EFFECT OF IMMUNOSUPPRESSION AND PASSAGE ON VIRULENCE OF LENTOGENIC VACCINE STRAIN OF NEWCASTLE DISEASE VIRUS

BY:

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Infectious Disease Diagnosis) in the school of pure and applied sciences of Kenyatta University

February, 2011

DECLARATION

I, Julius K. Kibe declare that this thesis is my own original work and has not been presented in any other University for the award of a degree or any other award.

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DEDICATION

This thesis is dedicated to my wife Nellius Mbuki and my children Kevin Kibe, Jane Wangui, Humphrey Munge and Samuel Waweru all of who persevered my frequent absence from the family and often arriving late at night.

ACKNOWLEDGEMENTS

I would like to acknowledge my supervisors Dr. Joseph. N. Ngeranwa Dr. John N. Mbithi and Professor Phillip N. Nyaga all of who tirelessly guided and encouraged me. I would also like to thank Dr. Jason Michieka, Dr. L. W. Njagi, Rose N. Gitari, Mary Mutune and Anne K. Munene for their technical assistance. In addition, I would like to thank Professor N. Maingi, Chairman of The Department of Veterinary Pathology Microbiology and Parasitology, University of Nairobi for allowing me to use Departmental Research facilities. Thank you to James Lidava and Isaac Matata who fed and took care of the birds. Finally, I cannot forget to thank DANIDA (Productivity of Small Holder Livestock in East Africa Project) for providing the research materials.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

APMV-1 : Avian Paramyxovirus type -1

CO_2: Carbon dioxide

DANIDA: Danish International Development Agency

END: Endemic Newcastle Disease virus

HA: Haemagglutination

HBSS: Hanks Balanced salt solution

ICPI: Intracerebral pathogenicity index

I-S: Immunosuppressed

KEVEVAPI: Kenya Veterinary Vaccine Production Institute

MDT: Mean death time

ND: Newcastle disease

NDV : Newcastle disease Virus

N-IS: Non-Immunosuppressed

OIE: Office of International Epizootic

PBS: Phosphate buffered saline

RBCs: Red blood cells

RNA: Ribonucleic acid

RPMI: Rose Parkwell Memorial Institute

RPM: Revolutions per minute

USA: United States of America

vNDV: Velogenic Newcastle disease virus

ABSTRACT

Newcastle disease virus (NDV) causes one of the most serious viral diseases of poultry. The virus is a member of the new A*vularvirus* genus in the family *Paramyxoviridae.* NDV has a negative stranded RNA genome which is approximately 15 Kb. NDV has a worldwide distribution, affects chicken, non-chicken poultry and wild birds. Mortality and morbidity of Newcastle disease (ND) in chicken may reach 100% in non immune birds. In Kenya, 70% of the thirty million poultry are indigenous chicken. These chickens are an important source of dietary protein and income from meat and eggs. This source of livelihood is constantly under threat due to frequent outbreaks (ND). The disease is controlled by vaccination of poultry using either live attenuated or inactivated vaccines. Following phylogenetic studies done in Australia and Ireland after NDV outbreaks, it was shown that wild non virulent strains of NDV that had reverted to virulence were responsible for the outbreaks. This supposedly occurred after the non-virulent viruses had circulated in poultry for some time. It has also been demonstrated that non virulent NDV strains turned virulent after passage in heterologous host cell lines, intramuscular inoculation in chicken and passage through intracerebral route in chicken. In Kenya, use of none virulent vaccine strains is widespread. It is not known if these vaccine strains revert to virulence after natural passage in chicken in the field thus establishing endemic ND foci. Neither is it known whether immunosuppression, as can be caused by poor nutrition, stress and unfavorable weather conditions in the field could contribute to this selection for virulence. This study was set to determine whether a lentogenic NDV would change to virulence after passage in leucocyte cultures of chicken and ducks. Forty NDV-free chickens and a similar number of ducks were used in the immunosuppression experiment. Half the bird in each group was used on passage experiments. One group in each species was immunosuppressed with dexamethasone while the other one was not. All the birds were treated with antibiotic to reduce bacterial contaminants in subsequent spleen cultures. Spleens were sampled each fifth day, leukocytes separated and cultured in 4 oz medical flat bottles and infected with a live attenuated Komarov NDV vaccine strain. Leukocyte cultures from immunosuppressed birds were treated with 20µl of dexamethasone per culture bottle. The cultures were

incubated under CO_2 atmosphere and progeny virus harvested amplified once in chicken embryonated eggs and then passaged into fresh leukocyte cultures. Virus virulence was tested by mean death time, (MDT) intracerebral pathogenicity, index ICPI and plaque assay in every passage. The result showed an increase in (ICPI) from 0 to 0.61 and 0.55 respectively for immunosuppressed chicken and ducks and was observed more in immunosuppressed than in the non immunosuppressed birds. Reduction in (MDT) was also observed from 154 hours to 81 hours and 88 in immunosuppressed ducks and chicken respectively. No plaque formation was observed in cultures infected with viruses passaged in both species of birds. These results show that passaging of the vaccine strain of NDV led to changes towards virulence. Further studies may need to be done in order to determine whether full virulence can be achieved. It was concluded that passaging of the vaccine strain of NDV in immunosuppressed chicken and duck leucocytes increased the virus ICPI and decreased its MDT, with increased potential of reverting to full virulence. It is recommended that use of live NDV vaccines in Kenya be reviewed with a view to using inactivated, or recombinant ND vaccine option and that causes of immunosuppression in poultry rearing environments be reduced or avoided.

CHAPTER 1

INTRODUCTION

1.1 Background

Newcastle disease (ND) is one of the major economically important poultry diseases in the world. In Kenya, the disease is widespread and has been reported in all Provinces (Nyaga *et al.,* 1985). Because chicken are the most susceptible birds, the disease is frequently responsible for devastating losses in poultry (Alexander, 2005). Newcastle disease virus (NDV) is a member of the order *Mononegavirales* in the family *Paramyxoviridae* in the genus *Avulavirus* (de Leeuw *et al*., 2005; Wakamatsu *et al.,* 2006 Tan *et al.,* 2008).

The virus has negative sense RNA genome of 15,186 – bases (Phillips *et al.,* 1998). Three pathotypes have been isolated all over the world. The avirulent strain is termed lentogenic, the low to medium virulence mesogenic and the highly virulent strain, the velogenic (Seal and King, 2001). The virus is pathogenic to chicken and other non – chicken poultry as well as many species of wild birds (Lancaster, 1966, Kadeta and Baldauf, 1998). The mortality and morbidity rates of non- immune chicken infected with the highly virulent strain of the virus can reach 100% (Alexander, 2003). All the three pathotype are indistinguishable serologically, structurally or morphologically. However, differences exist in their virulence (Slosaris *et al.,* 1989).

Recent incidences in Australia where velogenic NDV outbreaks had not been reported for a long time suggest that endemic lentogenic strains that are known to be non-virulent reverted to virulence under currently unknown circumstances leading to ND outbreaks (Gould, 2001). After two decades of strict ND prevention by vaccination in China, outbreaks occurred in sporadic version as opposed to epidemic nature. When molecular investigations were carried out the vaccine strain that was being used was identified as the most probable culprit through reversion because of the close identity with the NDV that caused the outbreaks (Yu *et al.*, 2001; Tan *et al*., 2008). Lentogenic strains have been shown to revert to virulence after passage in chicken brain and also in Mardin Darby Bovine Kidney (MDBK) cell lines (Islam *et al*., 1994).

Though it was suggested that the reversion occurs after the virus circulates in poultry for a long time, circumstances under which this reversion take place are hitherto obscure (Spradbrow, 2001). More recently, tagged lentogenic Lasota strain reverted to virulence after a single intracerebral inoculation into one day old chicken (Collins *et al*., 1994). Virulence of NDV is mainly determined by the amino acid sequence of the fusion protein (Fo) cleavage site (Seal *et al*., 2000). The virus becomes infective only when the precursor glycoprotein Fo is cleaved into F1 and F2 polypeptides. The ability of host proteases to cleave NDV glycoprotein Fo varies among different strains of NDV and this is the main determinant of mortality and morbidity in infected chicken (Gould e*t al*., 2001).

1.2 Problem statement and justification

Newcastle disease virus (NDV) is the cause of great economic loss due to its high mortality in poultry, and the cost of disease surveillance, control and vaccination. Evidence from Australia and China shows that non-virulent NDV strain can revert to virulence in the field. Possibility that lentogenic field strains and those of the live NDV strains being used as vaccines to control the disease may revert to virulence is a cause of great concern to the poultry industry. This is because it is not known whether these live vaccines might, after circulating in chicken over a period of time, revert back to virulence and infect some birds resulting in ND outbreaks. This could be the source of local disease outbreaks that eventually may establish endemicity in a country. Furthermore, the selective pressure leading to the reversion of NDV to virulence is unknown. This work sought to find out if immunosuppression, replication in leucocytes and passaging play any roles. Since there are many causes of stress in the poultry environment, immunosuppression was explored as a possible contributor to the reversion process.

The global economic impact of velogenic NDV (vNDV) is enormous and the virus represents a big drain on the world's economy. In developed countries where millions of poultry are kept in intensive systems, millions of dollars are lost when ND outbreaks occur. In developing countries where the main poultry population are indigenous chicken kept in free range and usually without vaccination, majority of these birds die out during disease epidemics. These chickens are in most cases the only source of animal protein in form of eggs and meat. Such mortality creates food deficit and economic losses.

1.3 Research questions

i. Does passage of lentogenic NDV in leucocytes cell culturesfrom normal or immunosuppressed chickens reduce MDT?

ii. Does passage of lentogenic NDV in leucocyte cell cultures from normal or immunosuppressed ducks reduce MDT?

iii. Does passage of lentogenic NDV in leucocyte cell culture from normal or immunosuppressed chicken increase ICPI?

iv. Does passage of lentogenic NDV in leucocyte cell culture from normal or immunosuppressed ducks increase ICPI?

v. Does passage of lentogenic NDV in leucocyte cell culture from normal or immunosuppressed chickens induce plaque formation?

vi. Does passage of lentogenic NDV in leucocyte cell culture from normal or immunosuppressed ducks induce plaque formation?

1.4 Hypotheses

i. Passaging of a lentogenic strain of Newcastle disease virus in normal chicken and duck leukocyte cultures would not affect its virulence.

ii. Passage in leukocyte cultures from immunosupressed chicken and ducks would not affect the virulence of a lentogenic strain of Newcastle disease virus.

1.5 Objectives

1.5.1 Main objective

To determine whether the lentogenic NDV would change to virulence after passage in leucocyte cultures of chicken and ducks.

1.5.2 Specific objectives

i. To determine the effect of successive passaging on virulence of lentogenic strain NDV in normal chicken and duck leukocyte cultures.

ii. To determine the effect of successive passaging on virulence of a lentogenic strain of NDV in immunosuppressed chicken and duck leukocyte cultures.

