuppression by dexamethasone wears off after day 6, in which case it would have no effect on the release of the virus in ducks thereafter. Immunosuppressed ducks had slightly higher NDV titers than non – immunosuppressed ones in their tissues and the virus persisted for over 3 weeks (29 days) in these tissues. Therefore, Newcastle disease virus appeared to persist for long periods in ducks' tissues, which could act as a source of infection to susceptible chickens in mixed and neighbouring flocks. In other studies involving inoculation of indigenous free – range chicks with infectious bursal disease virus or aflatoxin indicated prolonged ND faecal virus excretion up to 4 weeks, although the two immunosuppressants did not affect antibody immune response significantly (Ghosh *et al.*, 1991; Otim *et al.*, 2005). In the case of V4 NDV strain, there appears to be no records of the virus being isolated more than two weeks after exposure as observed in this study (French *et al.*, 1967).

In conclusion, the study has demonstrated that vNDV infected immunosuppressed ducks, shed the virus, transmitting it to in – contact chickens causing high mortality while the

ducks remained carriers for over 2 weeks indicating second endemicity model. This simulates the potential for disease transmission scenario in rural duck – chicken mixed flocks. This has not been demonstrated before and is being reported here for the first time. Although IS and NIS ducks transmitted NDV to chickens the NIS ducks appeared normal, healthy, showed no clinical signs, and chickens in - contact to them showed markedly less clinical signs. While 2 out of 5 IS ducks showed mild clinical signs, none of the NIS had any clinical signs. However, IS ducks showed clinical signs, though mild, and the in - contact chicken showed more clinical signs. Immunosuppression therefore seems to increase the ability of ducks to transmit NDV to in – contact chickens. Chickens should therefore be separated from domestic ducks to prevent NDV transmission to the chickens. All domestic poultry should be vaccinated against NDV.

CHAPTER 7

7.0. Experiment 5: Determination of types of pathological lesions in immunosuppressed and non – immunosuppressed Newcastle disease virus carrier ducks

7.1. Introduction

The clinical signs and gross or microscopic lesions observed in birds infected with NDV are not pathognomic or exclusive for ND. The clinical disease might range from subclinical infection to 100% mortality in a short period. This depends on many factors related to the host (species, age, and immune status); the virus (strain, pathotype, dosage, and route of infection), and environmental or social stress which can influence the severity and the course of the disease as well as the occurrence and distribution of the lesions (Kaleta and Baldauf, 1988; Alexander, 1998).

In natural infections, the disease may vary from peracute to inapparent. Several types of the disease have been recognized (Alexander, 1991). The most severe type of infection, caused by VVND virus originally described by Doyle (1927), is responsible for panzootics. It is characterized by gastrointestinal lesions and high morbidity and mortality rate in unprotected chickens. Hemorrhagic and necrotic lesions in the gastrointestinal tract affecting Peyer's patches and lymphoid aggregates have been consistently reported in both natural and experimental infections of chickens with VVND virus, and some strains cause severe respiratory signs (Alexander, 1991; Hamid *et al.*,

1991). Studies with VVND virus have shown some variations in the degree and extent of involvement of other tissues and organs in different species of birds (Alexander, 1991; Hamid *et al.*, 1991). In ducks, NDV causes mild clinical signs or no clinical signs at all (Nishizawa *et al.*, 2007), but it is not reported whether they develop any lesions. It is hypothesized that NDV carrier ducks do not manifest gross and histopathological lesions in their tissues. Thus, the aim of this study was to examine whether carrier ducks had any lesions and to record the type of pathology in immunosuppressed and non – immunosuppressed ducks.

7.2. Materials and methods

7.2.1. Experimental birds

One-day-old indigenous ducklings and chicks were hatched from the poultry flock maintained at the University of Nairobi premises. All the birds were reared in an isolation unit and then transferred to experimental units at the age of one year. They were wing tagged, tested and confirmed to be free of ND virus and antibodies.

7.2.2. Experimental design

Ninety-five ducks and 12 chickens free from NDV antibodies were used in this experiment. The 95 ducks were divided into 3 treatment groups and one control group as follows: (i) immunosuppressed and inoculated with NDV (38 ducks); (ii) non – immunosuppressed and inoculated with NDV (37 ducks); (iii) 10 ducks that were immunosuppressed only; and (iv) 10 control ducks. Twelve naïve chickens were used as infected positive controls. Ducks and chickens were housed separately according to their

treatment groups. The respective groups of birds were immunosuppressed using dexamethasone prior to inoculation with the velogenic NDV. The two respective infected groups of ducks and all the chickens were each inoculated intranasally with 0.2 ml of undiluted amnioallantoic fluids of vNDV with a titre of 1:1024 per bird, 24 hours after the last injection of dexamethasone. They were observed twice daily for clinical signs by the same person. Ducks were sacrificed on days 1, 4, 8, 14 and 28 p.i. and six tissues (liver, spleen, lung, cecal tonsils, kidneys and brain) sampled from each bird. All the chickens were sacrificed on day 4 p.i. on humane grounds since they were very sick.

7.2.3. Newcastle disease virus strain used

A velogenic Newcastle disease virus (strain L1) described in section 6.2.3 was used.

7.2.4. Immunosuppression of the ducks

Immunosuppression of ducks was carried out as described in section 6.2.4.

7.2.5. Post mortem examination and sample collection

Birds (ducks and chickens) were killed by cervical dislocation. Post – mortem was carried out as described by Bermundez and Steward – Brown (2003) and Chalton (2006). Birds were opened aseptically; tissues and organs were examined individually for gross lesions, findings and recorded. The severity of the lesions was scored as mild, moderate or severe after Shivaprasad and Droual (2002). From each bird, six tissues (liver, spleen, lung, cecal tonsils, kidneys and brain) were collected in 10% neutral buffered formalin for microscopical examination.

7.2.6. Histological processing of tissues

The tissues were kept fully immersed in 10% formalin solution for 24 hours. They were then transferred into 70 % alcohol, where they remained until trimming was done (Brown, 1998). The fixed tissues were manually trimmed to a thickness of 2 to 3 mm. The trimmed tissues were then placed in an automatic tissue processor for the following treatments: - (1) dehydration using: - (i) 80% ethyl alcohol for the first 4 hours, (ii) 96% alcohol for the next 4 hours, and (iii) 100% alcohol for 4.5 hours; (2) clearing with xylene for 5 hours; and (3) impregnating with molten paraffin wax at 60^0C for 6 hours. The tissues were then removed from the processor and were embedded into wax blocks using a molten wax dispenser. Individual tissue blocks were separated and fixed onto microtome chunks using a searing spatula, after which they were sectioned to 3 -5 µm thickness, floated on a water bath at 50^0C to flatten out, placed on a microscope slide and dried in an oven at 60^0C for about one hour. The sections were then quickly dewaxed in xylene and washed in alcohol before re – hydrating in water. They were then stained using haematoxylin and eosin, mounted in destrene 80, dibutylphthalate and xylene (DPX) and the slides left to dry before their examination under the microscope.

7.3. Results

7.3.1. Gross lesions observed in infected experimental ducks

Lesions encountered in the experimental ducks were congestion and mild haemorrhages in various organs, airsacculitis, lymphoid hyperplasia and splenomegally of the spleen, proventricular mucosal ulcers, and necrotic foci on the spleen. Airsacculitis, necrotic foci on the spleen and congestion of the small intestines were dominant in the

immunosuppressed as compared to the non – immunosuppressed ducks. Mild hemorrhages on the proventriculus mucosa were observed in immunosuppressed ducks. Splenomegally and haemorrhagic - enlarged cecal tonsils were observed in the non – immunosuppressed birds (**Figure 7.1**).

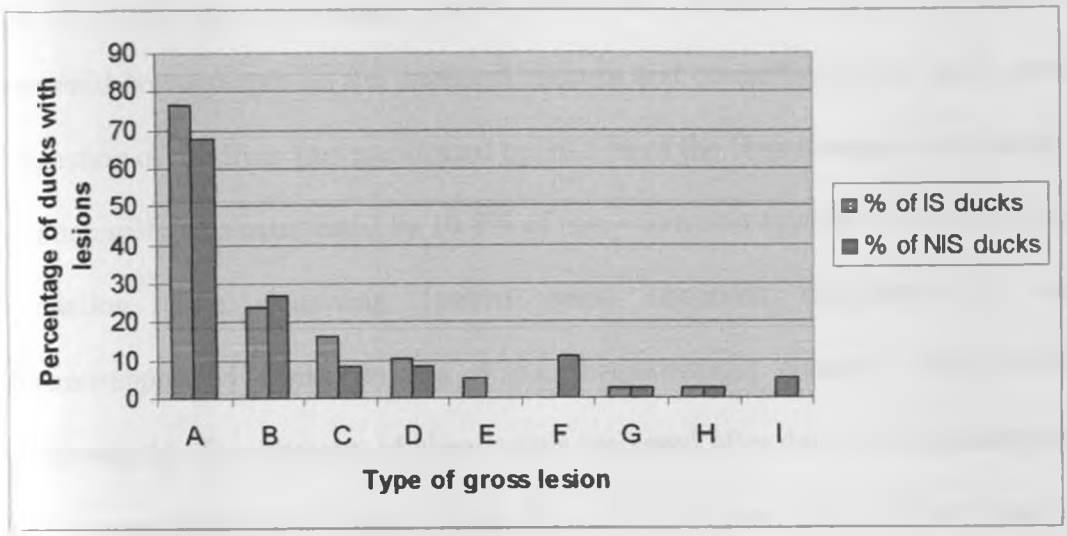

Figure 7.1: Percentage of immunosuppressed and non – immunosuppressed ducks showing different gross lesions

Legend:
A = Airsacculitis; **B**= Hemorrhages on the duodenal mucosa; **C**= Congestion of the small intestines; **D**= Congestion of the liver; **E**= Necrotic foci on the spleen; **F**= Splenomegally; **G**= Congestion of the trachea; **H**= Hemorrhages on the kidneys; **I**= Enlarged haemorrhagic cecal tonsils; **IS:** Immunosuppressed ducks; **NIS:** Non – immunosuppressed ducks; **%:** Percentage of ducks with lesions

The earliest (D_1 post challenge) lesions manifested by ducks in both treatment groups were mild hemorrhages on the duodenal mucosa and congestion of the small intestines. Congestion of the liver was manifested by 10.5 % of the immunosuppressed ducks while splenomegally was manifested by 10.8% of non – immunosuppressed ducks one day post inoculation. The following lesions were observed exclusively in non – immunosuppressed ducks on day 4 p.i.; haemorrhagic enlarged cecal tonsils and splenomegally. The intensity of airsacculitis increased after day 1 post challenge mainly in the immunosuppressed ducks (**Table 7.1**). All the control ducks did not manifest any gross lesions of Newcastle disease.

Table 7.1: Percentage of immunosuppressed and non – immunosuppressed ducks manifesting gross lesions with respect to days post – inoculation

Type of Gross Lesion	Days post – inoculation and percentage of NIS and IS ducks manifesting the gross lesion									
	D1		D4		D8		D14		D28	
	IS	NIS	IS	NIS	IS	NIS	IS	NIS	IS	NI
A	0	25	100	83.3	83.3	83.3	100	83.3	80	
B	60	25	0	33.3	33.3	66.7	33.3	33.3	13.3	
C	20	25	16.7	0	0	0	33.3	16.7	13.3	
D	10.5	0	33.3	16.7	0	0	16.7	0	0	
E	0	0	16.7	0	16.7	0	0	0	0	
F	0	10.8	0	33.3	0	0	0	0	0	
G	0	0	0	0	0	0	0	16.7	6.7	
H	0	0	16.7	16.7	0	0	0	0	0	
I	0	0	0	33.3	0	0	0	0	0	

Legend:

A = Airsacculitis; **B**= Hemorrhages on the duodenal mucosa; **C**= Congestion of the small intestines; **D**= Congestion of the liver; **E**= Necrotic foci on the spleen; **F**= Splenomegally; **G**= Congestion of the trachea; **H**= Hemorrhages on the kidneys; **I**= Enlarged haemorrhagic cecal tonsils; **D1, D4, D8, D14** and **D28** = Days 1 to 28 post – inoculation; **IS** = Immunosuppressed; **NIS** = Non – immunosuppressed

7.3.2. Microscopic lesions of non – immunosuppressed and immunosuppressed ducks

Microscopic lesions encountered in ducks were haemorrhages, congestion, lymphoid depletion of cecal tonsils and spleen; focal infiltration of mononuclear cells, perivascular cuffing in the brain and the liver; and necrosis and central chromatolysis of neurons. Majority of the immunosuppressed ducks manifested congestion in various organs and moderate lymphoid depletion in the spleen and cecal tonsils of immunosuppressed ducks at day 1 post inoculation. Focal infiltration of mononuclear cells was observed from days 1up to 8 post - inoculation in the lungs, liver, kidney and brain tissues especially in the immunosuppressed ducks. Most lesions were observed on day 4 and 8 post – inoculation. The following lesions were manifested by immunosuppressed ducks only: perivascular cuffing in the brain and iiver, focal necrosis of the spleen and central chromatolysis of neurons **(Table 7.2; Figure 7.2).**

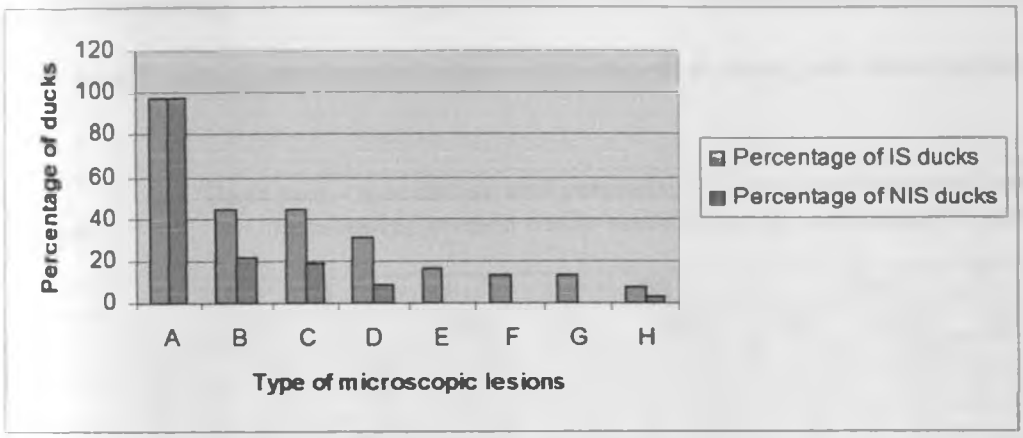

Figure 7.2: Percentage of immunosuppressed and non-immunosuppressed ducks manifesting various microscopic lesions

Legends:

A: Congestion of the liver; **B:** Lymphoid depletion of cecal tonsils; **C:** Lymphoid depletion of spleen; **D:** Focal infiltration of mononuclear cells; **E:** Perivascular cuffing in liver and brain; **F:** Necrosis of the spleen; **G:** Central chromatolysis of neurons; **H:** Haemorrhages in the kidney; **IS:** Immunosuppressed; **NIS:** Non – immunosuppressed

Table 7.2: Percentage of immunosuppressed and non – immunosuppressed ducks manifesting microscopic lesions with respect to days post –inoculation

Type of microscopic Lesion	Days post – inoculation and percentage of immunosuppressed and non – immunosuppressed ducks manifesting the microscopic lesions									
	D1		D4		D8		D14		D28	
	IS	NIS	IS	NIS	IS	NIS	IS	NIS	IS	NIS
A	80	100	100	100	100	0	100	100	100	93.3
B	80	0	50	16.7	33.3	16.7	16.7	33.3	46.7	26.7
C	60	25	66.7	16.7	33.3	0	0	50	53.3	13.3
D	60	75	83.3	0	66.7	0	0	0	0	0
E	40	0	33.3	0	33.3	0	0	0	0	0
F	20	0	33.3	0	16.7	0	0	0	6.7	0
G	0	0	33.3	0	16.7	0	0	0	13.3	0
H	0	0	0	0	16.7	0	16.7	16.7	6.7	0

Legends:

A: Congestion of the liver; **B:** Lymphoid depletion of cecal tonsils; **C:** Lymphoid depletion of spleen; **D:** Focal infiltration of mononuclear cells in the liver, lung, kidney and brain; **E:** Perivascular cuffing of liver and brain; **F:** Necrosis of the spleen; **G:** Central chromatolysis of neurons; **H:** Haemorrhages in the kidney; **D1, D4, D8, D14, D28:** Days 1 to 28 post – inoculation; **IS:** Immunosuppressed; **NIS:** Non – immunosuppressed

7.3.3. Gross lesions of the positive control indigenous chickens

The gross lesions manifested by chickens used as indicators of NDV pathogenicity were congestion of the small intestine; airsacullitis; hyperplasia of the spleen; necrotic hemorrhagic peyers patches; haemorrhagic cecal tonsils; haemorrhages and ulcers on the proventriculus mucosa; and ulcers and haemorrhages on large intestinal mucosa (**Figure 7.3**). The commonest gross lesions were; hyperplasia of the spleen (83.3%); ulcers on proventriculus mucosa (50%); haemorrhagic hyperplastic peyers patches (41.7%); and ulcerated hyperplastic peyers patches (33.3%) (**Figure 7.4).** The lesions were more severe compared to those observed in the ducks.