CHAPTER 2

LITRATURE REVIEW

2.1 Newcastle disease virus

Newcastle disease virus NDV is generally spherical particle of 100-500 nm in diameter. The surface of the particle is covered with projections about 8nm in diameter. The virus, which is a *Paramyxovirus*, consists of a single–stranded RNA molecule with a molecular weight of 5 x 10^6 daltons (Kolakofsky *et al.,* 1974) that makes up about 0.5 % by weight of the virus particle. Nucleotide sequencing of the NDV genome has shown it to consist of 15,186 nucleotides (Phillips *et al.,* 1998, Seal *et al.,* 2000). Virus particles have 20-25 % w/w lipid derived from the host cell and about 6% w/w carbohydrates. The density of average particle in sucrose is 1.18-1.20 g/ml.

2.2 Genomic structure

The genome of NDV codes for six proteins (Millar and Emerson, 1988), which were described by (Samson, 1988). Of the proteins, L protein is the RNA–directed RNA polymerase; HN is the haemagglutinin and is associated with the neuraminidase activities and forms the larger of the two types of projections seen on the surface of paramyxovirus particles; F is the fusion protein and forms the smaller of the surface projections; NP, is the nucleocapsid protein; P, the phosphorylated protein, and M the matrix protein. The order of the genes for these proteins in the virus genome is 3'N-P-M-F-HN-L5'. However, the P protein is edited during replication to produce two other proteins, namely

V and W Proteins. The V protein is associated with virulence. NDV edits its P gene by inserting one or two guanine residues at the conserved editing site in the genome sense and transcribes the P mRNA with a +2 frame shift (Huang *et al*., 2003).

2.3 NDV replication

The strategy for replication employed by NDV is that of the negative strand viruses (Peeples, 1988). The initial step is attachment of the virus to cell receptors mediated by the HN polypeptide. Fusion of the viral and cell membranes is brought about by action of the fusion (F) protein. Once the nucleocapsid complex enters the cell, intracellular virus replication takes place entirely within the cytoplasm. Since the virus has negative sense genome RNA the viral RNA directed RNA-polymerase (Transcriptase) must produce complementary transcripts of positive sense to act as messenger RNA and use the cells mechanisms enabling the translation into proteins and viral genome. The F protein is synthesized as a non-functional precursor Fo that requires cleavage to F1 and F2 by host proteases. The viral proteins synthesized in an infected cell are transported to the cell membrane, which becomes modified by their incorporation. Following the alignment of the nucleocapsid close to the modified regions of the cell membrane, virus particles mature by budding from the cell surface (McGinnes *et al*., 2002).

2.4 Transmission and epidemiology of NDV

Newcastle disease virus currently has a worldwide distribution (Seal *et al.,* 2000). In Kenya the disease is widely distributed and has been reported in all provinces (Nyaga *et*

*al.,*1985). National indigenous chicken which comprise about 70% of the 30 million poultry (Macharia, 2000) are frequently threatened by NDV outbreaks. Since the disease was first recognized in 1926, in Java, Indonesia four panzootics have probably occurred (Alexander, 2001). It was first reported in Kenya in 1935. The nature of spread of velogenic Newcastle disease virus (v-NDV) also affects the distribution. The first panzootic represented the initial outbreak in South East Asia. Doyle (1935) considered the disease to have moved slowly through Asia and caused isolated outbreaks such as in England in 1926 and took more than 30 years to spread worldwide (Doyle, 1935).

The second panzootic appears to have begun in the Middle East in the late 1960s and had reached most countries by 1973. This panzootic spread faster because the poultry industry had undergone a major revolution to a commercial industry and international trade. The panzootic seemed to have originated from a psittacine species through airborne shipment (Francis, 1973). Further seriousness of the second panzootic led to the development of vaccines and regimens that provided significant protection to poultry (Walker *et al*., 1973). According to antigenic and genetic evidence (Alexander, 1997; Lomniczi, 1998) the third panzootic in late 1970s was unclear presumably due to the almost universal use of vaccines since mid 1970s. A group of domesticated birds that was generally ignored as a potential source was the source of a fourth panzootic virus in the Middle East in the late 1970s (Kaleta *et al.*, 1985). By 1981, the virus had already reached Europe and then to all parts of the world. This source of viruses consisted of pigeons and doves (Columba livia) that are kept for racing, show or food purposes. The

variant nature of the virus enabled unequivocal demonstration of infection in 24 countries (Alexander, 2003).

2.5 NDV susceptible hosts

The virulence of NDV strains greatly varies with the host. Chickens are highly susceptible but ducks and geese may be infected and show few or no clinical signs even with strains lethal for chickens (Higgins, 1971). In chickens, the pathogenenicity of NDV is determined chiefly by the strain of virus although doses, route of administration, age of the chicken and environmental conditions all have an effect. In general, the younger the chicken, the more acute the disease. With virulent viruses in the field, young chickens may experience sudden deaths without any clinical signs. However, in older birds the disease may be more protracted and with characteristic clinical signs. Breed or genetic stock does not have a significant effect on the susceptibility of chickens to the disease (Cole and Hutt, 1961). Natural routes of infection are nasal, oral and ocular and they appear to emphasize the respiratory nature of the disease (Beard and Easterday, 1967). Intramuscular, intravenous and intracerebral routes are artificial inoculation routes and appear to enhance the neurologic signs (Beard and Hanson, 1984).

2.6 NDV pathogenicity

Isolates of NDV are distinguished or grouped in the laboratory by assessment of virulence. Hanson and Blandly (1955) suggested that strains of NDV can be grouped as "velogenic'', ''mesogenic'' and "lentogenic" based on chicken embryo mortality at <60

hr., 60-90 hr; and >90 hr respectively after allantoic inoculation (Hanson and Blandly, 1955). The values obtained provide a guide to the disease produced in infected chickens. The other tests used are the intracerebral pathogenicity index (ICPI) in day old chicks (with values of, <0.7 for lentogenic, 0.7-1.4 strains for mesoogenic strains and intravenous pathogenicity index (IVPI) in six – week old chicken (with values of 0.0-0.5 for lentogenic strains, 1.0-1.5 for the mesogenic and 2.0-3.0 for the velogenic strains. (Office of International Epizootics, (OIE) manual, 1996). The less the number of hours obtained in the mean death time, the more virulent the virus. However the higher the ICPI and IVPI values, the more virulent the virus, and ranges from 0.0 for the avirulent strains to 2.0 for the most virulent but 0.0-3.0.in the IVPI test (Alexander, 2003; Momayez *et al*., 2007).

Plaque formations in presence of trypsin, size and morphology of the plaques formed all have been used to characterize ND viruses (Hanson *et al.*, 1975). Low virulence NDV isolates do not form plaques in cell culture without addition of diethylaminoethyl (DEAE) and magnesium (Mg^{2+}) ions (Barahona and Hanson, 1968, 1988; Goodman and Hanson, 1988) or trypsin to the agar overlay. Plaques may be of two morphologic types; clear or red and the size produced appear to be related to the virulence of the virus for chickens (Reeve and Poster, 1971). Both the virulent viruses in the velogenic and mesogenic groups form plaques in chicken embryo fibroblast cell cultures without additives like (Mg+), (DEAE) or trypsin while the lentogenic strains do not, and the more virulent the virus, the larger the plaque size (Krishnamurthy *et al*., 2000).

2.7 Molecular basis of NDV pathogenicity

During the replication of NDV the functionally important fusion protein is produced as a precursor glycoprotein, Fo, which has to be cleaved to F1 and F2 for the progeny virus particles to be infective (Rott and Clenk, 1988). This post translation cleavage is mediated by host cell proteases (Nagai *et al*., 1976).

If cleavage fails to take place noninfectious virus particles are produced. Trypsin can cleave Fo for NDV strains, and *in vitro* treatment of noninfectious virus will restore infectivity (Nagai *et al., 1976*). The importance of Fo cleavage was easily demonstrated because viruses normally unable to replicate or produce plaques in cell culture systems were able to do both if trypsin was added to the agar overlay or culture fluid. Although all viruses could replicate and produce infectious progeny in the allantoic cavity, the viruses pathogenic for chickens could replicate in a wide range of cell types in vitro with or without added trypsin whereas strains of low virulence could replicate only when trypsin was added (Rott, 1979; Rott, 1985).

The Fo molecules of virulent viruses can be cleaved by a host protease or proteases found in a wide range of body organs. The Fo molecules in viruses of low virulence are restricted in their sensitivity to cleavage by specific host enzymes. Consequently, those viruses can grow only in certain host cell types, namely enterocytes. Early reports of the deduced amino acid sequences of the Fo precursor obtained from nucleotide sequencing of the F gene for a number of NDV strains (Chambers *et al.,* 1986; Mcginnes and

Morrison, 1986; Toyoda *et al.,* 1987; Glickman *et al.,* 1988; Millar and Emerson, 1988; Schaper *et al*., 1988; Collins *et al.,* 1993), enabled comparison of viruses of low virulence to those that were velogenic or mesogenic.

The amino acid at residue 116, the C- terminus of the F2 protein at the site cleavage was arginine. The viruses of low virulence all had leucine at residue 117, the N- terminus of the F1 protein and another basic amino acid at residue 113. In contrast all velogenic or mesogenic viruses had phenylalanine at residue 117 and with one exception basic amino acids at residues 115 and 112 in addition to those at 113 and 116. The exception was the pigeon variant Paramyxovirus type one (PMV-I) virus which was identical to the virulent viruses but lacked a basic amino acid at position 112. Further studies have identified that this variation was usual for pigeon variant PMV-I viruses but had no significance in the variability of pathogenicity to chickens recorded with these viruses (Collins *et al.,* 1994). It would appear that the mechanism controlling the pathogenicity of NDV is very similar to that described for influenza viruses (Webster and Rott, 1987). The presence of additional basic amino acids in virulent strains means that cleavage can be affected by protease or proteases present in a wide range of cell types in different host tissues and organs.

This ubiquitous enzyme has not been fully identified but as with avian influenza viruses, it is likely to be one or more protein processing subtilisin –related endoprotease (s) of which furin is the leading candidate (Fiji *et al., 1*991; Stieceke –Gober *et al.,* 1992). For

entogenic viruses, cleavage can occur only with proteases recognizing a single basic amino acid and is done by trypsin-like enzymes present in the gut. Lentogenic viruses therefore replicate only in cells where there are trypsin like enzymes. such as the intestinal epithelia whereas virulent viruses can replicate in cells located in a wide range of tissues and organs resulting in a fatal systemic infection (Rott, 1979).

2.8 Thermal stability of NDV

Thermal stability of NDV isolates varies (Hanson *et al.,* 1949) and has been used for virus characterization. This characteristic has been proven to be a useful tool in epizootiologic studies (Hanson and Spalatin, 1978) and rapid method for distinguishing between some avirulent and virulent viruses. The haemagglutinin of lentogenic strains is more easily inactivated at $56^{o}C$ while the virulent strains are more resistant at that temperature (Hanson and Blandly, 1955).

2.9 Cultivation of NDV

Newcastle disease virus can multiply in a range of non - avian (Lancaster, 1966) as well as avian (Kadeta and Baldauf, 1988) species, following laboratory infection. The chicken however, remains the most readily available and frequently used laboratory animal as well as the most important (Kessler *et al*., 1979) natural host of the disease. All avian paramyxoviruses replicate in embryonated chicken eggs. Embyonated eggs are generally used for virus propagation because of their sensitivity for virus growth and high titers to

which viruses grow. Newcastle disease virus strains vary in their capacity and time taken to kill chicken embryos.