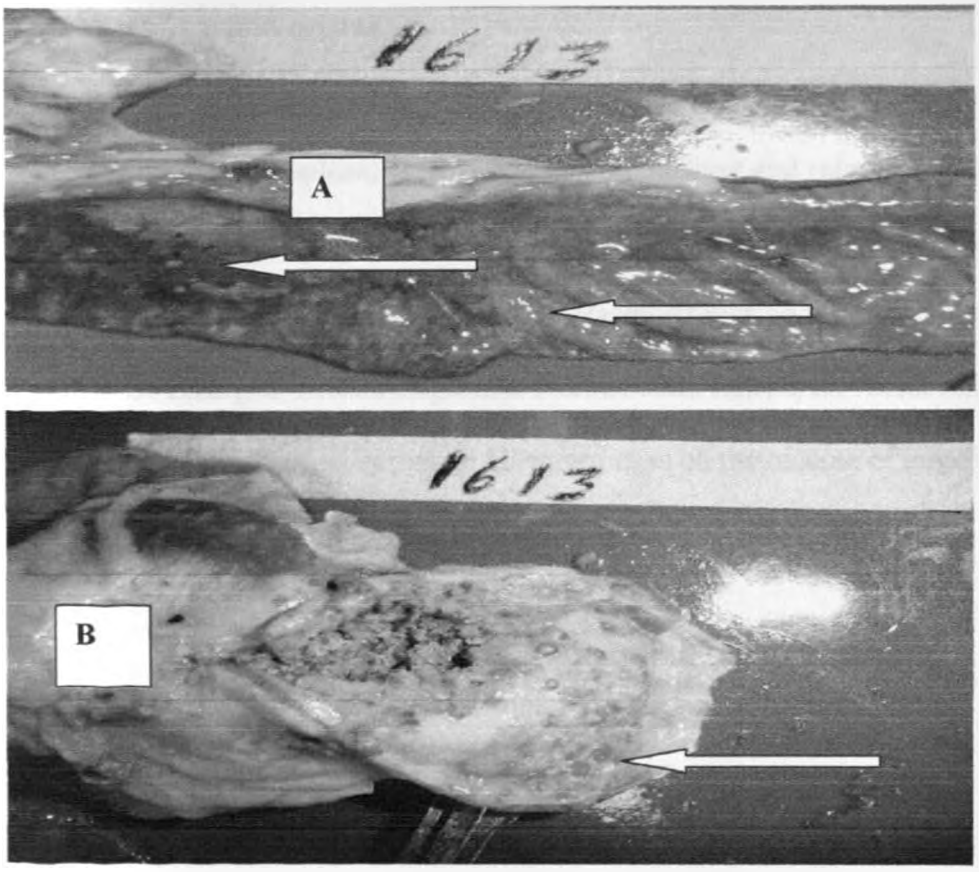

Figure 7.3: Duodenal mucosa (A) showing necrosis (arrows) and proventriculus (B) showing haemorrhages (block arrow) in a positive Newcastle disease control chicken

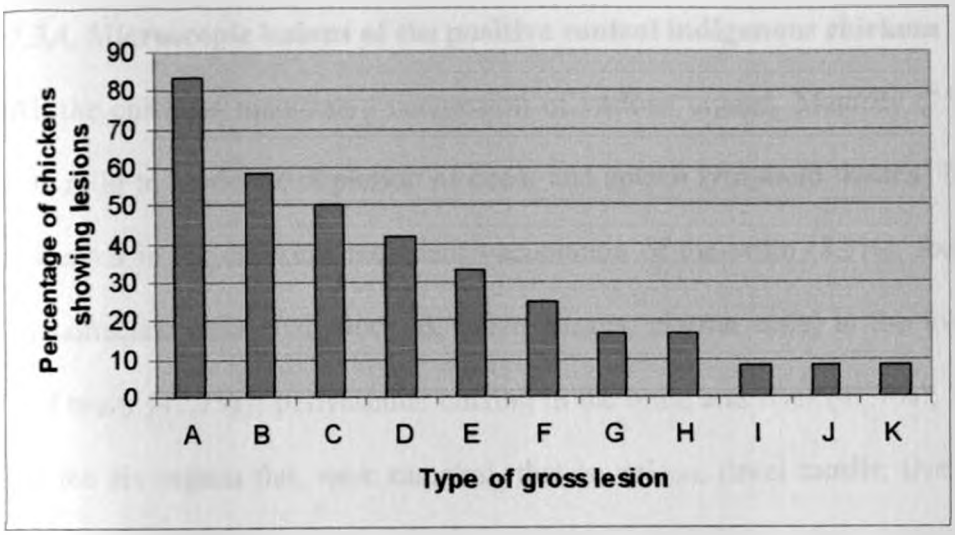

Figure 7.4: Percentage of chickens showing different gross lesions in various organs

Legends

A: Hyperplasia of the spleen; **B:** Necrotic foci on the liver and spleen; **C:** Ulcers on proventriculus mucosa **D:** Haemorrhagic hyperplastic peyers patches; **E:** Ulcerated, necrotic and hyperplastic peyers patches; **F:** Haemorrhages on the proventriculus mucosa; **G:** Enlarged, haemorrhagic and necrotic cecal tonsils; **H:** Airsacculitis; **I:** Congestion of the small intestines; **J:** Haemorrhages on the mucosa of large intestines; **K:** Ulcers on mucosa of large intestines

7.3.4. Microscopic lesions of the positive control indigenous chickens

All the chickens manifested congestion of various organs. Majority (75%) of the birds had mild to moderate depletion of cecal and spleen lymphoid tissues. The other lesions observed in the chickens included: vacuolation of the brain (8.3%); focal infiltration of mononuclear cells (lymphocytes, macrophages, plasma cells) in the liver, lung, kidney and brain {41.7%}; perivascular cuffing in the brain and liver (41.7%); focal necrosis on all the six organs that were sampled, that is; spleen, cecal tonsils, liver, lungs, kidneys and brain (58.3%); hemorrhages on cecal tonsils, liver, lungs and kidneys (41.7%); central chromatolysis (16.7%); neuronal degeneration (8.3%) and oedema of the lung, cecal tonsils and brain (33.3%) **(Table 7.3; Figures 7.5 and 7.6).**

Table 7.3: Percentage of chickens showing different types of microscopic lesions

Type of microscopic lesions	Percentage of chickens manifesting the lesions
A	100
B	75
C	58.3
D	41.7
E	41.7
F	41.7
G	33.3
H	16.7
I	8.3
J	8.3

Legend:
A: Congestion of various organs; **B:** Mild to moderate lymphoid depletion; **C:** Focal Necrosis on various organs; **D:** Focal infiltration of mononuclear cells (lymphocytes, macrophages, plasma cells); **E:** Perivascular cuffing; **F:** Hemorrhages on various organs; **G:** Oedema of brain and lungs; **H:** Central chromatolysis of the neurons; **I:** Neuronal degeneration; **J:** Vacuolation

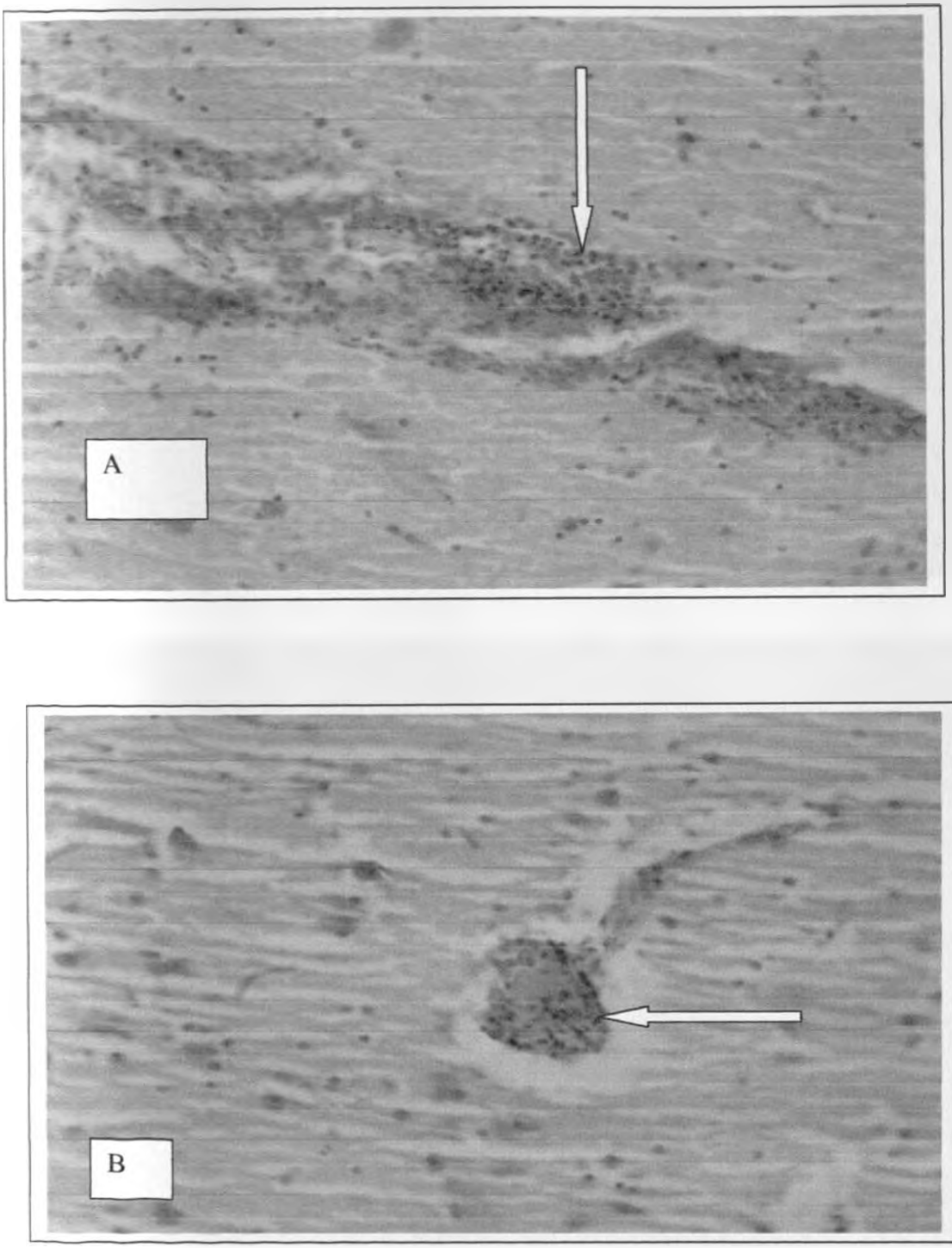

Figure 7.5: Photomicrographs of brain from a positive Newcastle disease control chicken showing perivascular cuffing (arrows) (HE, A: x10 and B: x25)

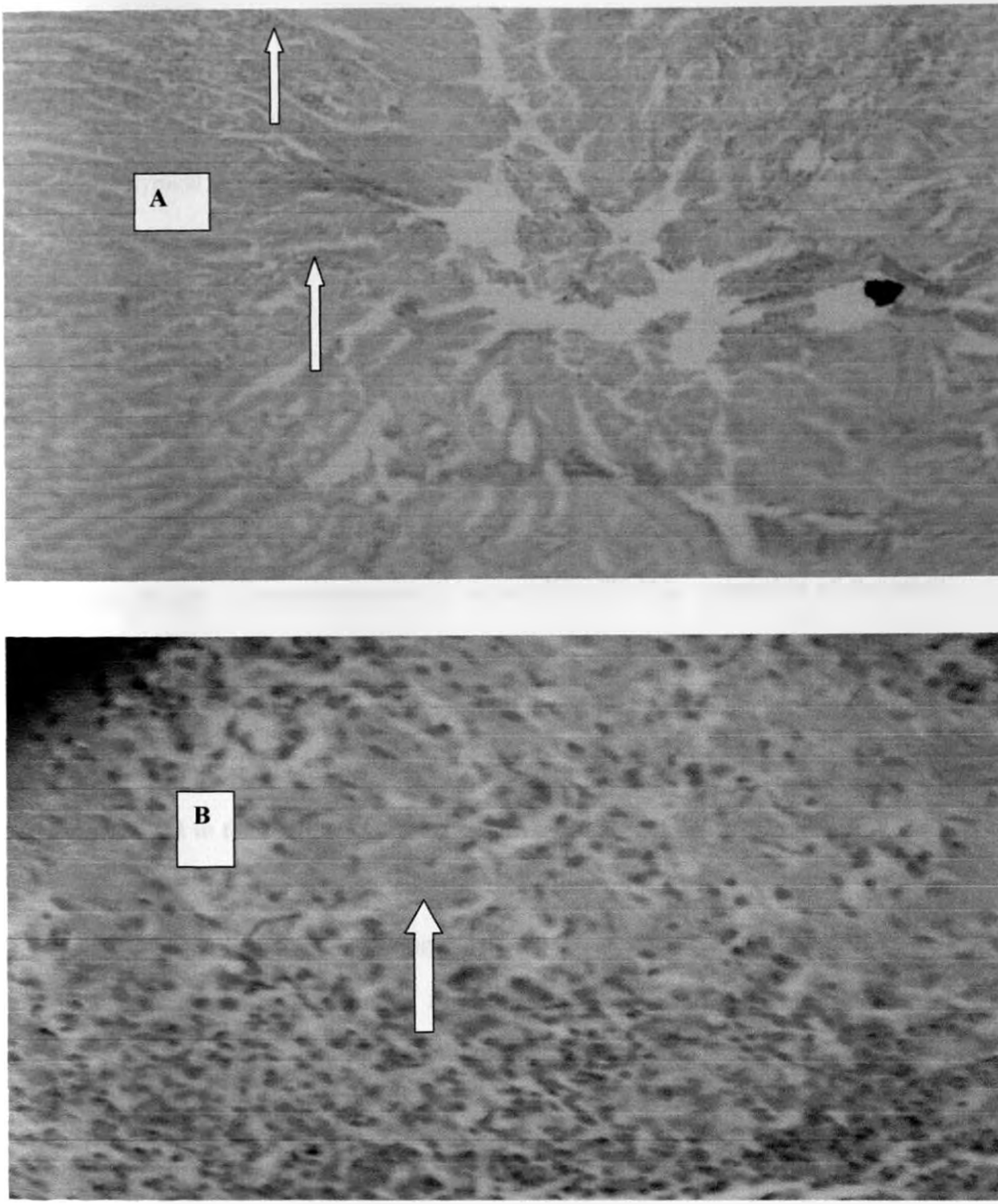

Figure 7.6: Photomicrographs of proventriculus (A) showing congestion (arrows) and spleen (B) showing necrosis (arrows) from a positive Newcastle disease control chicken (HE, A: 25x; B: 40x)

7.4. Discussion

The positive control chickens inoculated at the same time and same dosage as the ducks developed typical gross and histopathological changes characteristic of highly virulent NDV. This means that the virus used in the challenge of these birds (ducks and chickens) was highly virulent.