Virus titers are also influenced by strains, with the highest titers obtainable by those causing slow or no death (Gough *et al*., 1974). The presence of maternal antibodies in the yolk also affects virus multiplication in chicken embryo (French *et al*., 1976). The route of inoculation is also important (Beard and Hanson, 1984). Inoculation of NDV via the yolk sac compared with the allantoic cavity produced more rapid embryo deaths and caused deaths by strains that do not consistently kill by the latter route (Estupinan *et al.*, 1968).

The virus can replicate in a wide range of cells. Lancaster, (1966) listed 18 primary cell types and 11 cell lines as susceptible. Many more have been added to his list since his 1966 report. Macrophages and monocytes will support NDV replication (Seal *et al.*, 2000). The type of CPE produced by NDV is syncytia formation with subsequent cell death. The cytopathogenic effect CPE has some relationship to the virulence of strains for chickens. Plaque formation in chick embryo cells is restricted to velogenic and mesogenic viruses unless $Mg2^{+}$ ions, DEAE (Brahona and Hanson, 1968) or trypsin (Rott, 1985) are added to the overlay agar.

2.10 Genetic variations and mutations

During the intense investigations and surveillance work following outbreaks of NDV in Australia in the 1998-2000 several viruses showing variation in the amino acids at the F0 cleavage site were isolated (Westbury, 2001; Gould *et al*; 2001). The variations in those naturally occurring viruses confirm that the minimum requirement for ND viruses to show high virulence for chickens, appears to be the motif -113 R X R / KR* F 117. The reason for absolute requirement of phenyl alanine at position 117 is unclear and may not be part of the recognition motif of the ubiquitous protease.

The greater understanding of the molecular basis for virulence has given an insight into how viruses that causes velogenic NDV may emerge. Hanson, put forward three suggestions to account for the sudden emergence of virulent NDV (Hanson; 1972, 1978).

(i). The virus had always been in poultry but was unnoticed until the development of commercial poultry industries;

(ii). The virulent virus was enzootic in another species in which it showed less severe disease and

(iii). The virulent virus arose by mutations from virus of low virulence in poultry.

The first hint that the third explanation of virulent viruses arising from low virulence strains came from studies on viruses responsible for NDV outbreaks in Ireland in 1990. These variant viruses were closely related to variant viruses of low virulence usually

isolated from water fowls but both were genetically and antigenically distinct from all other NDVs (Alexander *et al.,* 1997; Collins *et al.,* 1998).

Much better evidence for mutations to virulence has come from the 1998-2000 v-NDV outbreaks in Australia (Kirkland, 2000; Westbury, 2001). Phylogenetic studies showed the virulent viruses responsible for the outbreaks in Australia in 1998 and 1999 to be very closely related to each other and to a virus of low virulence isolated earlier from chickens in the same geographical area (Gould *et al*., 2001). The overwhelming probability is that the virulent ND viruses emerging in Australia in 1998 were the result of mutations occurring viruses of low virulence and there is no reason to suppose that other similar mutations have not taken place in the past (Alexander, 2003). A recent study in China puts the situation into perspective where phylogenetic studies incriminated a lentogenic vaccine strain of NDV which had been used to vaccinate poultry for two decades when outbreaks of ND occurred. The virus responsible was very closely related to the vicinal virus (Tan *et al*., 2008).

2.11 Economic impact of NDV

The global economic impact of v-NDV) is enormous and probably represents a big drain on the world's economy. In late 2002 exotic Newcastle disease (END) invaded California in the United States of America (USA) (CIDRAP, 2003).More than 3.5 million birds at over 2,100 sites were affected. The cost of controlling the outbreak was US $160 million. Economic impact of ND outbreaks is not well documented but in Nairobi,

Kenya, there were 36 outbreaks mostly in exotic chicken recorded in 1989 and the loss due to mortality was estimated at US$ 0.6 million (Musiime, 1991).

Financial losses per bird in the event of ND outbreaks in rural chicken in Plateau state in Nigeria cost US $ 9,263 per anum (Musa *et al.,* 2009). In developed countries with established poultry industry, not only are outbreaks of NDV extremely costly but measures including vaccination represent a continuing cost to the industry. Countries free of NDV face the cost of repeated testing to maintain that status and for the purpose of trade (Leslie *et al.,* 2000). In many developing countries v-NDV is endemic and is a major constraint to commercial poultry production and establishment of trade links (Onapa, 2005).

2.12 Public health significance of NDV

Apart from its contribution to malnutrition NDV is a recognized human pathogen in its own right. Reports of disease have been anecdotal but the most substantiated clinical signs in human infections have been eye infections usually consisting of unilateral or bilateral reddening, excessive lacrimation, edema of the eyelids, conjunctivitis and subconjuctival haemorrhages (Zeydani *et.al.,* 1988). Infections are usually transient, and the cornea is not affected. There have been less well substantiated reports that a more generalized infection may sometimes occur resulting in chills, head aches and fever with or without conjunctivitis. Both vaccinal and virulent strains of NDV for poultry may

infect and cause clinical signs in humans. Human infections with NDV have usually resulted from direct contact with the virus such as from splashing infective fluids into the eyes with hands contaminated with the virus after handling infected birds or their carcasses and contamination of vaccination personnel especially when vaccination is given by aerosol. Such infections usually can be avoided by basic hygiene, appropriate clothing and eye protection. Casual contact with infected poultry represents a low risk of human infection. No reports exist of human-to-human spread.

More recently, a fatal human case of avian Paramyxovirus type-1 (APMV-1) infection associated pneumonia was described in a 42 year old man following a peripheral blood stem cell transplant. The Patient was suffering from a non Hodgkin's lymphoma and this was likely to be a unique case probably an opportunistic infection due to the patient's immunological condition. Nevertheless, this case shows that physicians and diagnostic laboratories should consider Newcastle disease virus (APMV-1) a possible source of disease especially in light of the significant segment of the population that is immunosuppressed owing to human immunodeficiency virus infection, cancer chemotherapy or organ transplant (Goebel *et al.,* 2007).

2.13 Control of NDV

Under industrial production conditions Newcastle disease has been successfully controlled through vaccination, biosecurity and different tailored policies (Higgins and Shortridge, 1988). Some countries like most Nordic countries banned the use of any

vaccines while others such as the Netherlands enforce vaccination of all poultry (Alexander, 1997). Nevertheless, ND outbreaks bear a considerable economic impact on

the poultry industry ranging from losses due to disease and expenses of vaccination to significant cost of diagnostic laboratory investigation and surveillance (Jørgensen *et al.*, 1999).

2.14 Vaccination issues

Live vaccines and inactivated vaccines from Master Seeds of ICPI of less than 0.4 and 0.7 respectfully, are currently used in countries that vaccinate against Newcastle disease (OIE, 1996). Mesogenic strains such as Strain H and Muketswar are attenuated in eggs and are used for secondary vaccination (Onapa, 2005). In Kenya, the ND vaccines used are LaSota and F (Asplin) strains, both of which are live naturally attenuated lentogenic pathotypes (Musiime, 1991). The Ministry of Livestock Development is in the process of introducing a thermal stable live attenuated NDV vaccine (I_2) which can be administered in feeds and its handling does not consider the cold chain as being very critical unlike for the former two strains. 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 (aerosol) spray although it is limited to secondary vaccination to avoid vaccine reactions (Alexander, 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental design

A Komarov live attenuated vaccine strain of NDV that had been stored frozen at -20^{o}C in the Kabete repository was thawed at 37^{o}C. The virus was identified as NDV with known NDV reference antiserum by a standard haemagglutination inhibition test (OIE, 1996). Initial virulence status was tested by (i) mean death time (MDT) in 9-10 day old chicken embryonated eggs, (ii) intracerebral pathogenicity index (ICPI) in one day old chicks and (i) plaque assay in chicken embryo fibroblasts to confirm the virus pathotype.

A group of forty indigenous chicken and forty ducks, six months old, was raised from a disease free flock, maintained at the Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi, Faculty of Veterinary Medicine. Good bio security measures were observed for the flocks including making sure that only the poultry attendant entered the premises and did not come into contact with any other poultry. A foot bath containing Omnicide^{R} (Glutaraldehyde and coco-benzyl-dimethyl ammonium-chloride, Cooper Kenya Ltd) disinfectant was maintained at the entrance to the main building and all pens inside the poultry house. Protective clothing to be used inside the poultry house only was also provided. The main building and poultry house clothing were provided. The birds were fed with growers mash (Unga^{R} Ltd Kenya) and clean water was provided from taps within the building. Clean wood shavings were

evenly spread in the rooms for bedding material. Waste litter was collected and disposed of safely regularly.

After acclimatization of the birds in the experimental isolation house, all the forty birds in each species were screened for anti-NDV antibodies. The birds were bled via the wing vein and the 3 ml blood obtained was allowed to clot in universal containers in a slanting position at room temperature, then placed at $+4^{o}C$ overnight. The serum was obtained by centrifuging the universal containers at 3000 R. P.M for 10 minutes and collected into bijou bottles and stored at $-20^{o}C$ until they were tested. All the sera were tested for anti-NDV antibodies by the standard haemagglutination inhibition test. Subsequently, the birds were immunosuppressed as described later.

3.2 Purification and identification of Komarov NDV

A Komarov live attenuated strain of NDV was obtained from the Kabete repository and identified by haemagglutination inhibition test using a reference polyclonal anti NDV serum obtained from the repository at the Department of Veterinary Pathology, Microbiology and Parasitology of the University of Nairobi. The virus was purified three times by limit dilution in 9-10 day old specific pathogen free embryonated chicken eggs obtained from Kenya Veterinary Vaccine Production Institute (KEVEVAPI). Dilutions used ranged from10^{-1}-10^{-12} and the highest dilution that showed positive haemagglutination reaction was taken as the pure virus. A stock of the purified Komarov

virus for future use was amplified by a further single passage in embryonated chicken eggs harvested and stored at –20^{o}C awaiting use in immnosuppression and other experiments.

3.3 Preparation of antiserum to purified Komarov NDV

Three NDV free cocks were kept in isolation and immunised in order to obtain anti-NDV serum. Allantoic fluid of the purified Komarov virus Sterilzed by filtration was inactivated with 37% formalin (0.025 ml of formalin per ml of allantoic fluid) Macharia, (1999) overnight at room temperature and each cock injected with 0.5ml into each thigh.

The birds were boosted with 0.25 ml per thigh with a similarly treated virus after one week. The cocks were then bled through the wing vein two weeks later. Serum was separated by allowing blood to stand at 37^{o}C and then left at +4^{o}C overnight and centrifuged at 3000 R.P.M for 10 minutes. The clear serum was collected using pasteur pipettes and titrated by haemagglutnation inhibition. The serum was stored at -20^{o}C in 4 ml aliquots until when used.

3.4 Initial determination of Komarov virus pathogenicity

Initial virulence status of the purified virus was determined by intracerebral pathogenicity index in one day old chicks (OIE, 1996), mean death time in 9-10 day old embryonated chicken eggs (OIE, 1996) and also by plaque assay in chicken embryo fibroblast monolayer agar overlays.