More immunosuppressed ducks manifested macroscopic and microscopic lesions than the non – immunosuppressed ducks as illustrated below. Of particular interests are the following gross lesions, which were observed more in immunosuppressed ducks compared to non – immunosuppressed: airsacculitis (76.3%), congestion of the small intestines (15.8%) and necrotic foci on the spleen (5.3%). It is possible that the immunosuppressed ducks in this study, had increased ND viral multiplication virus thus causing pathological lesions in various tissues; the lesions were very mild, compared to those observed in the positive control chickens. Otim *et al.* (2006) and Nishizawa *et al.* (2006) reported no pathological lesions following inoculation of non – immunosuppressed ducks with velogenic NDV. However, in an outbreak of ND in unvaccinated commercial duck farm, Roy *et al.* (1992) observed lesions in the intestinal tract, as reported in this study.

The most remarkable and consistent gross lesion observed was airsacculitis, observed as early as 4 days post inoculation in both duck treatment groups. Airsacculitis was more severe in immunosuppressed ducks (76.3%) than in non – immunosuppressed (67.6%)

counterparts. Severe airsacculitis has been reported in chickens experimentally infected with velogenic NDV (Brown *et al.*, 1999a) and was observed in this study. In this study, airsacculitis was more prevalent in ducks than chickens possibly due to species differences.

Oedema of the lungs and brain was seen in chicken but was not observed in ducks. Disseminated microscopic lesions in the lymphoid tissues indicated marked lymphotropism in ducks as previously described with other highly virulent NDV isolates in chickens (Kuiken *et al.*, 1999; Kommers *et al.*, 2001). This involved lymphoid depletion of the spleen and cecal tonsils. The depletion was more pronounced in the immunosuppressed ducks (44.7%) in all the treatment groups throughout the sampling period. In addition, lymphoid cell necrosis was observed in the spleen of immunosuppressed, infected ducks (13.2%) though not as severe as in positive control chickens (58.3%).

Significant brain lesions observed in this study included: central chromatolysis of neurons (13.2%) and perivascular cuffing (15.8%) in immunosuppressed ducks. Kaleta and Baldauf (1988) and Alexander (1998) reported that environmental or social stress could influence the severity, disease course, as well as the occurrence and distribution of the lesions in chicken. Perivascular cuffing resolved after day 8 p.i. while neuronal chromatolysis persisted through the entire sampling period in ducks. Unlike in chicken where Bhaiyat *et al.* (1994) indicated that the neuronal degeneration subsided after 21 days p.i., the perivascular cuffing was more prominent during the chronic stages of the

disease. The type of lesion and course of infection within the central nervous system depends upon a number of factors. These include age and immunocompetence of the host at the time of exposure and the neurotropic and immunosuppressive properties of the virus (Summers *et al.*, 1984). These factors may explain the difference between the immunosuppressed and non – immunosuppressed ducks in this study.

Vascular lesions in the brain sections observed in IS ducks and chickens in this study are consistently seen in ND viral encephalitis in chickens though not documented in NIS ducks. The nonsuppurative inflammatory response in the brain is associated with a proliferative vasculitis (Kommers *et al.*, 2002). Perivascular cuffing occurred predominantly in areas of general parenchyma cell infection where a high proportion of the infiltrating cells were also infected.

In conclusion, NDV infected ducks show different pathological lesions in various organs of IS and NIS birds. However, infected chickens show more severe lesions, which are distributed in more organs, compared to ducks. In this study, immunosuppressed ducks manifested more lesions. Although there were no gross lesions in the brains of ducks, the histological lesions in the brain were present in control chickens but less severe. Thus, this study has demonstrated that immunosuppression aggravates gross and histopathological lesions of NDV in ducks demonstrating the third component of the endemicity model.

CHAPTER 8

8.0. Experiment 6: Localization of Newcastle disease viral antigen in tissues of immunosuppressed and non – immunosuppressed ducks

8.1. Introduction

Newcastle disease virus occurs in several forms of virulence, these forms are classified into pathotypes known as velogenic, mesogenic, and lentogenic (Alexander, 1997). Velogenic strains are those causing severe disease with high mortality. Velogenic viruses may be carried inapparently in wild birds such as pigeons and cormorants (Collins *et al.*, 1994; Seal *et al.*, 1995; King, 1996), chickens and ducks. Such ducks are able to spread the virus to chickens in a duck – chicken mixed flock. Therefore, there is concern over the location of the virus in the carrier ducks tissues and organs that may lead to recrudescence when they are immunosuppressed leading to periodic emergence of disease. Thus, it is hypothesized that NDV multiplies and is sequestered in specific tissues of carrier ducks.

The use of immunohistochemistry to detect ND infections in the tissues of chickens has been reported by several workers (Kommers *et al.*, 2002; Kuiken *et al.*, 1999). This technique offers a rapid means of identifying various antigens, including viruses. It can be applied to formalin – fixed, paraffin – embedded tissues, potentially providing a diagnosis even in cases in which fresh tissue or serum is unavailable (Lockaby *et al.*,

1993). The purpose of the present study was to determine the location of NDV in carrier ducks, using immunoperoxidase staining technique.

8.2. Materials and Methods

8.2.1. Experimental birds

One-day-old indigenous ducklings and indigenous chickens were hatched from the poultry flock maintained at the University of Nairobi premises. All the birds were reared in an isolation unit and then transferred to experimental units at one year of age. They were wing tagged, tested and confirmed to be free of ND virus and antibodies.

8.2.2. Experimental design

A total of 57 ducks were used in this study. They were divided into 3 groups: (i) immunosuppressed and inoculated with NDV (23 ducks); (ii) non – immunosuppressed and inoculated with NDV (22 ducks); and (iii) 12 control ducks. The respective groups were inoculated intranasally with 0.2 ml of undiluted amnioallantoic fluids of VNDV with a titer of 1:1024.

All the ducks were transfered into the experimental units at the age of 12 months. Birds were sacrificed on day 1, 4, 8 and 14 post inoculation (p.i.) and 6 tissues (liver, spleen, lungs, kidneys, cecal tonsils and brain) sampled from each bird. On day 1 p.i., five immunosuppressed, viral challenged ducks together with four non – immunosuppressed ducks and 3 control ducks were sacrificed through cervical dislocation. Six ducks from immunosuppressed and non- immunosuppressed challenged groups, and 3 control ducks

were sacrificed on days 1, 4, 8, and 14 p.i. The tissues were preserved in 10% neutral formalin, processed and immunoperoxidase labelling done.

8.2.3. Newcastle disease virus strain used

A velogenic Newcastle disease virus (strain L1) obtained from the repository maintained at the University of Nairobi was used in this study as described in section 6.2.3.

8.2.4. Immunosuppression of the ducks

Dexamethasone (Dexamethasone sodium phosphate and Sodium methyl hydroxybenzoate, Coophavet, St Herblon – BP 7- 44153 Ancenis Cedex – France) was used to induce stress in ducks for this study as described by Miller *et al.* (2003) and in section 6.2.4.

8.2.5. Histological processing of tissues

The tissues were kept fully immersed in 10% formalin solution for 24 hours and processed as described by Brown (1998) and briefly in section 7.2.6. Teflon coated microscope slides (positive slides) were used and dried in an oven at 60^0C for about one hour to enhance adhesion of tissue sections and minimize loss of sections during immunostaining.

8.2.6. Immunohistochemistry labeling of Newcastle disease viral antigen

Immunoperoxidase staining was performed to detect viral nucleoprotein (NP) as described by Boenisch (2001) and summarized here below.

8.2.6.1. Deparaffinization of the tissues

The tissue sections on teflon positive coated slides were heated to 70^0C for 10 minutes. They were put on holding racks and deparaffinized in coplin jars by two changes of xylene for 5 minutes each step, dehydrated twice with 99% ethanol for 5 minutes at each step, re - hydrated in 96% ethanol for 5 minutes, followed by 70% ethanol for 5 minutes and finally three changes of tris buffered saline (TBS) for 5 minutes in each washing (Boenisch, 2001).

8.2.6.2. Antigen retrieval from the tissues

The sections were placed in a plastic slide holder and then put into 200ml Tris/ ethylenediaminetetraacetic acid (EDTA) buffer, pH 9.0. They were subjected to antigen retrieval by microwaving at full power until the buffer boiled, after which, the heat was reduced to approximately 450^0C and timed for another 5 minutes. After five minutes, the container was refilled with distilled water up to the mark and then microwaved for a further 5 minutes. After the second treatment, the sections were left in the Tris/EDTA buffer for 20 minutes at room temperature. They were rinsed after 20 minutes in distilled water and then washed in TBS for 5 minutes (Boenisch, 2001).

8.2.6.3. Blocking the tissues

The tissue were circled using a hydrophobic pen (Dako® PAP – pen, DAKO A/S, Glostrup, Denmark) and 300µl of 20% of Roche blocking buffer added on each tissue section, put in a humidified moist chamber and incubated for 20 minutes at room temperature. The buffer was poured off by tapping the slides on absorbent tissue papers.

8.2.6.4. Application of primary antibody on the tissues

A mixture of two mouse monoclonal antibodies: Anti - nucleoprotein and anti - hemagglutinin neuraminidase, donated by Prof. Ronald Iorio, Machassuchetts University (USA), each diluted 1:5 in Roche blocking buffer, were applied on each tissue section and incubated overnight in a humidified chamber at 4^0C. Antibody was poured off, tissue sections transferred to two changes of Tris buffer solution (TBS) for 1 minute in each step and put into one change of the same TBS for 5 minutes at room temperature.

8.2.6.5. Application of secondary and tertiary antibodies on the tissues

The secondary antibody was a rabbit anti – mouse (DAKO® Z259) diluted 1: 25 in 20% swine serum in TBS. Tissue sections were placed in humidified moist chambers, applied 300 µl of the antibody per section, and incubated at 37^0C for 30 minutes. The antibody was poured off, and tissues were transferred to two changes of TBS, 1 minute each step, and one change of TBS for 5 minutes at room temperature. The tertiary antibody, mouse (DAKO® D651) tagged to alkaline phosphatase – antialkaline phosphatase (APAAP), diluted 1:50 in 20% swine serum in TBS was applied on the tissue sections in the humid moist chambers and incubated at 37^0C for 30 minutes. The tertiary antibody was poured off and the slides washed in TBS.

8.2.6.6. Staining using Fast Red (KemEnTec)

Fast red substrate was prepared by adding 1 tablet of Fast Red to 2 ml of the substrate buffer (0.1 M TRIS/ HCl, pH 8.2) and mixed thoroughly for 3 minutes. The solution was filtered using Whatman No. 2 paper, applied on each tissue section in humidified

chambers and incubated at 37^0C for 30 minutes. The colour development was monitored by examining the sections every 10 minutes at low magnification (x4), for minimum background staining and maximum signal detection. Tissues were washed in TBS at room temperature as described in section 8.2.6.4.

They were then counterstained with Mayer's haematoxylin for 10 seconds, the stain was removed in running water for 1 minute; washed in deionised water for 4 minutes and cover slip applied using glycergel before examination.

8.2.6.7. Negative and positive control tissues

Tissues from normal chickens and ducks that were negative for NDV were used as negative controls. These tissue sections were subjected to similar treatments throughout the immunoperoxidase staining procedure. The isotype – matched monoclonal antibody (bovine respiratory synthial virus) diluted 1:5 was applied to positive tissues from NDV – inoculated ducks and used as positive controls.

8.3. Results

8.3.1. Location of Newcastle disease viral antigens in tissues of carrier ducks

The ND viral antigens were detected in the cytoplasm of macrophages of cecal tonsils of 13 ducks (28.9%). The viral antigens were in areas where vast majority of cells were lymphocytes. In the kidney, they were found in the tubular epithelial cells of one duck only (2.2%). The viral antigens were located in the cytoplasm and nucleolus of the respective cells. The ND viral antigen was not detected in the liver, lungs, spleen and

brain of all the infected ducks. All the control chicken and duck tissues were negative for viral antigen (**Figure 8.1**).