3.5 Intracerebral pathogenicity index (ICPI)

The chicks were provided with clean drinking water, commercial chick mash and clean wood shavings used for bedding. An infrared lamp was provided as a source of heat. Each of the chicks was injected into the left cerebral hemisphere with 0.05ml of the purified Komarov Newcastle disease virus of titer >1:16 diluted 1:10 in sterile physiological saline (Figure 3.1). The chicks were also labeled serially from number 1-10 with blue marker pen on the back and also with a tape marked with pen on the right leg for ease of individual chick identification and monitoring. It was ensured that each chick drank some water by dipping the beak into the drinker and then released into the brooder. The chicks were observed for eight days and its health state scored and recorded once daily. The health state was scored as zero for normal, one for sick and two for a dead chick. The ICPI was calculated as the total score per bird per day for the eight days (OIE, 1996).

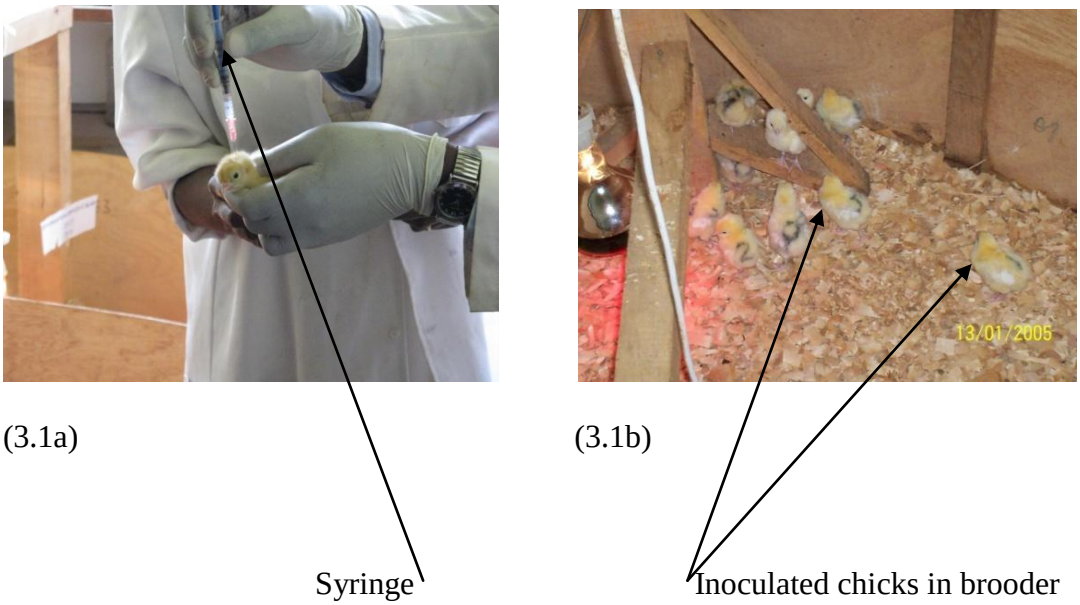


Figure 3.1: Intracerebral inoculation of one day old chicks (Figure 3.1a) and the chicks immediately after inoculation in the brooder (Figure 3.1b)

3.6 Mean death time (MDT)

Dilutions of the purified virus harvests were made in sterile physiological saline and 0.1ml of the dilutions 10^{-6}-10^{-9} inoculated into each of five 9-day old pencil labeled embryonated eggs per dilution and incubated at 37^{o}C. A similar number of eggs was infected eight hours later and incubated in the same way (OIE manual, 1996). The eggs were candled twice daily for seven days. Any deaths of embryos and the time of death were recorded. Eggs in which embryos died were chilled at +4^{o}C and tested for haemagglutination activity at the end of the MDT test. Mean death time was calculated

from the dilutions in which all the embryos died. The mean death time in hours was taken as the highest dilution where all the embryos died.

3.7 Screening of chicken and ducks for NDV antibodies

Forty, adult indigenous chicken and a similar number of ducks of both sexes bred in isolation in the Department of Veterinary Pathology, Microbiology and Parasitology were screened for NDV antibodies by standard haemagglutination inhibition (HAI) test (OIE, 1996). To each row of a microtitre plate was dispensed 25µl of phosphate buffered saline and 25µl of the chicken serum was also dispensed into the first well only. Serial dilutions of the sera were made with the last 25µl discarded. A volume of 25µl of Komarov NDV containing 4 haemaglutination units was dispensed into all the wells. A positive control reference serum was included on the last row of the micro plate. The plates were incubated at room temperature for 30 minutes and 25µl of 1% chicken RBC dispensed into each well. The plates were shaken gently and incubated at room temperature. The test was read after 45 minutes. Red blood cell and antigen control wells were read first and when satisfactory, other wells were read. Any well that showed positive haemagglutination was considered NDV antibody negative while no agglutination was interpreted as antibody positive and the reciprocal of the end point taken as the titer.

3.8 Immunosuppression experiments

Chicken and ducks sero-negative for NDV were isolated into their respective rooms previously prepared with clean wood shavings ready for immunosuppression experiments (Figure 3.2 a, b).The birds were fed with growers mash and clean drinking water was also provided. Four ducks and a similar number of chicken were weighed and the weights recorded against their respective wing band numbers to ensure correct identity during the immunosuppression experiments.

(3.2a)

(3.2b)

Figure 3.2: Chicken (Figure 3.2 a) and ducks (Figure 3.2b) in their respective experimemntal rooms after immunosuppression

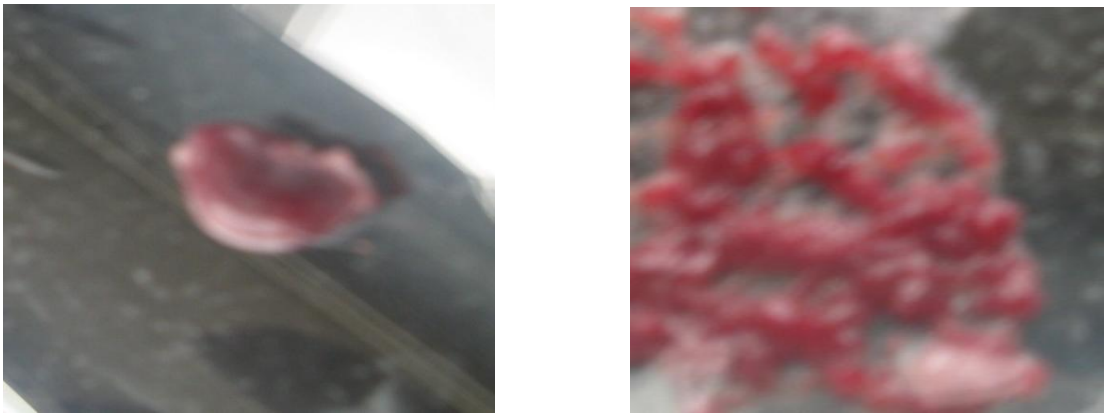
Dexamethasone (Dexacoryl^{R} Coophavet, St. Herblon-44153 ANKENIS cedex, France) is known to suppress immunity. It has also been shown to induce cell mediated immunosuppression and decrease resistance to infection in avian species (Huff *et. al*., 1999, Huff *et al*,. 2000). Two birds from each species were injected intramuscularly daily with the Dexamethasone via the breast muscle using 21 gauge needle at the rate of

4mg/kg body weight for chicken and half the dose for the ducks for four days (Figure 3.3). Each bird including the non-immunosuppressed group was disinfected with 70% ethanol at the injection site before it was injected with QuinAbic^{R} injectable solution (Teva Phamaceauticals (Pty) Ltd (Edms) Bpk 26 Blumberg straat 26 South Africa) intramuscularly at the same time daily at the dose rate of 0.1mg/kg body weight to reduce contamination in the subsequent leucocyte cultures.

Figure 3.3: Inoculation of a duck with dexamethasone through the intramuscular route into the breast muscle

3.9 Sampling of chicken and ducks spleens for preparation of leucocyte cultures

On each fifth day post immunosuppression, the four chicken and four ducks were sacrificed and spleens sampled (figure 3.4a) aseptically into labeled sterile beakers covered with aluminium foil. The spleens were taken to the laboratory and teased in a sterile petri dish after decapsulation (figure 3.4b). Each teased spleen was placed into a sterile siliconised centrifuge tube containing 10 ml-chilled RPMI 1640 medium and shaken gently to dislodge the cells.



(3.4a) (3.4b)

Figure 3.4: Intact chicken spleen (Figure3.4a) and the same spleen teased on a petri dish (Figure 3.4b) during spleen leucocyte culture preparation

The mixture was placed on an ice bath and subsequently layered onto 10 ml of ficoll-paque density gradient according to the method of Pei *et al*., (2001) and centrifuged at 1000 R. P.M for 15 minutes. Cells at the interphase between the density gradient and the

RPMI 1640 were pipetted out with siliconised 5ml pipettes and washed three times by centrifugation at 1000 R. P.M in cold RPMI 1640 medium for 10 minutes. A sample of the cell suspension was stained in trypan blue, counted in a Neuber chamber and cell concentration adjusted to $1.0x10^6$ cells/ml in medical flat bottles followed by addition of 20µl of Dexacoryl^R per medical flat bottle in 10ml volumes. The cultures were infected with the purified Komarov virus and incubated in a CO_2 atmosphere for four days. Virus harvest was obtained in the supernatant after low speed centrifugation at 2500 RPM for 10 minutes in a bench centrifuge. Presence of the virus progeny was tested in the culture supernatant by haemagglutination test and also by culturing of supernatants in 9-day old chicken embryonated eggs. Virus harvests from cultures that had undergone similar treatment were pooled and titer determined by haemagglutination test and stored at –20^{o}C awaiting next passage.

3.10 Mean death time test for NDV passages

Dilutions of the virus harvests from the four respective categories of birds leucocytes were made in sterile physiological saline and amounts of 0.1ml for each of the 10^{-6}-10^{-9} dilutions inoculated into each of five 9-day old chicken embryonated eggs per dilution and incubated at 37^{o}C. A similar number of eggs was infected eight hours later and incubated in the same way (OIE manual, 1996) as in section 3.6.

3.11 Intracerebral Pathogenicity index test for NDV passages

Four brooders were prepared for four groups of ten-one day old chicks in respective

brooders according to bird species and treatments. Clean drinking water, commercial chick mash and clean wood shavings used for bedding were provided in each brooder. An infrared lamp was provided as a source of heat for each brooder. Each of the ten chicks per group was injected with one of the four leucocyte culture harvests into the left cerebral hemisphere with 0.05ml of the Komarov NDV of titer >1:16 diluted 1:10 in sterile physiological saline (Figure 3.1). The chicks were also labeled serially from number 1-10 with blue marker pen on the back and also with a tape marked with pen on the right leg for ease of individual chick identification and monitoring. It was ensured that each chick drank some water by dipping the beak into the drinker and then released into the brooder. The chicks were observed for eight days and their health state scored and recorded once daily. The health state was scored as zero for normal, one for sick and two for a dead chick. The ICPI was calculated as the total score per bird per day for the eight days (OIE, 1996).