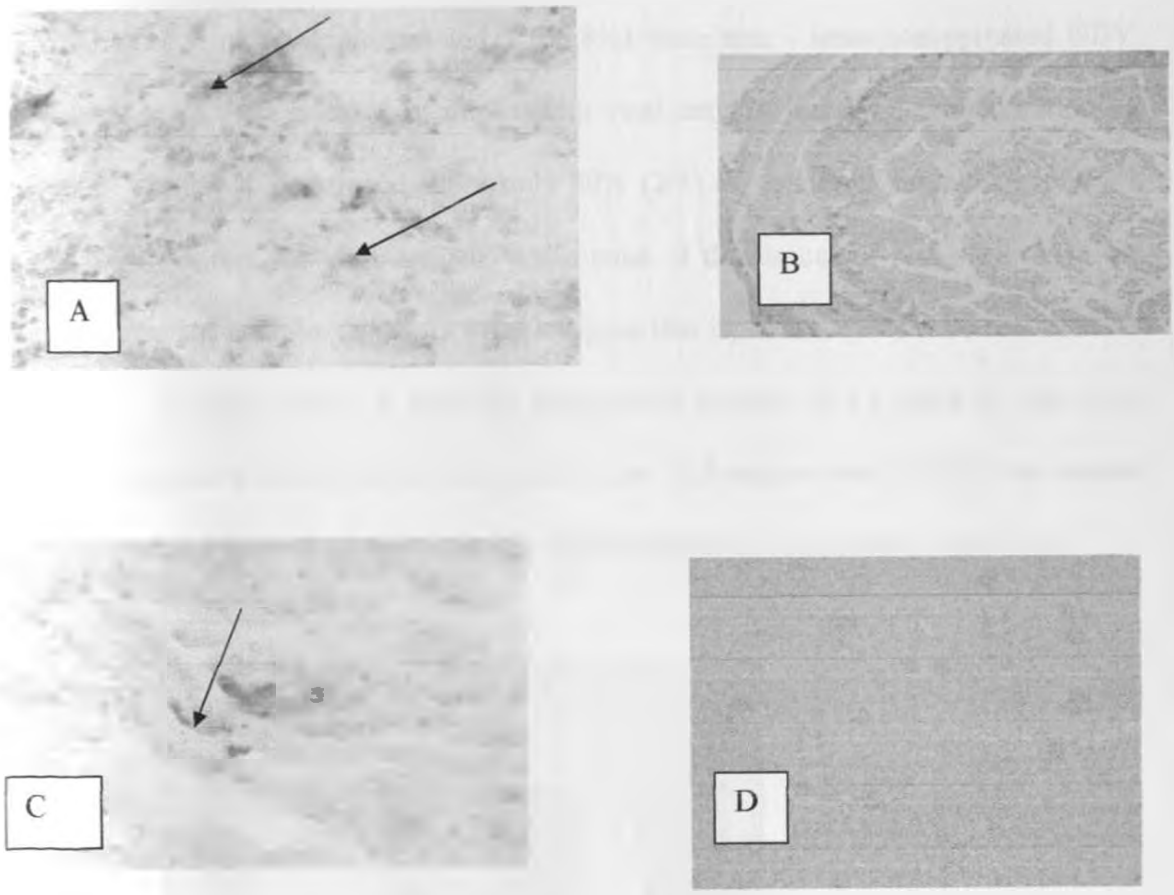

Figure 8.1: Photomicrographs of cecal tonsils (A): of a duck number 1725 showing Newcastle disease viral antigen (arrows), magnification x40; **(B)**: Newcastle disease viral antigen negative caecal tonsils from a control duck, magnification x10) and kidneys; **(C):** of a duck number 1877 positive for Newcastle disease viral antigen (arrows), magnification x40; **(D):** Newcastle disease viral antigen negative kidneys from a control duck, magnification x10) (Mayer's haematoxylin)

8.3.2. Percentage of ducks positive for Newcastle disease viral antigen

In total 13 ducks out of 45 (28.9%) were positive for ND viral antigen out of which 6 (46.2%) were immunosuppressed and 7 (53.8%) were non – immunosuppressed NDV inoculated birds. The number of ducks with viral antigen increased with duration of infection. On day 1 post inoculation, only 40% (2/5) of tissues of immunosuppressed ducks were positive for viral antigen, while none of the tissues of NIS were positive. Equal number of positive ducks for viral antigens that is, 33.3% (2/6) were recorded on day 8 p.i., in tissues from IS and NIS ducks while on day 14 p.i. more of the non-immunosuppressed ducks (50%) were positive for viral antigen and 33% for the tissues from IS ducks (**Figure 8.2**). However, the difference was not statistically significant.

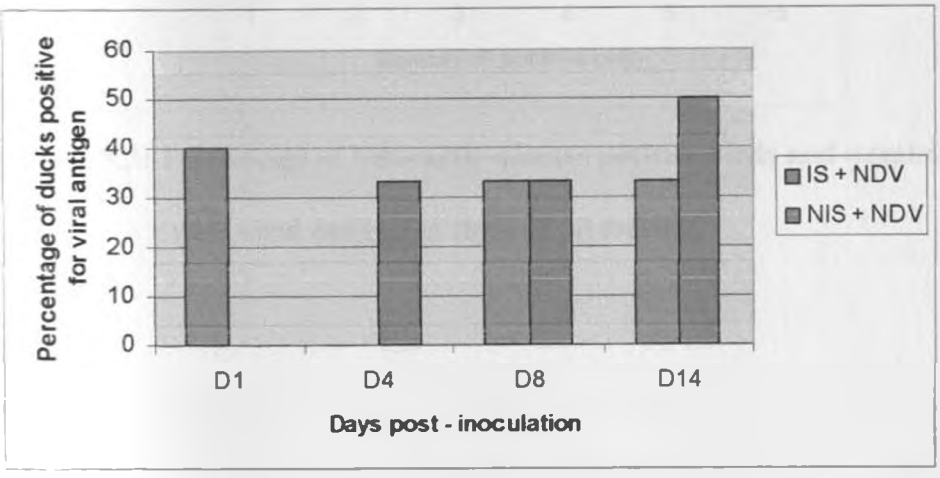

Figure 8.2: Percentage of experimentally infected immunosuppressed and non-immunosuppressed ducks positive for Newcastle disease viral antigen in their tissues, with respect to days post inoculation

Legend

IS: Immunosuppressed, **NIS:** Non – immunosuppressed, **NDV:** Newcastle disease virus, **D1,D4,D8, and D14:** Days 1 to 28 post – inoculation

8.3.3. Intensity of the viral antigen in the cecal tonsils of individual birds

The intensity of viral antigen pooled for all ducks was as follows: 15.4% had 4 cells; 53.8% had 5 cells and 30.8% had more than 5 cells infected per tissue section of cecal tonsils (**Figure 8.3**). In the kidneys, more than 5 positive cells were recorded.

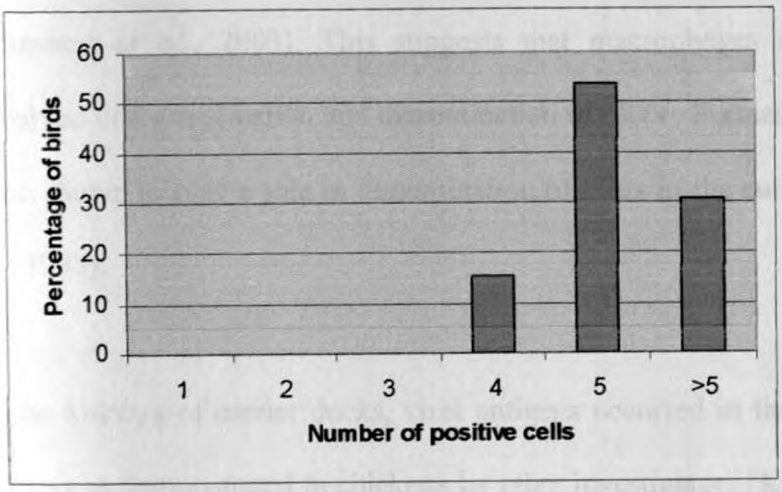

Figure 8.3: Percentage of Newcastle disease positive birds and number of cells with viral antigen in their cecal tonsils

8.4. Discussion

In the present study, immunoassaying demonstrated NDV antigens in macrophages and in areas where vast majority of the cells are lymphocytes. This suggests that these cells are involved in viral replication in carrier ducks. Lymphoid cells of cecal tonsils are reported to have viral nucleoprotein in infected chickens and in carrier ducks (Lam, 1996; Kommers *et al.*, 2003). This suggests that macrophages and lymphocytes may be involved in the replication and dissemination of NDV. Furthermore, lymphoid cells have been shown to play a role in dissemination of virus in the entire host system (Bhaiyat *et al.*, 1995).

In the kidneys of carrier ducks, viral antigens occurred in the tubular epithelium of the kidneys as demonstrated in chickens by other investigators (Kommers *et al.*, 2001). Viral replication in the tubules may damage and compromise the renal epithelia (Kommers *et al.*, 2002; 2003). This will result in impaired excretion, ionic imbalance and could allow the entry of secondary infectious agents (Kommers *et al.*, 2002; 2003). Interestingly, other workers (Brown *et al.*, 1999 a) who utilized commercial chickens that had clinical disease did not demonstrate any positive immunohistochemistry labelling in the kidneys. May be in ducks, this is a predilection site for NDV replication unlike in the chicken.

In view of the type of tissues that were positive for viral nucleoprotein on immunohistochemical staining, NDV in carrier ducks appears to spread and localize mainly in cecal tonsils and kidneys in IS and NIS ducks unlike in commercial chickens where, the spread occurs rapidly throughout the body tissues and localizes in many of

the carrier ducks, unlike domestic chickens where abundant viral replication in the brain of infected birds occurs (Bhaiyat *et al.*, 1994; Kommers *et al.*, 2002). Other workers Brown *et al.* (1999a) and Kommers *et al.* (2003) associated the absence of detectable nucleoprotein in the brain of chickens with delayed occurrence of remarkable brain lesions. The dissemination of the virus to the central nervous system occurs through a cell – associated viraemia with migration of infected, circulating cells into the tissues (Bhaiyat *et al.*, 1994). Viral antigens were not demonstrated in the other tissues (liver, spleen and lungs) of infected duck. This may be due to low viral load in the infected cells in these tissues or low sensitivity of the test and may be these organs are not the replication sites for NDV in this poultry species.

In conclusion, the study has shown for the first time that the ND virus multiplies in macrophages in cecal tonsils and tubular epithelial cells of kidneys of the carrier ducks. The virus in such carrier birds can be excreted into feces leading to periodic outbreak of ND in rural flocks. In addition, NDV may also be shed into the environment through renal discharge leading to transmission to susceptible chickens. This forms the fourth component of endemicity model in which NDV may be sequestered in some organs and is released following immunosuppression of carrier ducks. Thus, in suspected NDV carrier state in ducks, the kidneys and cecal tonsils need to be sampled for virus isolation besides other tissues.

CHAPTER 9

9.0. Experiment 7: Determination of the effect of immunosuppression on the virus persistence in ducks with different levels of Newcastle disease antibodies

9.1. Introduction

Newcastle disease (ND) is a highly contagious disease of domestic poultry, caged birds and wild birds. Newcastle disease virus (NDV) is synonymous with avian paramyxovirus type 1 (APMV-1) and has been classified in the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Avulavirus* (Alexander, 1998; Mayo, 2002).

Village indigenous birds are constantly exposed to immunosuppressive conditions (aflatoxicosis) and infections like infectious bursal disease virus (IBDV) and Marek's disease virus (Mazija, 1990; Sharma, 1997; Otim *et al.*, 2005). In addition, management and ecological factors such as confinement, climatic and seasonal fluctuations, lack of feed supplementation and worm infestations have been associated with stress and reduced immune response (Hørning *et al.*, 2003). Stressful factors have been reported to cause functional and morphological changes in chickens (Graczyk *et al.*, 2003).

Newcastle disease virus is present in poultry worldwide, but there is comparatively little information on factors that lead to the maintenance or release of virulent virus in carrier

birds. Studies on carrier status of NDV show that there are several forms of carrier status, namely: carriage in healthy appearing birds (village chicken, non – chicken hosts, wild birds, and birds in captivity), vaccinated birds that continually shed the virus and contaminated eggs that transmit the virus to the offsprings (Pospisil *et al.*, 1991; Alexander, 1995). Studies of steroid treated chickens indicated that there was a higher viral multiplication in treated groups than non-treated birds (Asdell and Hanson, 1960).

Previous studies have shown that strains of NDV virulent for chickens were also isolated from ducks (Spradbrow, 2000). Ducks may be infected by NDV and show few or no clinical signs even with strains lethal to chickens but are capable of spreading the virus (Spradbrow, 2000; Alexander, 2001a; chapter 6 of this thesis). In Tanzania, it was observed that ND was a greater problem in villages where ducks are also kept (IAEA/FAO, 1999). Earlier reports indicated that NDV persisted for along time in a flock of ducks in a village situation in Indonesia (Kingston and Dharsana, 1979). However, the factors leading to the shedding of the virus by the carrier ducks are not well documented.

It was hypothesized that immunosuppression of immunised carrier ducks does not influence persistence of Newcastle disease virus in the ducks. In this experiment, dexamethasone was used to simulate stress in village indigenous ducks. Thus, the aim of the present study was to determine the effect of immunosuppression on the viral persistence and potential of spread to chickens and effect on immune status of ducks. It

was designed to simulate field situation where ducks that have varying levels of NDV antibodies undergo immunosuppression in the presence of high NDV challenge.

9.2. Materials and methods

9.2.1. Experimental birds

One-day-old indigenous ducklings and chicks were hatched from the duck flock maintained at the University of Nairobi premises. All the birds were reared in isolation and transferred to experimental units at one year of age. They were wing tagged, tested and confirmed to be free of ND virus and ND antibodies.

9.2.2. Experimental design

Sixty-four ducks were inoculated with 1ml of inactivated ND vaccine intramuscularly and 14 days later, they were bled from the brachial vein and sera prepared. They were later boosted with a single dose of 0.5 ml of the ND inactivated vaccine and bled 7 days later. All sera were tested for presence of Newcastle disease antibodies. Seven days after the booster dose, the ducks were divided into two groups, each of 32 birds, namely: low antibody level group ($\leq$1: 32) and medium antibody level group ($\geq$1: 64). Each group of 32 ducks was further subdivided into 4 minigroups, as follows: (i) immunosuppressed and challenged (1a, 2a), (ii) immunosuppressed only (1b, 2b), (iii) vaccinated and challenged (1c, 2c), and (iv) vaccinated only (1d, 2d). Another group of 30 non – immunized ducks were subdivided into 4 groups. Two groups of 12 ducks each were challenged. The other two groups, of 3 ducks each were used as control birds. Twelve indigenous chickens were also transferred into the isolation units, and challenged with

virus. They were used as positive controls for Newcastle disease clinical signs. The respective groups were immunosuppressed as described in chapter 7 before being inoculated intranasally with 0.2 ml of undiluted amnioallantoic fluids of vNDV having a titer of 1:1024.

Five birds from each of the challenge groups and all the 3 ducks from each control group were sampled throughout the experimental period (28 days). The samples were taken on days 0, 1, 4, 8, 14, and 28-post inoculation. Blood for serum, cloacal and oropharyngeal swabs were sampled each time from the five ducks in each challenge group, and the three ducks from each of the controls. Further, two ducks from each of the NDV challenged groups were sacrificed serially and the following 6 tissues (brain, kidney, lung, cecal tonsils, liver and spleen) collected separately from each bird. The swabs and tissues were processed for ND viral recovery using chicken embryo fibroblasts while serum samples were tested for NDV specific antibodies by hemagglutination inhibition (HI) test. **Table 9.1** shows the experimental design used. The experimental birds were observed twice daily for clinical signs.

Table 9.1: Groups of ducks used to evaluate the effect of immunosuppression on persistence of Newcastle disease virus under different treatments

Antibody grouping of ducks	Group code of ducks	Number of ducks	Treatments		
			Dexamethasone	VNDV	Vaccination
Low antibody level	1a	13	+	+	+
	1b	3	+	-	+
	1c	13	-	+	+
	1d	3	-	-	+
Medium antibody level	2a	13	+	+	+
	2b	3	+	-	+
	2c	13	-	+	+
	2d	3	-	-	+
Non – immunized	3a	12	+	+	-
	3b	3	+	-	-
	3c	12	-	+	-
	3d	3	-	-	-
Chickens (non - immune)	Positive controls	12	-	+	-

Legend:

+: Respective treatment administered; - : No treatment; **VNDV:** Velogenic Newcastle disease virus; **Groups 1a, 2a:** Vaccinated immunosuppressed ducks and challenged with Velogenic Newcastle disease virus; **Groups 1b, 2b:** Vaccinated and immunosuppressed ducks only; **Groups 1c, 2c:** Vaccinated non – immunosuppressed ducks and challenged with NDV; **Groups 1d, 2d:** Immunised only; **3a:** Immunosuppressed ducks and challenged with Velogenic Newcastle disease virus; **3b:** Immunosuppressed only; **3c:** Non – immunosuppressed ducks and challenged with NDV; **3d:** Control ducks (naive)

9.2.3. Newcastle disease virus strain used to infect birds

A velogenic Newcastle disease virus (strain L1) obtained from the repository maintained at the University of Nairobi was used in this study as described in section 6.2.3.