3.12 Plaque assay test for NDV passages

Specific pathogen free 9-day old chicken embryos were used to prepare chicken embryo fibroblast monolayers according to standard methods. After reaching confluence the growth medium was aspirated out and fibroblasts washed with warm (37^{o}C) Hanks balanced salt solution (HBSS). The fibroblast cell cultures were then infected with 0.2ml of varying virus concentrations (neat, 1/5, 1/10, 1/100, 1/1000) and adsorbed for one hour at 37^{o}C. Excess virus was washed and aspirated out and the monolayers overlaid with an

equal volume (1:1) of 1.4%agarose in (HBSS) and minimum essential medium containing 5% fetal bovine serum in six oz medical flat bottles. The agarose overlay was allowed to solidify and the bottles incubated at 37^{o}C with the agarose overlay side uppermost in a CO_2 atmosphere with caps loosened. The cultures were observed for plaque formation daily for seven days. A positive control reference NDV was included.

3.13 Haemagglutination profile for Komarov NDV with mammalian and avian RBCs

Blood was collected from various animals of mammalian species and also birds into screw capped universal bottles containing Alsever's solution. The red blood cells were washed three times in phosphate buffered saline and finally a 1% red blood cell suspension was prepared in the PBS and stored at +4^{o}C. Each of the Komarov virus passages was then tested for haemagglutination activity with a suspension of 1% red blood cells on a micro titer plate using the methods of Kasiiti (2000) and Wambura *et al*. (2006) as described before.

Phosphate buffered saline (PH7.2) was dispensed into wells of a U bottomed micro titer plate in volumes of 25µl. A volume of 25µl of each of the Komarov virus passages was also dispensed into each first well of the micro titer plate and mixed. Serial doubling dilutions of the virus were then made. A volume of 25µl of the 1% RBC was dispensed into each well. Negative control wells containing only PBS and the 1%RBC suspension were also set at the end of each row.

3.14 Thermal inactivation profile for Komarov NDV passages

Haemagglutination test was performed on all the Komarov NDV virus passages to determine initial titers before inactivation commenced using a modification of the method of Wambura *et al*., (2006). Water bath temperature was set at 56^{o}C and a volume of 1.0 ml from each of the 40 passaged chicken and duck leucocytes Komarov amplified stock viruses was dispensed into an equal number of labeled bijou bottles and then tightly capped. The titers were determined, recorded and subsequently vials containing the viruses were immersed in the water bath removed and placed in an ice bath to stop continued inactivation. The experiment was repeated with 40 more vials which were incubated for 2, 3, 4 and 5 minutes respectively. At the end of the incubation period the vials were removed and placed in an ice bath and titrated for haemagglutination activity (Wambura *et al*., 2006).

3.15 Data analysis

Data analysis was done by instat version 3.36 computer programme. T-test was used to compare the difference between two means for the immunosuppressed and non-immunosuppressed chicken leucocyte passaged virus titers and similarly for the ducks. Where the data was not normally distributed, wilcoxon non-parametric test was used. All tests were two tailed with α=0.05. The graphs were drawn by micosoft excel.

CHAPTER 4

RESULTS

4.1 Purification of Komarov NDV

The original Komarov virus had an initial haemagglutination titer of 1:32 and haemagglutinaton inhibition titer of 1:16. After the three purifications by limit dilution and passage in specific pathogen free chicken embryonated eggs, the virus titers increased as shown in Table 4.1. By the end of the third passage, the titers had reached 1:4096 in dilution 10^{-11} as shown in Table 4.1.

Table 4.1: Komarov virus haemagglutination titers in different purification stages showing highest virus titer in the third stage, taken as the pure virus

	Limit dilution purification stages		
Virus Dilutions	First purification (HA titers)	Second purification (HA titers)	Third purification (HA titers)
10^{-1}	1:512	1:256	1:2048
10^{-2}	1:1024	1512	1:2048
10^{-3}	1:1024	1:128	1:4096
10^{-4}	1:1024	1:1024	1:4096
10^{-5}	1:1024	1:512	1:4096
10^{-6}	1:1024	1:1024	1:4096
10^{-7}	1:1024	1:512	1:4096
10^{-8}	1:1024	1:512	1:4096
10^{-9}	-ve	1:512	1:4096
10^{-10}	-ve	1:512	1:4096
10^{-11}	-ve	-ve	1:4096
10-^{12}	-ve	-ve	-ve
-ve Control	-ve	-ve	-ve

Key: -ve =No virus was detected in the haemagglutination test for that dilution

HA= Haemagglutination

4.2 Preparation of anti-NDV serum

Serum from two of the cocks (1 and 2) showed similar NDV antibody titer (1:256) after haemagglutination inhibition test, but the third cock had higher antibody levels (1:512 as shown (Figure 4.1).

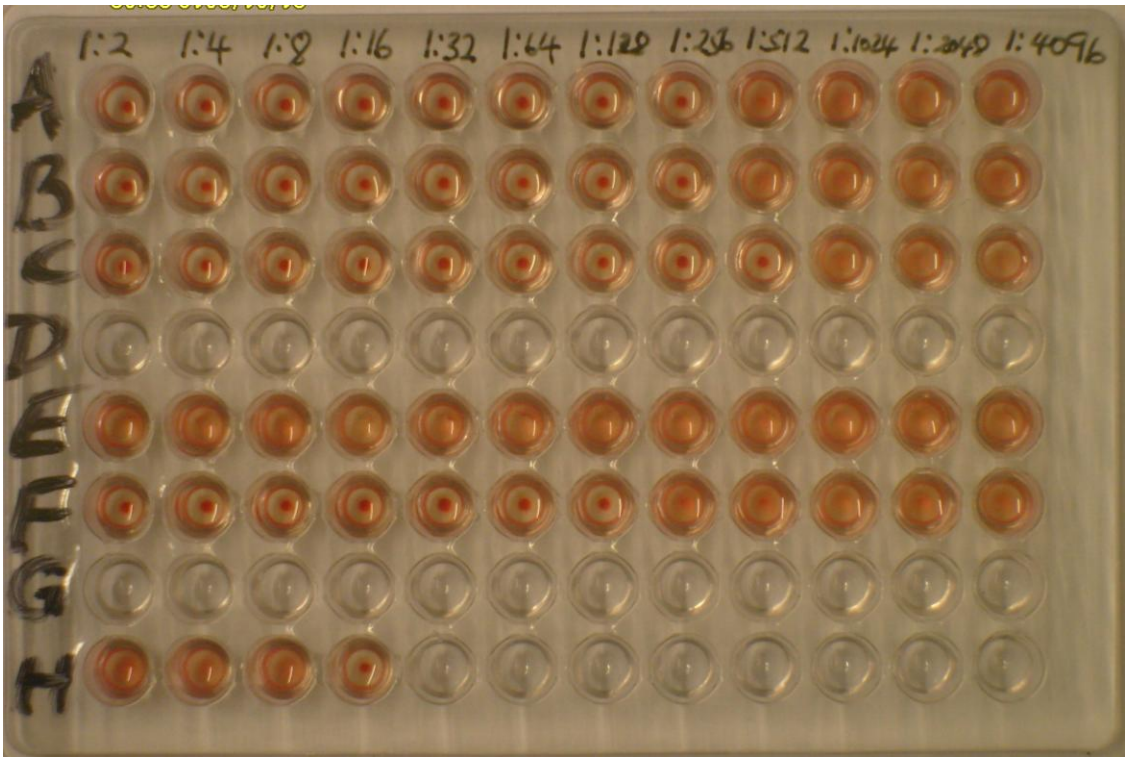


Figure 4.1: Haemagglutination inhibition test during the testing of anti- NDV serum. Row E shows negative serum control indicated by positive haemagglutination of the chicken red blood cells) while Row F is the positive serum control (showing complete haemagglutination inhibition up to the seventh well). Row H is the back titration of the four haemagglutination units showing no RBC agglutination in the forth well.

4. 3 Screening of chicken and ducks for NDV antibodies

Chicken and ducks that were to be used for Immunosuppression experiments were screened for NDV antibodies. Haemagglutination inhibition test was performed and an end point was indicated by a red button of RBC at the bottom of the well while a negative test was indicated by a matt at the bottom of the well. All the birds were found to be free of antibodies to NDV.

4.4 Initial NDV virulence tests

Before the viruses could be used for the various tests, following three tests were performed on the purified viruses: mean death time, intracerebral pathogenicity index and plaque assay (OIE, 1996). The purified Komarov NDV had an intracerebral index (ICPI) of 0.0, mean death time of 154 hours and showed no signs of plaque formation as shown in Table 4.3. The three virulence tests for determining virus status, mean death time, intracerebral pathogenicity index and plaque assay were performed according to the protocol previously described in materials and methods.

Table 4.2: Showing initial virulence status of the original unpassaged Komarov NDV

Test	**Test results**
Intracerebral pathogenicity index (ICPI)	0.0
Mean death time (MDT)	154 Hours
Plaque assay	-ve

Key: -ve = No plaque formation; 0 = Lowest ICPI value which classifies the virus in the Lentogen pathotype

4.5 Virus titers in leucocyte cultures

Both chicken and duck leucocytes remained suspended in the cell culture medium throughout the culture period and did not attach on to the inner surface of the cell culture bottle. Leukocyte culture virus harvest that were harvested in most passage levels had low virus haemagglutination titers (1:4 -1:64) except in passage 7 where both non-immunosuppressed chicken and duck leucocyte cultures showed much higher levels (1:128) and (1:256) respectively, as compared to both non-immunosuppressed bird species both of which showed HA titer of only 1:16. Immunosuppressed chicken leukocytes showed lower virus titers at more passage levels (6 out of 10 passages) than in the non immunosuppressed chicken leukocytes (2 out of 10). There were also more passage levels where virus titers were higher in immunosuppressed duck leukocytes (5 out of10) than in the non-immunosuppressed (2 out of 10).

Immunosuppressed chicken leukocyte cultures had higher virus titers at more passage levels (4 out of 10) than duck leukocyte cultures (2 out of 10) while virus titers from non-immunosuppressed chicken leucocytes had higher titers at more passage levels (7 out of 10) than in non-immunosuppressed ducks(1 out of 10) (Table 4.3). There was however no statistically significant differences between the immunosuppressed and non-immunosuppressed chicken passaged virus titers ($p>0.05$) and similarly for the duck leucocyte passaged viruses.

Table 4.3: Leukocyte culture supernatant harvest virus HA titers before amplification

Passage Level	**CHICKEN**		**DUCKS**	
	IS	N-IS	IS	N-IS
1	1:16	1:4	1:4	1:4
2	1:32	1:32	1:64	1:16
3	1:32	1:16	1:16	1:4
4	1:4	1:16	1:4	1:4
5	1:8	1:32	1:8	1:32
6	1:16	1:16	<1:2	<1:2
7	1:16	1:128	1:16	1:256
8	1:8	1:32	1:16	1:8
9	1:8	1:16	1:32	1:8
10	1:8	1:16	1:16	1:8

Key: IS = immunosuppressed chicken or ducks: N-IS = non –immunosuppressed chicken or ducks.

4.6. Komarov NDV passage titers after a single amplification in chicken embryonated eggs

Each of the leucocyte culture virus passages was amplified in 9-10 day old chicken embryonated eggs, by a single passage. The resultant virus haemagglutination titers ranged from 1:128 - 1:2048. In seven out of the ten passages titers of viruses passaged in immune-suppressed leukocytes were slightly higher than those from non-immunosuppressed chicken leukocytes (Table 4.4). Only five out of the ten virus passage

titers from immunosuppressed ducks were higher than those from non-immunosuppressed ducks (Table 4.4).The virus titers were not statistically different ($p>0.05$).