9.2.4. Preparation of inactivated vaccine

Inactivated vaccine was prepared by mixing 40% formalin and allantoic fluid with a titer of 2^9 of vNDV in a ratio of 1: 40 (formalin to virus). The preparation was kept at room temperature (24°C to 26°C) for 24 hours before use. The inactivation of the virus was confirmed through inoculation of embryonated eggs. All the birds were vaccinated via an initial dose of 1ml of the vaccine intramuscularly on the thighs and a booster of 0.5 ml of the same vaccine 16 days later.

9.2.5. Immunosuppression of the ducks

This was done as described in section 6.2.4.

9.2.6. Collection and processing of samples

Swabs and blood samples were collected and processed as described on section 4.2.3.

The tissues from each individual bird were homogenized to make a 10 % suspension in transport media (containing antibiotics (2000units/ml of penicillin; 2000 µg/ml of streptomycin and 2500 µg /ml of amphotericin B). The homogenate was then centrifuged at 1000 xg for 10 minutes and the supernatant stored at -20^0C until used.

9.2.7. Preparation of chicken embryo fibroblasts

Primary chicken embryo fibroblast cultures were prepared as described by Kumanan and Venkatesan (1994) and has been briefly described in section 4.2.5.

9.2.8. Virus propagation and harvesting

Newcastle disease virus isolation was carried out on confluent CEF cultures following the method described by Kumanan and Venkatesan (1994) and has been briefly described in in section 4.2.6.

9.2.9. Preparation of red blood cells

The RBCs were prepared as described by Hsiung (1973) and briefly described in section 4.2.7.

9.2.10. Virus detection using haemagglutination test

The protocol for the hemagglutination test is as described by OIE (2000) and briefly described in section 4.2.8.

9.2.11. Haemagglutination inhibition test

The hemagglutination inhibition test is as described by OIE (2000) and briefly described in section 4.2.9.

9.2.12. Data analysis

Data on HI titres for sera were analyzed using SAS software (SAS Institute Inc., Cary, NC, USA, 2002 -2003). The HI titres were log transformed before analysis. Analysis of variance of repeated measures was performed in the SAS software to determine the treatments' main effects and the interaction between time (days) and treatment, on various responses. The Waller – Duncan K – ratio t test and Ryan – Einot – Gabriel – Welsch multiple range test (Steel and Torrie, 1980) were used to analyse the data sets antibody responses.

9.3. Results

9.3.1. Clinical signs manifested by the ducks

The NDV infected ducks showed mild depression. The percentage of ducks that manifested clinical signs per treatment were as follows: 30.8, 0.0, 30.8, 0.0, 58.3, 8.3 for groups 1a, 1c, 2a, 2c, 3a, and 3c, respectively. Mild depression was the first clinical sign to be manifested and was evident on day 4 p.i. There was no mortality recorded and no clinical signs were observed in the non -infected control ducks.

All the NDV infected positive control chickens were severely depressed, had nasal discharge, greenish diarrhoea, nervous tics and ataxia. All the chickens were sacrificed on day 4 p.i. as they were terminally ill and examined at post mortem for lesions.

9.3.2. Serological responses of ducks under different treatments

Immunosuppressed – virus challenged ducks (group 1a) had low mean antibody levels (5.0) up to day 4 p.i. compared with day 0 (4.5). Thereafter, there was marked increase (from 4.5 to 7.0) in antibody titers up to 14 days p.i. After day 14 p.i., there was a slight decrease (6.9) in antibody levels up to 28 dpi although the levels were still higher than any period between day 0 and 8 p.i. **(Figure 9.1)**. The non – immunosuppressed – virus challenged group (1c) had a moderate increase (5.0 to 6.0) in antibody levels from day 1 up to day 14 p.i., after which there was a decrease to day 0 level titers by day 28 p.i. **(Table 9.2; Figure 9.1)**. The immunosuppressed group (1b) had marked decrease in antibody titers from day 1 to 4 and gradual decrease (5.0 to 3.8) up to day 28 p.i.

The immunosuppressed – virus challenged group for the medium antibody level ducks (2a), had a gradual decline (6.0 to 5.7) in antibody titers up to day 4 followed by an increase in antibody titers (6.9) up to day 14 p.i. This was followed by a marked decrease (6.1) and by day 28 p.i. the antibody level was almost equal to the day 0 level titers (**Table 9.2; Figure 9.2**). The non – immunosuppressed virus challenged group (2c) showed a slight decrease (from 6.0 to 5.9) in the antibody titer followed by a gradual decrease and then an increase up to the end of the experiment **(Table 9.2)**. From day 1 up to day 4 post- inoculation, the immunosuppressed, immunised non - infected (2b) group showed a more rapid decrease (6.0, 5.2, 4.8, 3.7, 2.3 and finally 2.0) in antibody levels as compared (6.0, 4.7, 4.5, 3.8, 2.7 and finally 2.2) to the non – immunosuppressed controls (2d). In general, all the non – challenged, but immunized control ducks showed decrease in antibody titers with time **(Table 9.2)**.

The immunosuppressed - virus challenged group (3a) had a gradual antibody response (from 0.0 to 6.5) up to the end of the experimental period (**Table 9.2; Figure 9.3**). The non – immunosuppressed - virus challenged group (3c) showed a massive increase (0.0 to 6.6) in antibody levels similar to immunosuppressed – virus challenged group 3a. The group 3c also had a marked decrease (from 6.6 to 4.6) in antibody titers after day 14 p.i. and by 28 days p.i., the titers were quite low **(Table 9.2)**. Negative control ducks (3b and d), sampled at the same time, and were negative for NDV antibodies.

For days 4, 8, 14 and 28 p.i. antibody titres of the following groups were found to be significantly different ($p<0.05$): between non- immunosuppressed low antibody level,

non – challenged ducks (group 1d) versus non- immunosuppressed medium antibody level, non – challenged ducks (group 2d) being lower in group 1d; immunosuppressed low antibody level, challenged with NDV ducks (group 1a) versus immunosuppressed low antibody level, non -challenged ducks (group 1b) the latter group had lower antibody levels; non -immunosuppressed low antibody level, challenged with NDV ducks (group 1c) versus (group 1d) being lower in latter group; immunosuppressed medium antibody level, challenged with NDV ducks (group 2a) versus immunosuppressed medium antibody level, non -challenged (group 2b), was lowest in the latter group; non-immunosuppressed low antibody level, challenged with NDV ducks (group 2c) versus 2d, the latter group had lower levels of antibodies. In addition, antibody titres of group 1a versus 1d were significantly different ($p<0.05$) on day 14, being lower in the latter. All the control naïve (groups 3b and 3d) birds did not seroconvert.

Table 9.2: Mean antibody titers for immunosuppressed and non-immunosuppressed experimentally infected ducks with respect to days post inoculation

Treatments Groups	Days post inoculation and Mean HI titers (log_2)					
	DO	D1	D4	D8	D14	D28
	Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE
Group 1a	5.0	4.3 ±0.1	4.5 ±0.4	6.4 ±0.5	7.0±0.5	6.9±0.7
Group 1b	5.0	5.0 ±0.4	3.8±0.4	3.3 ±0.5	1.5±0.5	1.0±0.4
Group 1c	5.0	5.0 ±0.2	5.5 ±0.3	5.8 ±0.4	6.0±0.4	5.0±0.4
Group 1d	5.0	4.5 ±0.2	3.7 ±0.5	2.5±0.6	1.8±0.5	1.7±0.4
Group 2a	6.0	5.3 ±0.3	5.7 ±0.4	6.4 ±0.4	6.9±0.3	6.1±0.7
Group 2b	6.0	5.2 ±0.6	4.8 ±0.3	3.7 ±0.4	2.3±0.7	2.0±0.7
Group 2c	6.0	5.9 ±0.3	6.2 ±0.3	5.5 ±0.4	5.8±0.3	5.9±0.3
Group 2d	6.0	4.7 ±0.2	4.5 ±0.3	3.8 ±0.2	2.7±0.5	2.2±0.6
Group 3a	0.0	0.0	0.0	6.4 ±0.5	6.4±0.6	6.5±0.7
Group 3b	0.0	0.0	0.0	0.0	0.0	0.0
Group 3c	0.0	0.0	0.0	6.6 ±0.5	6.6±0.4	4.6±0.7
Group 3d	0.0	0.0	0.0	0.0	0.0	0.0

Legends

Groups 1a, 2a, 3a: Vaccinated immunosuppressed ducks and challenged with Velogenic Newcastle disease virus; **Groups 1b, 2b, 3b:** Immunosuppressed only; **Groups 1c, 2c, 3c:** Ducks NIS challenged with NDV only; **Groups 1d, 2d:** Immunised only; **3d:** Control ducks; **HI:** Heagglutination inhibition; **D1, D4, D8, D14 and D28:** Days 1 to 28 post – inoculation; **SE:** Standard error

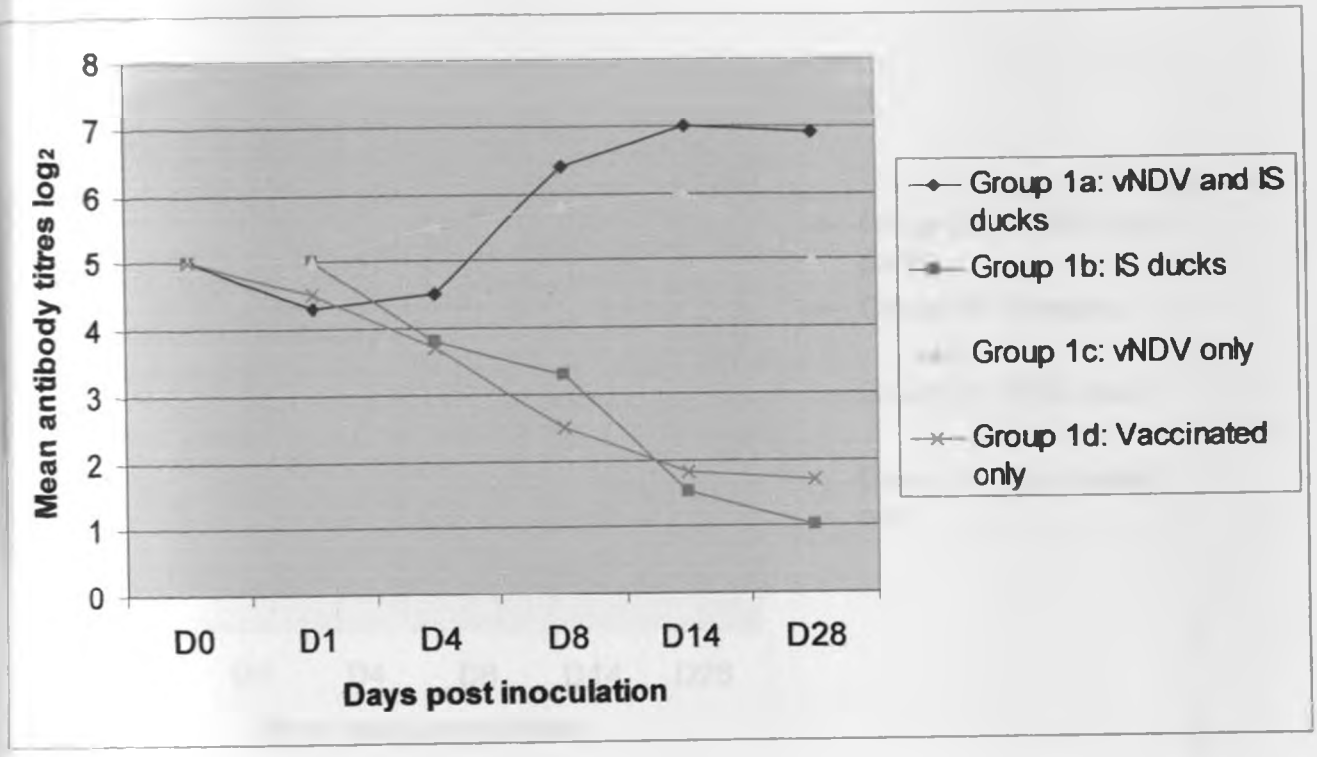

Figure 9.1: Mean antibody titre responses in vaccinated, immunosuppressed and control ducks with low antibody levels (≤1:32) with respect to days post challenge

Legends

vNDV: Velogenic Newcastle disease virus; **IS:** Immunosuppressed; **Groups 1a:** immunosuppressed ducks and challenged with vNDV; **Groups 1b:** Immunosuppressed only; **Groups 1c:** Non- IS ducks challenged with NDV only; **Groups 1d:** Immunised only; **D1, D4, D8, D14 and D28:** Days 1 to 28 post – inoculation;

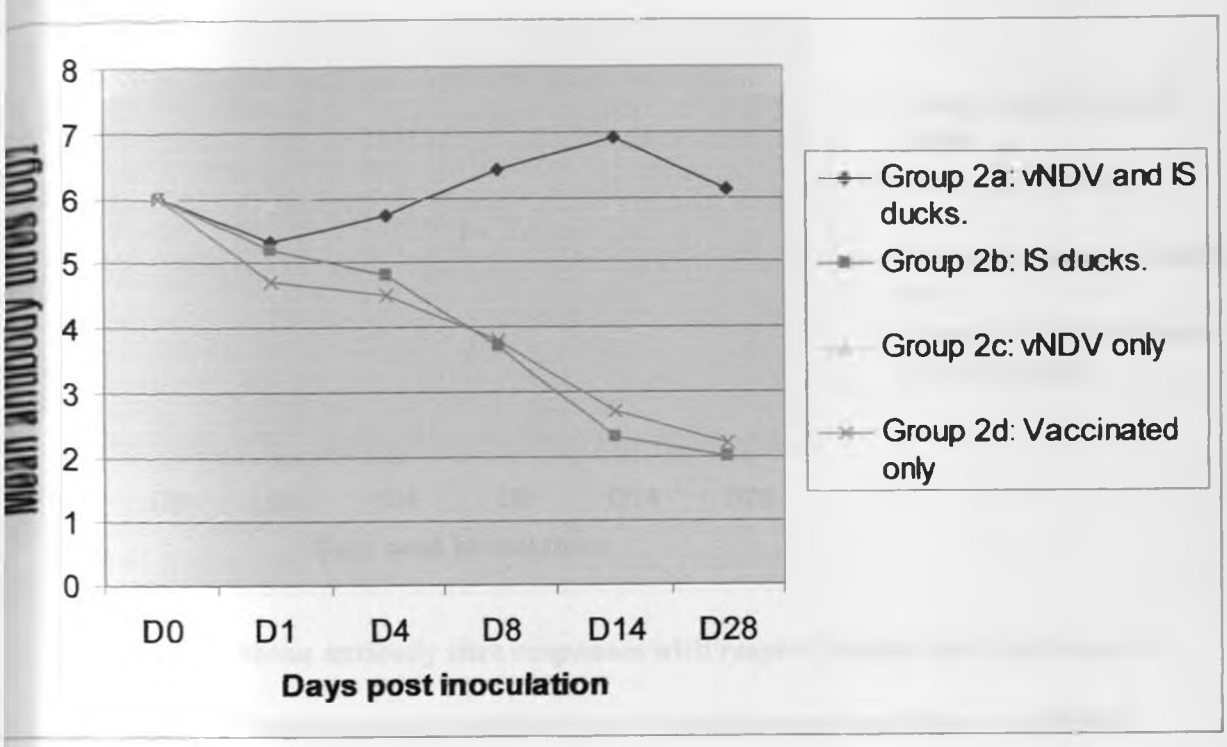

Figure 9.2: Mean antibody titre responses in vaccinated, immunosuppressed and control ducks with medium antibody levels (≥1:64) with respect to days post challenge

Legends

vNDV: Velogenic Newcastle disease virus; **IS:** Immunosuppressed; **Groups 2a:** immunosuppressed ducks and challenged with vNDV; **Groups 2b:** Immunosuppressed only; **Groups 2c:** Non- IS ducks challenged with NDV only; **Groups 2d:** Immunised only; **D1, D4, D8, D14 and D28:** Days 1 to 28 post – inoculation;

and challenged with vNDV (1c) groups and 2a (medium antibody level group, immunosuppressed and challenged with vNDV). Newcastle disease virus was isolated from majority of the duck tissues on day 14 and 28 post inoculation **(Figure 9.4)**.