Table 4.4: Komarov NDV haemagglutination titers after a single amplification in chicken embryonated eggs at different passage levels

PASSAGE LEVEL	CHICKEN		DUCKS	
	IS	N-IS	IS	N-IS
1	1:512	1:128	1:128	1:128
2	1:512	1:512	1:512	1:256
3	1:256	1:1024	1:512	1:1024
4	1:1024	1:2048	1:512	1:1024
5	1:128	1:512	1:256	1:1024
6	1:256	1:1024	1:512	1:256
7	1:128	1:256	1:256	1:512
8	1:256	1:2048	1:128	1:512
9	1:512	1:128	1:512	1:128
10	1:128	1:512	1:256	1:1024

Key: IS = immunosuppressed chicken or ducks: N-IS = non –immunosuppressed chicken or ducks.

4.7 Mean death time after NDV passages

Mean death time of the original virus was 154 hours and subsequent passage indicated a downward trend right from the first passage and in all the experimental groups for both chicken and ducks. Immunosuppressed ducks indicated the highest value in MDT reduction in passages 1, 2, 6, and 9. In passage level 2 and 6, the lowest MDT value of 81 and 89 hours which were within mesogenic level were realized in immunosuppressed ducks (Table 4.5). No-immunosuppressed duck leucocytes passaged virus showed reduction in MDT to mesogenic value of 90 hours in passage level 6 only. The non-immunosuppressed chicken leukocytes also showed reduction in MDT in passages 4, 8, 9 and 10 where mesogenic values of 90 and 86 were reached in passages 9 and 10 respectively. In immunosuppressed chicken leukocyte culture viruses showed reduction in MDT of 88 and 89 hours to mesogenic level in passages 8 and 10 (Table 4.5).The differences in mean death times between the viruses passaged in immunosuppressed and non- immunosuppressed chicken leucocytes were not significantly different ($p>0.05$) and the same case applied for the duck leucocytes passaged viruses.

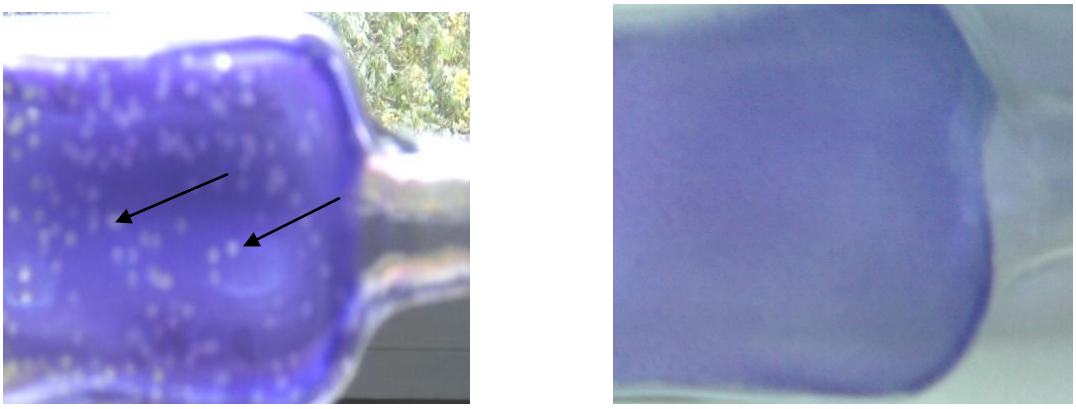
4.8 Intracerebral Pathogenicity index after NDV passages

Immunosuppressed chicken showed a sharp rise in icpi in passage 5, there was a drop in passages 6 and 7 and reached higher values of 0.61 in passage 9 as shown in Figure 4.a,b. The non-immunosuppressed duck leukocytes ICPI increased but with lower values, the highest being 0.51. There was then a sudden drop in ICPI in passage 10 in all the passage categories (Table 4.5) but the corresponding MDT for chicken decreased further to

mesogenic level for both immunosuppressed and non-immunosuppressed chicken at the same passage level. The percent increase in ICPI for viruses passaged in duck leucocytes was correspondingly progressive as the ICPI while in the chicken leucocytes it was eratic

4.9 Plaque assay after NDV passages

In all the ten passages no plaque formation was observed (Figure 3.6b) despite repeated infection of the chicken embryo fibroblasts cell cultures from respective virus passages but a control reference NDV (Figure 4.2a) showed clearly visible plaques.



(4.2a) Cell culture sheet showing NDV plaques (4.2b) Plain cell culture sheet

Figure 4.2: Positive control reference NDV in primary chicken embryo fibroblasts cell cultures (Figure 4.2a) showing plaque formation stained with methyl violet (arrows) while the Komarov NDV infected test also similarly stained (Figure 4.2b) does not show any plaques.

Table 4.5: Showing Mean death time, Intracerebral pathogenicity index and their % increase in normal and Immunosuppressed ducks and chicken leucocyte cultures

Passage level	DUCKS								CHICKENS							
	Immunosuppressed				Non-immunosuppressed				Immunosuppressed				Non-immunosuppressed			
	ICPI	%IN	MDT	%IN	ICPI	%IN	MDT	%IN	ICPI	%IN	MDT	%IN	ICPI	%IN	MDT	%IN
P1	0	0	130	16	0	0	118	23	0	0	105	32	0	0	106	48
P2	0	0	81	47	0	0	143	7	0	0	141	8	0	0	120	34
P3	0	0	108	29	0	0	105	32	0.15	21	120	22	0	0	120	34
P4	0.15	21	113	27	0	0	119	23	0.26	37	99	36	0	0	110	44
P5	0.25	36	116	25	0.15	21	90	42	0.48	69	104	32	0.33	47	122	32
P6	0.35	50	89	42	0.3	43	100	32	0.3	45	97	37	0.5	71	93	61
P7	0.46	66	124	19	0.2	28	124	19	0.3	43	109	29	0.3	43	109	45
P8	0.48	69	118	23	0.5	71	108	30	0.37	53	88	43	0.45	64	126	28
P9	0.55	79	94	39	0.51	73	108	30	0.61	87	112	27	0.53	76	90	64
P10	0.26	37	100	35	0.38	54	113	27	0.33	47	89	42	0.12	17	86	86

Key:IS = Immunosuppressed chicken or ducks; N-IS= Non–immunosuppressed chicken or ducks MDT= Mean death time; ICPI= Intracerebral pathogenicity index; IN = % increase in ICPI or MDT

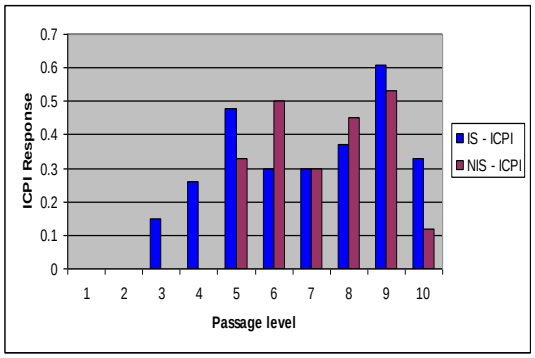


Figure 4.3a

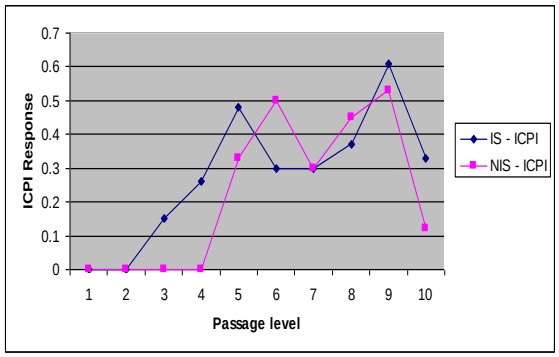


Figure 4.3b

Figure 4.3: Effect of passaging Newcastle disease virus in leucocytes from immunosuppressed chicken (blue bars) and that from non-immunosuppressed chicken (red bars). Note the early increase in ICPI from passage three to nine which after a drop in passages five and six rises to a near mesogenic value of 0.61. ICPI = Intracerebral pathogenicity index; IS=Immunosuppressed chicken ICPI; NIS= Non-immunosuppressed chicken ICPI).

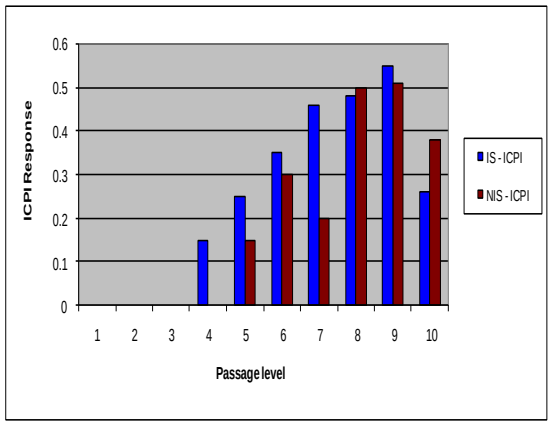


Figure 4.4a

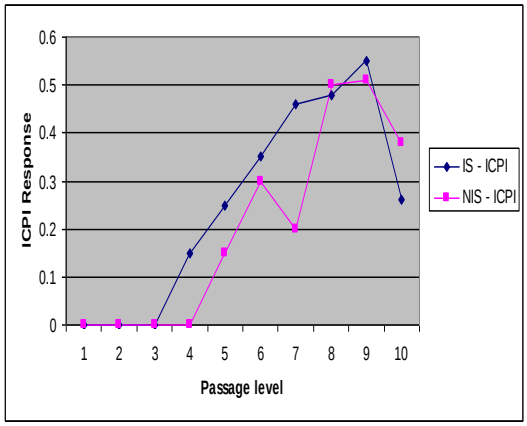


Figure 4.4b

Figure 4.4: Effect of passaging Newcastle disease virus in leucocytes from immunosuppressed ducks (blue bars) and non-immunosuppressed ducks (red bars).Note the progressive rising pattern of the ICPI for the immunosuppressed ducks up to passage 9. **(**ICPI) =Intracerebral pathogenicity index; IS=Immunosuppressed ducks ICPI; NIS= Non-immunosuppressed duck ICPI).

4.10 Komarov NDV haemagglutination profile with different avian and mammalian 1% RBCs

A total of eleven avian and mammalian species red blood cells were tested for haemagglutination activity as previously described, with original Komarov and also all the ten virus passages. The RBC were from chicken, turkey, duck, camel, dog, guinea pig, rabbit, horse, mouse and rat. The original Komarov NDV agglutinated all the RBC except the cat and rabbit RBC. Komarov viruses passaged in both chicken and duck at passage level one agglutinated all types of RBC except those from the cat and rabbit.

The rabbit RBCs were never agglutinated by the NDV throughout the passages. The RBCs from duck, chicken, rat and mouse were agglutinated by the NDV in all passages. Some RBCs like those from dog, horse, turkey, camel and guinea pig showed erratic pattern of agglutination at some passage levels and failing in others.

The RBCs from cat were only agglutinated at passage levels1 and 6 for both chicken and ducks. In passages 7 and 8 these RBCs were only agglutinated by viruses passaged in normal bird leucocytes either chicken or ducks (Table 4.6).