On day 1 post inoculation, NDV titers were recorded in liver tissues of group 1a (low antibody level group, immunosuppressed and challenged with vNDV) ducks only. On day 4 p.i., high titres of the NDV were recorded in the kidneys than any other organ **(Figure 9.5)**. On day 8 p.i., NDV was isolated in the liver, kidneys, cecal tonsils and lungs of all treatment groups **(Figure 9.6)**. The highest NDV titers, were recorded in the liver and kidney tissues of immunosuppressed medium (2a) and non – immune (3a) challenged ducks and non – immunosuppressed, low antibody level challenged ducks (1c). No NDV was isolated by day 14 p.i. in the brain and spleen from any of the groups.

On day 14 p.i. high NDV titres were recorded from the liver tissues of ducks in all treatment groups. However, titres were recorded in the cecal tonsils only in group 2a on day 14 p.i. and in groups 1a and 1c at day 28 p.i. **(Figures 9.7 and 9.8)**. Other organs that were positive for NDV were kidneys and cecal tonsils. In addition, the immunosuppressed ducks of groups 1a (low antibody level group, immunosuppressed and challenged with vNDV) and 2a (medium antibody level group, immunosuppressed and challenged with vNDV) yielded the highest NDV titres as compared to other treatment groups. Newcastle disease virus was recovered from many other organs such as, brain and lung on day 28 p.i from immunosuppressed ducks only (groups 1a and 2a).

The highest NDV titres were recorded in the liver, cecal tonsils and lung tissues (**Figure 9.8**).

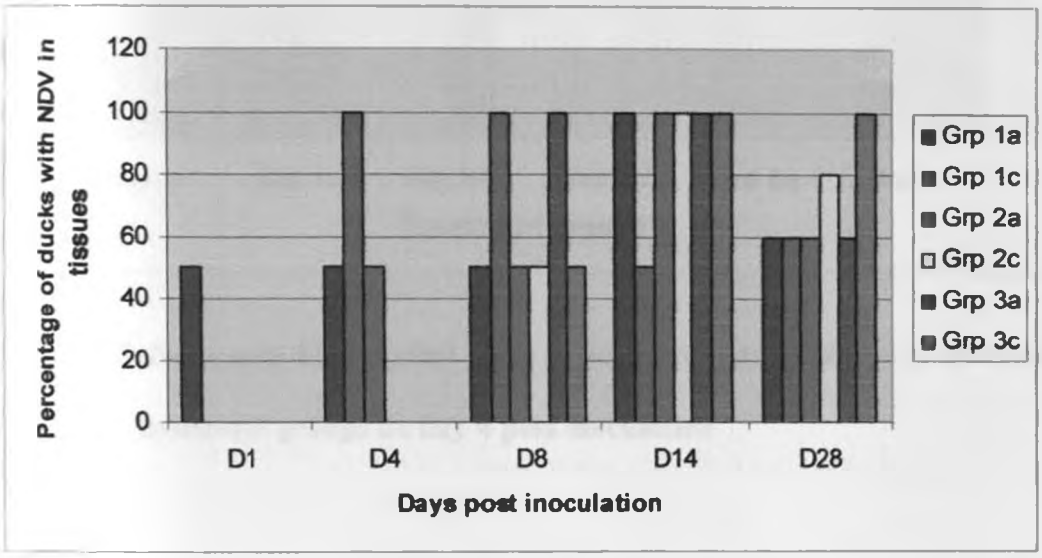

Figure 9.4: Percentage of ducks with Newcastle disease virus in their tissues, with respect to different treatment groups and days post inoculation

Legends

NDV: Newcastle disease virus; **IS:** Immunosuppressed; **Groups 1a, 2a,3a:** immunosuppressed ducks and challenged with vNDV; **Groups 1c, 2c,3c:** Non- IS ducks challenged with NDV only; **D1, D4, D8, D14 and D28:** Days 1 to 28 post – inoculation

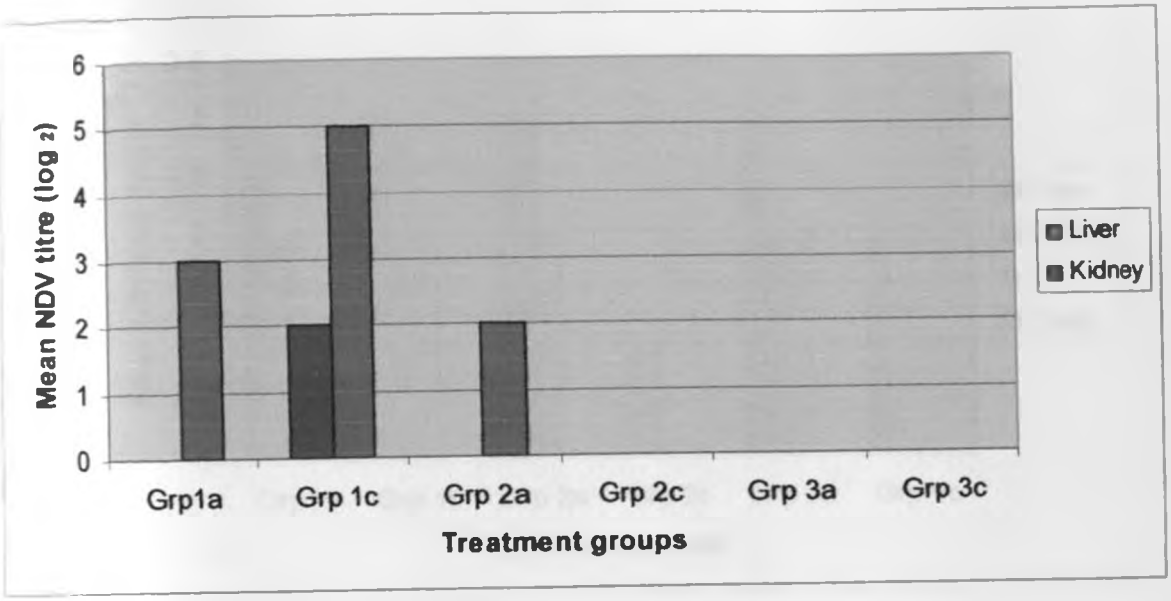

Figure 9.5: Newcastle disease viral titres in duck livers and kidneys in different treatment groups on day 4 post inoculation

Legends

NDV: Velogenic Newcastle disease virus; **IS:** Immunosuppressed; **Groups 1a, 2a,3a:** immunosuppressed ducks and challenged with vNDV; **Groups 1c, 2c,3c:** Non- IS ducks challenged with NDV only

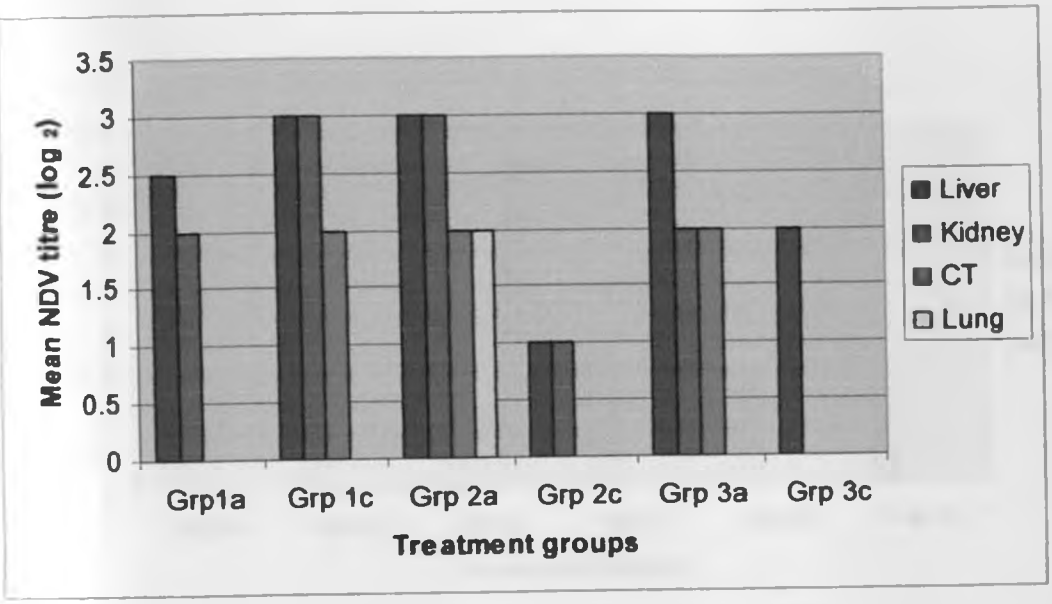

Figure 9.6: Newcastle disease viral titres in duck liver, kidneys, cecal tonsils and lungs in different treatment groups on day 8 post inoculation

Legends

NDV: Velogenic Newcastle disease virus; **CT:** Cecal tonsils; **IS:** Immunosuppressed; **Groups 1a, 2a, 3a:** immunosuppressed ducks and challenged with vNDV; **Groups 1c, 2c, 3c:** Non- IS ducks challenged with NDV only

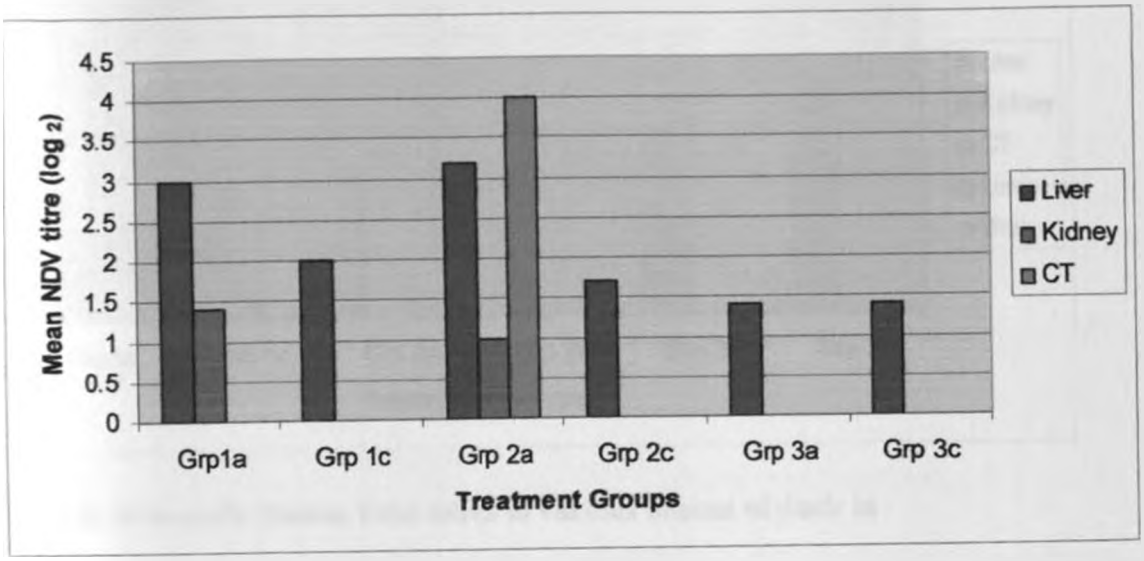

Figure 9.7: Newcastle disease viral titres in duck livers, kidneys and cecal tonsils in different treatment groups on day 14 post inoculation

Legends

NDV: Velogenic Newcastle disease virus; **CT:** Cecal tonsils; **IS:** Immunosuppressed; **Groups 1a, 2a, 3a:** immunosuppressed ducks and challenged with vNDV; **Groups 1c, 2c, 3c:** Non- IS ducks challenged with NDV only

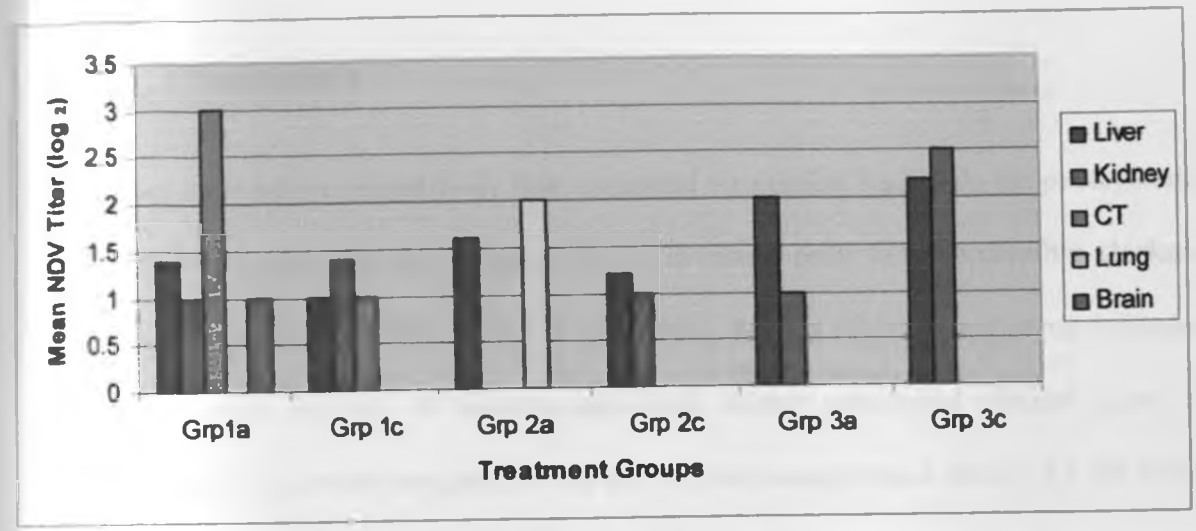

Figure 9.8: Newcastle disease viral titres in various tissues of duck in different treatment groups on day 28 post inoculation

Legends

NDV: Velogenic Newcastle disease virus; **CT:** Cecal tonsils; **IS:** Immunosuppressed; **Groups 1a, 2a, 3a:** immunosuppressed ducks and challenged with vNDV; **Groups 1c, 2c, 3c:** Non- IS ducks challenged with NDV only

9.4. Discussion

There have been comparatively few sequential virological studies on the pathogenesis of ND in ducks and the reported studies involved only fully susceptible chickens (Parede and Young, 1990; Brown *et al.*, 1999a). Results of the present study indicated that, a high number of immunosuppressed ducks manifested clinical signs of Newcastle disease as compared to the non – immunosuppressed ducks. All the ducks with low to medium antibody level and the non –immunosuppressed ones that were challenged with vNDV did not manifest any clinical sign of ND. While ducks infected with vNDV have been reported to show few or no clinical signs, even with strains lethal to chickens (Higgins, 1971; Spradbrow, 2000; Alexander, 2001a), this work reports for the first time on the effects of immunosuppression on the expression of clinical signs in ducks. Reports in other studies have documented frequent isolation of virulent NDV from captive caged birds (Senne *et al.*, 1983; Alexander, 2000). In some cases, the ducks expressed clinical ND as a result of confinement stress (Kaleta and Baldauf, 1988; Carter, 2005). The present study also demonstrates that, ducks that are carriers of velogenic NDV can come down with clinical disease under stressful conditions. Our findings support the proposed conceptual framework of NDV carrier status in village chickens; that the non – natural hosts such as ducks which would be carrying velogenic virus, under stress, recrudescence virulent virus from sequestered sites in the kidney, liver and cecal tonsils, leading to virus release in fecal and respiratory exudates.