Table 4.6: Komarov haemagglutination profile with various mammalian and avian RBCs

Passage level	Bird species	Treatment	Chicken	Duck	Turkey	Guinea pig	Cat	Camel	Dog	Horse	Rat	Mouse	Rabbit
0	Original Komarov	NIL	512	512	512	512	-ve	256	128	256	256	128	-ve
1	Chicken	I-S	128	512	2	512	16	1024	2048	128	512	256	-ve
		N-IS	64	128	128	256	16	64	128	32	256	512	-ve
	Ducks	I-S	256	512	1024	128	16	1024	4096	64	512	1024	-ve
		N-IS	512	128	256	1024	4	64	1024	128	64	512	-ve
2	Chicken	I-S	512	128	512	256	-ve	512	1024	64	256	512	-ve
		N-IS	512	128	512	128	-ve	256	1024	-ve	512	128	-ve
	Ducks	I-S	128	64	64	32	-ve	64	256	-ve	16	64	-ve
		N-IS	256	128	128	-ve	-ve	64	512	-ve	128	256	-ve
3	Chicken	I-S	128	256	256	-ve	-ve	64	1024	16	1024	256	-ve
		N-IS	128	64	128	8	-ve	64	256	32	1024	128	-ve
	Ducks	I-S	512	512	512	4	-ve	128	2048	-ve	1024	512	-ve
		N-IS	256	128	256	-ve	-ve	64	2048	-ve	1024	16	-ve
4	Chicken	I-S	256	128	256	-ve	-ve	256	512	-ve	2048	32	-ve
		N-IS	256	256	-ve	-ve	-ve	128	128	1024	16	512	-ve
	Ducks	I-S	64	128	64	256	-ve	64	1024	-ve	128	1024	-ve
		N-IS	64	256	128	64	-ve	256	256	256	1024	1024	-ve
5	Chicken	I-S	1024	512	1024	128	-ve	1024	-ve	256	1024	2048	-ve
		N-IS	256	512	128	256	-ve	-ve	-ve	-ve	1024	256	-ve
	Ducks	I-S	512	64	256	64	-ve	256	-ve	128	1024	128	-ve
		N-IS	512	128	256	512	-ve	256	-ve	256	2048	512	-ve

Table 4.6: continued

Passage level	species	Treatment	Chicken	Duck	Turkey	Guinea pig	Cat	Camel	Dog	Horse	Rat	Mouse	Rabbit
6	Chicken	I-S	256	256	512	128	32	64	1024	128	512	256	-ve
		N-IS	2048	1024	1024	512	64	512	-ve	16	512	64	-ve
	Ducks	I-S	1024	512	512	1024	128	512	1024	32	1024	256	-ve
		N-IS	2048	512	1024	512	-ve	1024	2048	-ve	1024	512	-ve
7	Chicken	I-S	32	4	8	16	-ve	-ve	-ve	-ve	32	512	-ve
		N-IS	64	16	16	64	-ve	32	256	-ve	128	256	-ve
	Ducks	I-S	64	32	32	32	-ve	64	-ve	-ve	1024	256	-ve
		N-IS	16	16	-ve	-ve	32	-ve	-ve	-ve	16	16	-ve
8	Chicken	I-S	1024	512	1024	512	-ve	2048	1024	64	1024	128	-ve
		N-IS	2048	1024	1024	1024	32	2048	2048	1024	1024	1024	-ve
	DUCKS	I-S	64	128	16	128	-ve	64	512	256	128	512	-ve
		N-IS	512	512	1024	64	32	2048	2048	64	1024	128	-ve
9	Chicken	I-S	64	32	32	32	-ve	16	256	-ve	64	256	-ve
		N-S	512	256	256	128	-ve	1024	2048	-ve	512	512	-ve
	Ducks	I-S	2048	1024	2048	256	-ve	2048	1024	16	1024	1024	-ve
		N-IS	128	128	256	256	-ve	512	-ve	-ve	128	128	-ve
10	Chicken	I-S	256	64	256	512	-ve	256	-ve	16	64	256	-ve
		N-IS	128	32	64	64	-ve	256	512	512	32	512	-ve
	Ducks	I-S	1024	512	512	128	-ve	4096	-ve	-ve	512	128	-ve
		N-IS	512	128	128	64	-ve	2048	-ve	128	64	1024	-ve

Key: IS =immunosuppressed chicken or ducks; N-IS=non-immunosuppressed chicken or ducks

After the final passage was completed all the Komarov passages were subjected to heat inactivation to determine whether heat inactivation as a marker would indicate change of virulence since virulent NDV are heat resistant unlike the heat labile avirulent strains, (Hanson, 1955). The original Komarov as well as viruses in passage levels 1, 2,4,5,6,7,8,9 were inactivated within two minutes except viruses passaged in immunosuppressed chicken in passage levels 1 and 2 which were inactivated in three minutes.

Viruses passaged in non-immunosuppressed chicken leucocytes in passage levels 3 and level 10 took the longest time of 4 minutes to be inactivated. Both viruses passaged in immunosuppressed and non-imnmunosuppressed ducks in passage level 3 and that passaged in non-immunosuppressed chicken in passage level 10 were inactivated within 4 minutes as shown in Table 4.7.

Table 4.7: Heat inactivation profile for Komarov passages at 56^{o}C

Passage level	Bird Species	Treatment	Time (in minutes) post inactivation					
			0	1	2	3	4	5
0	Original Komarov	None	1024	512	256	-ve		
1	Chicken	I-S	2048	1024	64	-ve		
		N-IS	256	128	64	-ve		
	Ducks	I-S	4096	2048	256	-ve		
		N-IS	512	128	32	-ve		
2	Chicken	I-S	2048	1024	512	-ve		
		N-IS	1024	128	128	-ve		
	Ducks	I-S	256	128	64	-ve		
		N-IS	256	256	256	-ve		
3	Chicken	I-S	256	256	256	64	-ve	
		N-IS	512	128	128	8	4	-ve
	Ducks	I-S	64	64	64	4	-ve	
		N-IS	128	128	128	64	-ve	
4	Chicken	I-S	64	64	64	-ve		
		N-IS	256	128	128	-ve		
	Ducks	IS	128	128	64	-ve		
		N-IS	512	256	256	-ve		
5	Chicken	I-S	256	128	128	-ve		
		N-IS	64	64	4	-ve		
	Ducks	I-S	128	128	128	-ve		
		N-IS	512	128	128	-ve		
6	Chicken	I-S	512	512	128	-ve		
		N-IS	2048	512	256	-ve		
	Ducks	I-S	2048	128	128	-ve		
		N-IS	2048	256	256	-ve		
7	Chicken	I-S	2048	256	256	-ve		
		N-IS	1024	256	256	-ve		
	Ducks	I-S	1024	512	64	-ve		
		N-IS	1024	256	256	-ve		
8	Chicken	I-S	2048	512	32	-ve		
		N-IS	4096	256	256	-ve		
	Ducks	I-S	2048	512	256	-ve		
		N-IS	2048	64	64	-ve		
9	Chicken	I-S	1024	128	128	-ve		
		N-IS	2048	128	128	-ve		
	Ducks	I-S	2048	1024	1024	-ve		
		N-IS	256	128	128	-ve		
10	Chicken	I-S	1024	256	256	16	-ve	
		N-IS	1024	256	256	16	4	-ve
	Ducks	IS	2048	128	128	-ve		
		N-IS	256	128	64	-ve		

Key: IS =immunosuppressed chicken or ducks; N-IS=non-immunosuppressed chicken or ducks

CHAPTER 5

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Newcastle disease virus strains are classified into three pathotypes, namely velogenic, mesogenic and lentogenic strains. This classification is based on determination of mean death time (M DT) in chicken embryonated eggs and intracerebral pathogenicity index (ICPI) in day old chickens (Islam *et al.,* 1994; Seal and King., 2001, de Leeuw *et al*., 2005). Thus the pathogenicity of any newly isolated strain of NDV can be classified by determining the intracerebral pathogenicity index and the mean death time (Yu *et al*., 2001). In this study, the objective was to determine whether immunosuppression would influence development of a lentogenic strain passaged in avian splenic leucocyte cultures.

The trend observed in this study of the Komarov NDV was reduction in M DT in chicken embryonated eggs in both immunosuppressed and non-immunosuppressed ducks and chicken. Reduction in M DT in chicken embryonated eggs is associated with increased virulence of NDV (Slosaris *et al.,* 1989, Kommers *et al*., 2001). A similar trend was observed by other researchers working with Newcastle disease virus (Islam *et al*., 1994, King and Seal 1997; Peeters *et al.,* 1999). Slosaris *et al.,* (1989) passaged two strains of lentogenic Newcastle disease virus in Baby hamster kidney cell line and Mardin Darby Bovine kidney (MDBK) cell line and demonstrated changes in virulence by means of decrease in M DT. Dexacoryl (immunosuppressant) was used in the present study, in

chicken and ducks, to simulate immunosuppression. In the field, it is taken that stress would lead to immunosuppression in both chicken and ducks. Immunosuppressed ducks showed higher decrease in M DT (from 154 to 81) hours than the non immunosuppressed birds where at least in two instances the virus mean death time values indicated the virus had reverted to mesogenic level of pathogenicity at 81and 88 values. Both immunosuppressed and non-immunosuppressed chicken leucocyte virus passages showed mesogenic values of MDT in two passages each. According to the MDT obtained, viruses passaged in immunosuppressed ducks leucocytes reverted to mesogenic earlier in passages 2 and 6 and also in passages 5 in non-immunosuppressed duck leucocytes. In passage 8, immunosuppressed chicken showed decreased MDT values first followed by passage nine where non-immunosuppressed chicken leucocytes showed decreased MDT values. Reversion towards virulence via MDT and close to virulence values for intracerebral pathogenicity index were seen at the same passage level.

The ratio of the immunosuppressed duck leucocyte passaged virus in passage level 9 MDT to that of the original virus was 1:1.6 while that of the non–immunosuppressed duck leucocyte was 1:4. The reverse was observed in chicken leucocytes where the MDT ratios were 1:3 and 1:7 for immunosuppressed chicken and non-immunosuppressed chicken leucocytes respectively. The NDVs passaged in both immunosuppressed and non-immunosuppressed chicken leucocytes in passage 10 showed values that had reverted to mesogenic level of pathogenicity.

Another pathogenicity test, intracerebral pathogenicity index test (ICPI) was also employed to test the pathogenicity status of the Komarov NDV passages. The trend observed was an increase in ICPI in one day old chicks from value of zero towards virulence at 0.61. Increase in ICPI of NDV strains signifies increased virulence (Kommers *et al*., 2001, de Leeuw *et al.*, 2003). The results showed that the highest ICPI value of 0.61 reached by the NDV passaged in immunosuppressed chicken leukocytes at passage 9 was only 0.9 ICPI units away from reaching mesogenic level of 0.7 (OIE, 1996). In immunosuppressed duck leucocytes, the ICPI value was only 0.15 units away from reaching the mesogenic level of pathogenicity but MDT for the isolate had not reached mesogenic state. Although the difference between the ICPI of viruses passaged in immunosuppressed and non- immunosuppressed chicken leucocytes was not significant ($p>0.05$) and similarly for ducks, the increase of ICPI from 0.0 to 0.61 through passages seems worthy giving some attention.