Immunosuppression that was induced by injection of dexamethasone in the three treatments influenced the manifestation of the clinical disease, the pattern of antibody response and the NDV recovery rate. The immunosuppressed ducks that had low and

medium antibody level showed a decrease in antibody titers up to day 4 after challenge with NDV. The non – immunosuppressed – virus challenged ducks of low to medium antibody level developed an increase in antibody titres up to day 14 p.i.. The non – immunized ducks manifested increased antibody titres after day 4 p.i. and had a massive increase in antibody levels as compared to immmunosuppressed – challenged group. In the present study, the number of immunosuppressed ducks that yielded the ND virus was higher compared to the non – immunosuppressed. The pre – challenge antibody titers may play a role in the shedding of the virus as well as clinical manifestation of the disease. Unvaccinated birds in this study had the highest number (58.3% and 8.3%) of sick birds. Gessani *et al. (*1988) noted that a few hours of treatment with low concentrations of synthetic glucocorticoid (analogue dexamethasone) are sufficient to inhibit significantly the synthesis of interferon, a virus inhibitor. This may account, in part, to the observation that treatment with glucocorticoids increases virus yield and lethality in infected mice. Our present study using ducks, concur with those of Asdell and Hanson (1960) who showed that prior treatment of chickens with dexamethasone lead to massive ND virus multiplication.

There was significant difference in geometric mean antibody titers between the immunosuppressed ducks of group 1a and non – immunosuppressed counterparts (group 1d) and also between immunosuppressed ducks of group 3a and non – immunosuppressed group 3d. This means that whereas dexamethasone seems to have an effect on immune system of NDV – infected ducks, the pre – challenge titres also play a major role in the immune response of immunosuppressed birds in that immunosuppression of ducks with high viral titers allows virus multiplication making ducks better carriers and thus form the fifth part of the endemicity model.

Immunosuppression effects of dexamethasone in ducks appears to be the same as that induced by aflatoxin in chicks as far as NDV infections are concerned. Chickens fed on aflatoxin produced lower antibody levels when compared to the uninfected ones (Otim *et al.*, 2006).

The non – challenged pre – immunized ducks had a progressive decrease in antibody levels suggesting that if they were to be exposed to the virus, they could come down with the ND or if the antibody titers were within the protective levels (2^4 to 2^7), they might not develop clinical disease but instead may remain as virus carriers. The fact that the ducks in these experiments had high levels of antibodies may not necessarily prevent sub-clinical infection and excretion of virulent virus as supported by other studies elsewhere (Stone *et al.*, 1981).

Based on these results, it is clear that immunosuppressed ducks carrying NDV are likely to shed virus under stress. Furthermore, they are likely to have low levels of antibodies and may be susceptible to the virus, leading to clinical disease and excreting the virus. The excreted virus will contaminate the birds' environment and be transfered to susceptible chickens and other birds. Thus, the pre – challenge antibody levels affect the immune response in NDV carrier ducks. This forms the fifth component of the endemicity model in which the actual immune response in ducks may be affected by immunosuppression.

CHAPTER 10

10.0. General discussion and conclusion

10.1. Discussion

The study dealt with the role of carrier birds in the epidemiology of Newcastle disease and factors that are involved in the maintenance of the virus in the village indigenous poultry population. Although there is extensive literature on ND and NDV, most of it is based on commercial poultry production systems, leaving a knowledge gap in the village poultry system, which is the predominant management system in the developing countries. The free – range village chickens are believed to keep the virus in circulation and act as reservoirs and carriers to other village and exotic poultry breeds but the mechanism of how this occurs are not known (Martin, 1992; Binta *et al.*, 1996). These issues were therefore investigated in this study.

This study entailed investigation of risk factors associated with ND outbreaks, its endemicity under field conditions, antibody profiles and virus carrier status in village indigenous hens, viral transmission from ducks to chickens, pathological lesions and location of NDV antigens in tissues of carrier birds using immunohistochemistry and sero – immunological assessment on the effects of immunosuppressants.

Several risk factors were identified to be associated with the occurrence of ND in village indigenous chickens. These were confinement of birds, lack of feed supplementation, cold temperatures, winds, all of which could induce stress in birds, and restocking farms with market birds. Restocking chickens from the market and neighbourhood flocks was found to be a major risk factor for ND outbreaks, as reported in Vietnam (Nguyen, 1992). During ND outbreaks, flock owners commonly

recover some money through slaughter of the sick, sale of apparently healthy appearing chickens to the market and to neighbours, and giving them out as gifts to friends and relatives. The latter would perpetuate the disease at village level by spreading the virus between flocks. Outbreaks were significantly associated with climate and agro – ecological zones. There were more outbreaks in dry season in LM5 and cold wet season in LH1 and UM3.

The role of asymptomatic carrier birds in the spread and epidemiology of ND in the study area was studied by evaluating the prevalence of antibodies and ND virus in village indigenous chickens of different age and sex in two agro - ecological zones as indicators of NDV endemicity. It was established that healthy chickens carried virulent virus while chickens that were not previously vaccinated had antibodies to NDV. The prevalence of NDV was significantly higher in LM5 than LH1. This corresponds to the observation that flock owners in LM5 managed ND outbreaks by selling off the affected birds, carrying them by hand to the market and that there were more reports of NDV outbreaks in LM5 than in any other zone. They also disposed chicken faecal waste in rubbish heaps where it was accessible to other birds unlike in LH1.

The NDV carriage seemed to have a sex preference and female birds had higher mean NDV titers than males, as reported in the risk factors study. Hens had the highest survival rate after ND outbreak. This was also the case in Bangladesh where more cocks died than hens (Kutubuddin, 1973). There is a possibility, therefore, that the surviving hens remained carriers of the velogenic NDV thereafter. However, the actual mechanism responsible for this sex related resistance to ND and carriage of the

virus is still unknown. Age of the chicken was also shown to influence the carrier status of the NDV in poultry population as reported by Beard and Hanson, (1984).

There was no significant difference in the seroprevalence between the warm and cold zones. The mean NDV HI titres were uniformly low in the two zones. This could mean that ND was occurring in a classical epizootic pattern, with high fatality rates. Furthermore, following pathogenicity testing of the isolates recovered, it was found that all NDV isolates recovered from the healthy birds were velogenic and these could result in clinical disease in infected birds. Why overt disease was absent in these birds is still unclear. Birds that yielded the virus did not have antibodies in their serum. Perhaps cell mediated immunity played a protective role in this case (Sharma, 1997).

In an attempt to unfold the mechanism behind hens being better reservoirs of NDV in multi-age flocks, the association of antibodies in sera and egg yolks of the village chickens and virus prevalence in them was evaluated. The mean antibody titers were found to be consistently lower in serum than in the egg yolks of laying hens in all the AEZs. This agrees with reports in migratory cormorants (Farley *et al.* 2001). However, in the latter ND virus isolation was from dead birds and not from healthy birds. Hens with high antibody titers did not yield NDV.

Birds of UM2 zone were all seronegative but had high mean egg yolk titers. This could have been due to lack of continuous exposure to virus. Moreover, antibodies are known to be preferentially passed from serum to the yolk (Schade *et al.*, 1991) which, if it occurred over a laying period with many eggs and perhaps the third or so laying clutch since the exposure to the viruses, the antibodies may decline to zero in the

serum due to the extensive sequestration of antibodies as in the eggs. This concurs with observation by Martin (1992) that NDV HI antibodies generally decline after 3 to 4 months and disappear by 8 to 12 months. Hens in our experiments were thus reported to be seronegative and infected, seropositive with antibodies in eggs / ovules; or seronegative but with antibodies in eggs and ovules. If the hens with high antibody levels in their sera were infected by NDV they could probably survive. However, when antibodies in the serum waned off, the birds would be susceptible to infection leading to clinical disease. This could then complete one component of the endemicity model suggested in this study.

Since, the mean antibody titers were higher in mature egg yolks than in ovules of the same hens, the disparity may possibly be due to differences in stages of exposure to NDV. Chicks hatched from such hens are likely to have very high levels of maternal antibodies. This should be considered when designing vaccination programmes such that the first vaccine dose is administered after the maternal antibodies have waned (Allan *et al.*, 1978).

There was insufficient evidence to support the role of turkeys, doves, geese and guinea fowls reared with chickens as contributors to increased risk of ND outbreaks in village indigenous chickens (Otim *et al.*, 2007). Ducks and other poultry are frequently reared together with chickens under village management. Ducks are suspected to spread the ND virus to chicken (Spradbrow, 2000) which has been supported by data reported in this study. Newcastle disease virus is speculated to persist long in ducks under village conditions posing great problems in mixed flocks (IAEA/FAO, 1999; Spradbrow, 1999). In this study, it was shown that viral shedding

was detectable using sentinel chickens for over 2 weeks while the virus was isolated from the tissues of infected ducks for a period of up to 29 days. The other studies were designed to unravel the role of ducks in NDV endemicity in indigenous village chickens.

The sentinel indigenous chickens kept in contact with vNDV – inoculated ducks seroconverted, had 80% and 100% mortality for those mixed with non-immunosuppressed (NIS) ducks and immunosuppressed (IS) ducks, respectively. The virus was recovered from chicken tissues and from the cloacal and oropharyngeal swabs of both NIS and IS ducks up to day 15 p.i. and in duck tissues upto day 29 post inoculation. This was similar to the virus persistence reported in a village situation in Indonesia (Kingston and Dharsana, 1979). Most probably, the transmission occurred through aerosol and coprophagia. The study demonstrated that infected ducks shed the virus and transmitted it to chickens, indicating that ducks are carriers of NDV. Since chickens mixed with IS ducks showed more clinical signs and that only IS ducks showed clinical signs, it seems, the cross-transmission of NDV was more readily facilitated from IS ducks to chickens compared to the NIS ducks. This demonstrates a second component of the endemicity model suggested in this study. The model simulates the potential for disease transmission scenario in rural duck – chicken mixed flocks. Once exposed to stress or immunosuppressants, ducks would be more likely to release more virus from their tissues, shedding it to the environment infecting the contact susceptible birds reared together in rural mixed flocks.

On post mortem examination, the experimental ducks showed varied gross and microscopic lesions. The pathological lesions in positive control chickens in this study

were as classically reported for typical NDV infections (Brown *et al.*, 1999a; Kommers *et al.*, 2002; 2003). However, lesions in ducks were mild and less severe than those in sick chickens. More immunosuppressed ducks manifested macroscopic and microscopic lesions compared to the non – immunosuppressed birds; although splenomegaly and enlarged hemorrhagic cecal tonsils were observed in NIS ducks only which would be expected to be a normal reaction of the lymphoid tissues to the virus in uncompromised bird. On the other hand, only IS ducks manifested necrosis of the spleen in addition to more ducks showing lymphoid depletion of the cecal tonsils and spleen. Treatment of infected birds with dexamethasone lead to increased virus yield (Gessani *et al.*, 1988). Stressed ducks in this study may have released more virus causing pathological lesions in various tissues (though milder than those observed in the positive control chickens). Central chromatolysis of the neurons, vacuolation and perivascular cuffing in the brain were manifested in IS ducks only. Immunosuppression therefore exacerbated lesions in ducks. This demonstrated the third component of the model in which immunosuppression increases the extent of tissue damage in infected ducks.

The six different tissues that were assayed by immunohistochemistry demonstrated presence of NDV antigens in the kidneys and cecal tonsils only. In the cecal tonsils, antigen labelling occurred in macrophages and in areas where vast majority of the cells were lymphocytes. This could suggest that these cells are involved in viral replication in carrier ducks. Lymphoid cells of cecal tonsils are reported to have viral nucleoprotein in infected chickens just like these carrier ducks (Lam, 1996; Kommers *et al.*, 2003). Macrophages and lymphocytes may therefore be involved in the replication and dissemination of NDV. It would be interesting to find out whether the

kidneys and cecal tonsils have any special affinity or mechanisms that make them preferentially susceptible to NDV infection. Perhaps they have special receptors for NDV or posses Neuraminidase enzyme or protease that allow ready virus multiplication and release.

In the kidneys of carrier ducks, viral antigens occurred in the tubular epithelium as demonstrated in chickens by some investigators (Kommers *et al.*, 2001; 2002) although others could not demonstrate the same (Brown *et al.*, 1999a,b). Viral replication in the tubules may damage and compromise the renal epithelia. Newcastle disease virus may also be shed into the environment through renal excretion. This finding suggests that renal epithelium is probably a predilection site for NDV replication in ducks unlike in chicken.

Viral nucleoproteins were not detected in the brain of all the carrier ducks, unlike domestic chickens where abundant viral replication in the brain of infected birds occurs (Bhaiyat *et al.*, 1994; Kommers *et al.*, 2002). Other workers (Brown *et al.*, 1999a; Kommers *et al.*, 2003) associated the absence of detectable nucleoprotein in the brain of chickens with delayed occurrence of brain lesions. Based on the results of this study, it seems that the viral antigens are not restricted to the mononuclear phagocytic system but there is parenchymal cell involvement. This study demonstrated for the first time that NDV localized and possibly multiplied in cecal tonsils and kidneys of the carrier ducks, where it can be excreted leading to periodic outbreaks of the disease in duck – chicken mixed rural flocks.

Newcastle disease virus was isolated from the brain, cecal tonsils, kidney, liver and lungs of ducks 28 days p.i. in ducks. The mean titer of NDV was also consistently high in the kidneys and liver from 4 dpi upto 28 dpi. This means that although the shedding of the virus by the ducks could not be demonstrated by day 29 p.i., they retained the virus in the kidneys and the liver. Two organs, namely the kidneys and cecal tonsils, are thus probably the principle sites for viral multiplication and sequestration in carrier ducks. This observation together with the location of NDV antigen completes the forth model of Newcastle disease endemicity whereby virus seems to be sequestered in particular organs from where immunosuppression can induce virus release and persistence as was observed in the virus recovery studies.

Ducks that were immunosuppressed with dexamethasone showed clinical signs and severe pathological lesions of Newcastle disease virus than non – immunosuppressed ones. Therefore, ducks that are carriers of velogenic NDV are likely to come down with clinical disease under severe stressful conditions and release virus to in contact chicken. Captive caged birds that are carriers of virulent NDV can show clinical ND due to stress induced by confinement (Bruning – fann *et al.* 1992; Carter, 2005). This study has shown that ducks yield more virus under simulated stress.

Immunosuppressed NDV infected ducks had lower mean antibody titers as compared to those of control birds. On day 4, the titers were higher in vaccinated and infected ducks compared to the vaccinated only. This could be due to progressive antibody decline with time for ducks that were vaccinated only in absence of challenge whereas challenge induced anamnestic response in ducks that were vaccinated and subsequently challenged. This could be due to dexamethasone impairment of the

immune system (Giambrone *et al,* 1978). However, at day 8 p.i., the antibody levels were higher in immunosuppressed ducks than in controls, possibly due to waning off dexamethasone effect and corresponding compensatory lymphoid tissue mechanism resulting in massive production of antibodies to neutralize the high NDV titres in the body. Interestingly, the high levels of antibodies achieved after challenge of ducks with vNDV in the immunised groups was not protective against infection possibly due to high multiplication of the virulent NDV compared to the neutralisation late. Similarly, Kapczynski and King (2005) observed that infection, shedding, and transmission of virulent NDV in vaccinated birds may occur without overt disease signs.

There was significant difference in mean antibody titers between low and medium antibody levels - challenged NIS ducks, in addition to having more ducks with no pre – challenge antibody titers manifesting clinical signs of ND compared to the other immunized counterparts. Thus, the pre – challenge antibody levels affect the immune response in NDV carrier ducks. This forms the fifth component of the endemicity model in which the actual immune response in ducks is modurated by stress. Thus, antibody levels could at times be reduced. This may lead to less virus neutralisation and higher quantities of viruses being released into the environment to infect in – contact chickens.

10.2. Conclusions

Based on this study, it can be concluded that:

1. Factors such as confinement, feed deprivation, and climatical change create stress that influences NDV persistence, and prevalence in village indigenous chickens.
2. Newcastle disease virus is present in healthy village chickens in Embu and Mbeere districts, Kenya and more outbreaks occur in warm dry climates and in wet humid climates than other agro- ecological zones.
3. Hens that survive outbreaks or have antibodies from previous exposure to Newcastle disease virus may maintain NDV endemicity in village chickens, though they appear healthy.
4. Ducks under stress yield more virus, show clinical signs and more lesions compared to non – stressed ducks and are better reservoirs of NDV, to be transmitted to in – contact chickens.
5. Newcastle disease virus seems to localize and replicate in the kidneys, liver and cecal tonsils of the carrier ducks from where immunosuppression can induce fresh virus release into the environment.
6. The pre – challenge antibody levels affect the immune response in NDV carrier ducks in that immunosuppression of ducks having high viral antibody titers allows virus multiplication making ducks better NDV carriers.
7. From the proposed conceptual framework of an NDV carrier status, in village chickens, virulent virus in carrier ducks is released into the flocks by stress in carrier ducks, imbalance of antibody levels in hens, virus sequestration in carrier ducks, climatic changes, and husbandry practices like confinement. All

these factors that could induce stress play a role in the endemicity of the ND virus.

8. From the data in these experiments, the endemicity of NDV can be explained in a five component model, as elaborated in the discussion in this study and and includes many other factors, which is part of a wider model.

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12.0. APPENDICES

Appendix 1: A questionnaire on: A survey of village poultry production in the Eastern province (Embu and Mbeere districts) of Kenya.

A: Background Information

1. Questionnaire NO.....
2. Farmer's name ..... Village .....

   Division..... AEZ..... District.....

3. Interviewer's name.....Date of interview.....
4. What is your main occupation? (1) farming, (2) trading, (3) civil service,

   (4) others,.....

5. Livestock kept by the farmer,

	Number	Reason for raising (1-7)
1. Chicken		
2. Cattle		
3. Goat		
4. Sheep		
5. Pig		
6. Ducks		
7. Pigeon		
8. Guinea fowls		
9. Turkeys		
10. Others		

Key: 1= as family food; 2= for manure; 3 = for selling produce, 4= for ceremonies; 5= to earn money; 6 = for prestige, 7= others, specify.....

6. Which crops do you grow?

Type of crop	Hectares	Main use (1-3)

Key: 1= for food; 2= for selling; 3= others,

specify.....

B. Management

1. For how long have you been keeping local chicken?

(a) 1 – 4 years, (b) 5 - 8 Years, (c) 9 – 14 years, (d) 15 or more

2. What type of chicken do you keep? local breeds, (b) cross breeds

3. What are the reasons for keeping chicken? (a) availability, (b) high growth rate,

(c) resistance to diseases, (d) easier to manage

4. What are the sources of your local chicken? (a) purchase, (b) gift, (c) inheritance,

(d) contractual agreement, (e) others,.....

5. Number of chickens kept,

(a) Male adults.....(b) male growers..... (c) female adults.....

(d) female growers..... (e) chicks.....

6. What type of management system do you practice? (a) free range system, (b)

backyard system (semi intensive)

7. Do you confine your birds at particular season? (a) Yes, (b) No

8. (i) If yes, which period of the year? January to March, (b) April to June, (c) July to

September, (d) October to December

8. (ii) Why do you confine the chickens? (a) to prevent them from destroying crops,

(b) others.....

8. (iii) What are the chickens fed on during confinement?

(a) commercial feeds, (b) kitchen leftovers, (c) cereal grains, (d) brans

(e) household refusal, (f) any other .....

9. Which diseases occur only during confinements?

(a) Newcastle disease (coughing and high mortality), (b) Diarrhoea and worms,

(c) Fowl pox (closed eyes with wounds on combs), (d) Others.....

10. Which diseases occur during confinement and also when birds are not confined?

(a) Newcastle disease (coughing and high mortality), (b) Diarrhoea and worms,

(c) Fowl pox (closed eyes with wounds on combs), (d) Others.....

11. Who does a day to day management of chicken?

(a) women, (b) children, (c) husband, (d) all, (e) none, (f) Others,.....

12. Do you house your chicken at night? (a) Yes, (b) No

13. If YES, what is the type of housing? (a) part of a kitchen, (b) part of a sleeping

house, (c) a separate shelter, (d) others,.....

14. How many times per week do you clean the chicken house? (a) Once, (b) twice,

(c) thrice, (d) more than four times

15. What do you do with manure from the chicken house?

a= use as fertilizer

b= thrown away as rubbish

c = given free to neighbors

d= others, specify.....

16. Do you provide supplementary feeds? (a) Yes, (b) No

17. If YES in question 16 above, what types of feeds do you supplement?

(a) commercial feeds, (b) kitchen leftovers, (c) cereal grains, (d) brans

(e) household refusal, (f) any other,.....

18. When do you feed the chickens? (a) in the morning, (b) in the afternoon,

(c) in the evening, (d) at any time,

19. What problems do you face in keeping chicken?

(a) diseases

(b) predation, (c) accidents, (d) lack of feed, (e) lack of market,

(f) lack of medication / vaccines

(g) others, specify.....

20. Which months of the year is Newcastle disease outbreak commonest?

(a) January to March, (b) April to June, (c) July to September,

(d) October to December

21. (i) Does Newcastle occur before or after confinement? (a) before, (b) after

21. (ii) How many weeks before or after confinement? (a) 1 to 2 weeks, (b) 3 to 4 weeks, (c) 5 to 6 weeks, (d) above 7 weeks

22. Which birds get sick first? (a) hens, (b) cocks, (c) growers, (d) chicks

23. Which die faster or quicker? (a) hens, (b) cocks, (c) growers, (d) chicks

24. Which survive mostly? (a) hens, (b) cocks, (c) growers, (d) chicks

25. When there is Newcastle disease outbreak what do you do to the chickens?

(a) Sell them, (b) Kill the sick, (c) give some to the relative or neighbour,

(d) treat them

26. If, treatment, what do you use? (a) conventional medicine/ drugs, (b) herbs,

(c) others.....

27. When is Newcastle disease more common?

(i) Wet season – (a) before rains, (b) after rains

(ii) Dry season – (a) middle, (b) before rains

28. What else can be seen in the season when Newcastle is occurring e.g. in the flocks?

(a) flowering of trees

(b) lots of wind and no dust

(c) very hot temperature

(d) dust, storms

(e) ceremonies

(f) visiting more often

(g) flowering beans

(h) Sukuma wiki and other greens

(i) Wild birds visiting homes,

(j) New bird introduced to the flock (bought from the market)

(k) New birds as gift from friends

(l) Very cold temperatures

(m)Cold and hot for few days then outbreak

(n) When there are other domestic birds in the flock (turkeys, ducks, guinea fowls, others.....)

29. If new birds, is it during the confinement period? (a) Yes, (b) No

30. Do you vaccinate your local birds against NDV? (1) Yes, (2) No

31. If yes, who does the vaccination? (a) Veterinary personnel, (b) the farmer, (c) others.....

32. What measures do you take to control:
(a) predators.....

(a) diseases.....

(b) parasites.....

33. What are the ways to improve feeding and management of chicken?

.....

.....

.....

C. Productivity and marketing

1. What is the age (in months) at which the birds start laying? (a) six months,

   (b) 7 months, (c) others,.....

2. Where do chickens / ducks/ guinea fowls/ turkeys lay?

   (a) in the kitchen, (b) in the sleeping house, (c) separate shelter,

   (c) any other place.....

3. Do you provide nests for laying hens? (a) Yes, (b) No.....

4. How many eggs are produced per hen per

   clutch?.....

5. What is the length of laying period in days before sitting on

   eggs.....

6. How many clutches per hen per year? (a) once, (b) twice, (c) thrice,

   (d) others,.....

7. How many eggs are set (for incubation) per hen?.....

8. Of the eggs set, how many hatch?.....

9. What is the source of eggs for hatching? (1) from the flock, (2) purchased,

   (d) others,.....

10. What is the number of hens with chicks in your

    flock?.....

11. Is there any change in eggs production with season? (a) Yes, (b) No

12. If YES in question 11 above, what are the reasons?

(a) availability of feed, (b) confinement of birds, (c) Disease outbreaks,

(d) others, specify.....

13. Do you eat eggs from your hen? (a) Yes, (b) No

14. If answered yes in question 13 above how many per hen?.....

15. Do you sell eggs? (a) Yes, (b) No

16. If yes in question 15 how many per hen?.....

17. Where do you sell the eggs? a= market, b= at home, c= to hotels and restaurant,

d= others, specify.....

18. What is the price of an egg?.....

19. Is the market for eggs reliable? (a) Yes, (b) No

20. If No what are the reasons?.....

21. How long does it take before chicks are weaned?.....

22. Is there any chick mortality up to weaning? (a) Yes, (b) No

23. Do you separate the chicks from the hen (wean)? (a) Yes, (b) No

If No, Why.....

24. If Yes, in question 24 above, how do you wean? (a) confining the hen,

(b) confining the chicks, (c) others.....

25. Where do you sell your birds? (a) at home, (b) local market, (c) market far away,

(d) others.....

26. How do you determine which birds to sell? (a) health, (b) weight, (c) sex, (d) sick,

(e) age, (f) others.....

27. Which birds do you sell more frequently? (a) cocks, (b) old hens, (c) growers 4 to

6 months, (d) chicks 2 to 4 weeks.

28. How do you transport your birds to the market place?

(a) using hands, (b) bicycle in a basket, (c) vehicle in a basket, (d) vehicle without a basket, (e) others.....

29. Records on chicken prices

Birds	**Average Price**
1. Cock	
2.	
3	
1.Hen	
2.	
3.	
1. Pullet	
2.	
3.	

30. Do you face any problem in marketing your birds? (0= No, 1 = Yes)

31. If yes, what are the major problems of marketing local birds?

a= prices are too low

b= there are few customers

c = there is transport problems

d= high market levy

e = there is no specific place for selling

f= others, specify.....

Appendix 2: Formulae for reagents used

(i) Hanks balanced salt solution

Solution A stock

Sodium chloride (NaCl)	40 g
Potassium chloride (KCl)	2.0 g
Magnesium sulphate ($MgSo_4.7H_2O$)	0.5 g

Dissolve in 200ml de-ionised water. Dissolve 0.7 g Calcium chloride ($Cacl_2$) in 30 ml de – ionizedd water. Mix and make up to 250 ml with de- ionized water. Add 0.5 ml chloroform. Store at $+4^0C$. Solution is stable for at least 1 year.

Solution B stock

Sodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	0.76 g
Potassium hydrogen phosphate (KH_2PO_4)	0.30 g
Dextrose	5.60 g

Dissolve in 200 ml de – ionised water. Make upto 250 ml with de – ionised water. Add 0.5 ml chloroform stored at $+4^0C$. Solution is stable for at least 1 year.

Working solution

Solution A	50.0 ml
Solution B	50.0 ml
Water	870.0 ml

0.4% Phenol red 2.0 ml

Mix solution A and B with 870 ml de – ionised water. Add 2.0 ml phenol red distributed into bottles in desired volumes and autoclave at 10 lbs for 15 minutes. Before use, adjust PH as desired with 7.5 % sodium bicarbonate ($NaHCO_3$).

(ii) Phosphate buffered saline (PBS)

Solution A

Sodium chloride (NaCl) 8.00 g

Potassium chloride (KCl) 0.20 g

Sodium phosphate ($NaPO_4$) 1.15 g

Potassium hydrogen phosphate (KH_2PO_4) 0.20 g

0.4% Phenol red 2.00 ml

Dissolve in de – ionised water. Add 2 ml of 0.4% phenol red. Make up to 800 ml and autoclave at 10 lbs for 15 minutes.

Solution B

Magnesium chloride ($MgCl_2.6H_2O$) 0.1 g

Dissolve in 100 ml de – ionised water. Autoclave at 10 lbs for 15 minutes.

Solution C

Calcium chloride ($CaCl_2$) 0.1 g

Dissolve in 100 ml de – ionised water. Autoclave at 15 lbs for 15 minutes.

Working solution of PBS

Add 8 parts of solution A to 1 part of solution B and 1 part of C.

(iii) 7.5% Sodium bicarbonate

7.5 g Sodium bicarbonate

100 ml distilled water

0.4% phenol red 0.2 ml

Saturate with CO_2 till orange in colour

Dispense in tightly stoppered bottles and autoclave at 10 lbs for 15 minutes.

(iv) 1 % Versene in EDTA

Versene EDTA	5 g
PBSA	500 ml
0.4% Phenol red	0.2 ml

Autoclave at 10 lbs for 15 minutes.

Trypsin	0.25 %
Trypsin 1:250	2.5 g
PBSA	1000 ml

Stir for 2 hours in magnetic stirrer

Add 1% phenol red 1.5 ml

Filter in Millipore filter membrane 0.2 µm. Store at +4^{O}C after adjusting pH to 7.6 –7.8.

(v) Trypsin versene (Mixture solution)

Trypsin 1 part

Versene	4 parts

(vi) Tincture of of iodine

96% Alcohol	76 ml
Distilled water	2 ml
Potassium iodide	2.2 g
Iodine	2.0 g

(vii) Tris / EDTA buffer, pH 9.0

Tris / EDTA buffer (10 Mm TRIS, 1 mM EDTA, pH 9.0)

1.21 g Tris base (like Calbiochem 648311 or Sigma T- 1503)

0.372 g EDTA (Sigma E- 5134)

Milli Q H_2O to 1 litre

Adjust the pH to 9.0

(viii) Tris Buffered Saline (TBS)

(0.05 M Tris, pH 7. 5, 0. 15 M Na Cl)

- For 1 litre

50 ml 1M Tris pH 7.5

30 ml 5M NaCl

dH_2O to 1liter