In addition, the other pathogenicity indicator, MDT, showed that the virus had already become mesogenic with MDT of 90 hours and below. This clearly indicates that in poultry flocks passaging of a vaccine lentogenic NDV strain over time in chickens could generate a mesogenic level virus which could become a velogenic virus with further passage as has been the case for field strains in Australia (Gould *et al*, 2001). It was also noted that the increase in ICPI in immunosuppressed duck leucocytes passaged virus was steady from passage four to nine and that the highest value obtained was more than for the non –immunosuppressed. The increase in ICPI in passage nine towards mesogenic

level of pathogenicity seemed to coincide with decreasing MDT values also towards near mesogenic level of pathogenicity. However; it was not clear why the ICPI suddenly dropped in passage ten in all categories of virus passages while MDT levels were still low. The 47% decrease in MDT in passage 2 from the original level of 154 hours mesogenic level MDT in passage 6 to as low as 81 hours and 42% in passage 6 is worthy to note.

According to the Office International Epizooties standards OIE, ND is a notifiable disease and belongs to list A disease in International Animal Health Code (E-mail: http//www.oie int) and any strain which has ICPI value of 0.7 or greater is reportable as virulent and this may lead to imposition of trade embargo or restriction of trade (OIE manual, 1996, Kommers *et al*,. 2002). Mohammed *et al*., (1994) noted that Japanese quails (*cortonix cortonix japonica*) are rather resistant to NDV; however, they become infected under stress condition. This might mean that stress plays a role in enabling the virus to thrive in the quails and therefore stress perhaps plays a role a role in enhancing NDV pathogenicity in chicken (Gross and Colmano, 1970). A similar situation may be taking place in the field in immunosuppressed birds any where wherever poultry flocks are kept especially in the rural Africa family poultry flocks.

Plaque assay is also another test for pathogenicity determination which was used in this study. Lentogenic NDVs do not form plaques on chicken embryo fibroblasts (CEF) in cell cultures the absence of additives like trypsin, magnesium salts and DEAE. When the

Komarov NDV passages were infected into the CEF and overlaid with agar, no plaque formation was observed up to passage ten which is in agreement with lentogenic NDV behavior in these types of cell cultures (Alexander, 2003).

The NDV passages in passage 9 showed mesogenic level pathogenicity, in MDT and not in ICPI and may explain why there were no plaques. Perhaps if both indicators of ICPI and MDT had showed the strain as being mesogenic, there would have been plaques. It remains to be shown whether the passage 9 isolates has any genomic changes at cleavage site.

Studies by de Leeuw *et al*., (2003) with tagged LaSota Vaccine strain showed that a single intracerebral inoculation of lentogenic LaSota vaccine virus into day old chicks induced reversion to virulence and the cleavage motif was similarly changed (de Leeuw *et al.,* 2003) .The avian and mammalian RBC haemagglutination profile showed no pattern of agglutination that could be associated with virulence changes in the passaged viruses. However, it was observed that RBCs showed higher virus haemagglutination titers for viruses in low passages in non-immunosuppressed bird leucocytes, in both chicken and ducks. In passage nine, the situation was reversed where viruses passaged inimmunosuppressed chicken leucocytes had lower titers than the non-immunosuppressed ones but the pattern reversed for the ducks. In passage ten, the situation in chicken reversed and viruses passaged in immunosuppressed chicken leucocytes had higher titers than the non- immunosuppressed and similarly for the ducks.

According to the results of the observations of the heat inactivation profile it was clear that the NDV passages were all heat sensitive. The heat inactivation profile may not be sensitive enough to detect the magnitude of the changes reached detectable by the other two techniques. Nevertheless, it was noted that viruses passaged in non-immunosuppressed chicken leucocytes in passage three took the longest time to become inactivated and similarly for passage ten where HA titer level patterns had also reversed compared to passage 9.

The primary molecular determinant for NDV pathogenicity is the fusion protein cleavage site amino acid sequence (Seal *et al*., 2001; de Leeuw *et al*., 2003, 2005). However, it has more recently been demonstrated that haemagglutinin neuraminidase gene, also contributes to virulence of NDV (Gould *et al*., 2001; Tan *et al*., 2008). Comparative results signified that the differences in virulence among NDV strains mainly correlate with the number and arrangement of the basic amino acid residues in the cleavage site. Direct evidence has been achieved by introducing three amino acids into the Fo cleavage site of LaSota/46 (Yu *et al.*, 2001). When the cleavage site ^{112}GRGGR/L^{117} was changed to ^{112}RRQRR/F^{117}, the ICPI of the modified LaSota rose from 0.0 to 1.28 (Yu *et al*., 2001). The cleavage site motifs for the passaged virus Komarov NDV strain in our study are yet to be determined. Studies by different researchers seem to agree that the reversion phenomena revolves around the fusion protein cleavage site amino acid sequence and the HN protein genes (Seal *et al*., 2000; Gould *et al*., 2001 Mcginnes *et al*., 2002 ; Tan *et al*., 2008).

However, the selective pressure or cause of the reversion of NDV to virulence is still unknown. After the investigation of the 1998-2000 of NDV outbreaks in Australia and in Ireland in 1990 failed to give evidence of exotic relationship of the epidemic NDVs, the similarity of outbreak strains to the endemic indigenous lentogenic strain was proven. It was suggested that the outbreak was as a result of a mutation of local lentogenic strains that had been circulating in chicken and water fowls for a long time (Gould *et al.*, 2001). The risk factors and the selective pressures leading to these changes are yet to be understood.

In China where NDV had been controlled by a strict vaccination programme with a lentogenic LaSota strain for two decades, occurrence of ND outbreaks changed from pandemic to sporadic nature in vaccinated flocks. It was thought that increasing poultry production and more immune pressure from the host could have enhanced the evolutionary process of NDV leading to emergence of virulent strains (Tan *et al*., 2008).

Virus reversion to virulence is not unique to NDV. Other viruses have been known to revert to virulence after passage while others become attenuated. For instance a vaccine strain of infectious larygotracheitis virus of chicken, a herpes virus, increased its virulence after serial passage in chicken (Guy *et al*., 1991). A dog kidney cell culture attenuated canine distemper virus, (Rock born strain), was shown to revert to virulence after serial passages in dogs (Apel, 1978). After only four serial passages of human

immunodeficiency virus (HIV) Type-2 in baboons (*papio cynocephalus*), the virus showed increased kinetics of viral replication and cytopathogenicity in both the peripheral blood mononuclear cells (PBMC) and the baboons (Locher, 2003). The Komarov NDV strain used in the present study was originally a virulent chicken strain and was attenuated in ducklings by serial intracerebral passages (Komarov and Goldsmit, 1946). This then means that passaging can reduce virulence but can also increase it depending upon the virulence and unknown risk factors. It is interesting to note that in our study in ducks, although ICPI values increased from zero to 0.51, the increase was steady and consistent in immunosuppressed ducks while it was sporadic in non-immunosuppressed. This may indicate that immunosuppresion plays a role in reversing the role in ducks where attenuation was expected and instead virulence was enhanced.

It has been stated earlier that the 1998-2000 Australian ND outbreaks were thought to have been due to a local lentogenic wild strain of NDV that reverted to virulence after it had been circulating in poultry for a long time as was the case when a virulent virus was passaged intracerebrally as originally done (Komarov and Goldsmit,1946). Phylogenetically the virus responsible for the outbreak was closely related to the local endemic strains (Gould *et al.,* 2000; Spradbrow, 2001). It therefore would seem that this process could be slow in nature and is only greatly accelerated under unknown conditions. In the case where a tagged LaSota, a lentogenic NDV vaccine strain reverted to virulence after only one intracerebral passage in chicken brain the mechanism is still unknown (Collins *et al*., 2003).

The recommended method of controlling ND is by vaccination, employing live naturally occurring lentogenic, live attenuated lentogenic or mesogenic NDV strains. These live vaccine strains are off-loaded into many poultry – rearing environments throughout the world including our Country, Kenya. The viruses then keep on circulating amongst poultry and possibly among wild birds (Kasiiti, 2000). By naturally passaging themselves in this manner the Newcastle disease viruses could be covertly reverting back to virulence and causing NDV outbreaks under field conditions under yet unknown circumstances. This might be the origin of the many foci of NDV outbreaks in Kenya and many other areas throughout the world (Gould *et al*., 2001; Yu *et al*., 2001; Tan *et al*., 2008).

5.2 Conclusions

1. In the present study MDT decreased with increasing passages in the two species of birds, i.e. chicken and ducks in both immunosuppressed and non-immunosuppressed conditions. However, in the immunosuppressed chicken and ducks, the decrease was greater and occurred earlier than in the non -immunosuppresed birds.

2. There were two instances for the immunosuppressed chicken and ducks where the MDT values reached mesogenic levels. A decrease in MDT of NDV to mesogenic level signifies increased potential for virulence. This might mean that the virus had the potential of reverting to overt virulence especially if further passages were realized.

3. The ICPI increased with passaging in both chicken and duck leucocytes with increasing passages. The increase in ICPI was more progressive and more steady for immunosuppressed ducks than in immunosuppressed chicken. Higher ICPI values were realized from immunosuppressed chicken than in non–immunosuppressed chicken, immunosuppressed ducks or non-immunosuppressed ducks. The highest level of ICPI reached was in immunosuppressed chicken and was only 0.09 units away from reaching the OIE minimum reportable mesogenic level of virulence of 0.7 while in ducks it was only 0.15 units away. The MDT at the same passage showed the virus had reached mesogenic level. Since increase in ICPI of NDV indicates increase in virulence, passaging and immunosuppression seem to play a role in influencing both decrease of MDT and increase in ICPI.
4. No plaque formation was observed throughout the passages and this was thought to be in line with the characteristic of lentogenic NDV which do not form plaques in chicken embryo fibroblast cell cultures without added trypsin and Mg^{2+} ions or DEAE.

5.3 Recommendations

5.3.1 Recommendations made from the study:

1. That inactivated NDV or recombinant vaccines may be used to control NDV and eventually reduce endemicity of NDV towards its eradication in Kenya and elsewhere.

2. That factors which may induce stress in chicken and ducks be reduced to prevent generation and promotion of virulent NDV strains from lentogenic and mesogenic strains.
3. Poor feed, intercurrent diseases be reduced to prevent development of virulent NDV strains from lentogenic and mesogenic strains.

5.3.2 Recommendations for future work:

1. Passage the virus to higher passage levels in vitro to find out whether a fully virulent virus would be achieved.
2. Passage virus in immunosuppressed birds to determine if virulence may emerge faster.
3. Determine other factors that might be exerting pressure in selection for virulence.
4. Determine if there are changes in the F- protein at molecular level of the passaged virus isolates.
5. Carry out a pilot vaccination programme with inactivated vaccine and asses if NDV endemicity will be reduced in a specific region of Kenya.

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APPENDIX

Solutions and Reagents

A . Phosphate buffered saline

Sodium chloride (Nacl) AR…….…….....8.00g

Potassium chloride (Kcl) AR…… 0.20g

Disodium hydrogen phosphate (AR) Na_2HPO4…….....1.15g

Potassium dihydrogen phosphate (AR) KH2PO4…….....0.20g

Disolve in de-ionised water. Make up to 1000 ml distilled water and autoclave at 15 lb for 15 minute and store at $+4^oC$.

B. Alsevers solution

1. Solution I

Sodium chloride (Nacl) …….4.2g

Citric acid …….. …….0.55g

Sodium citrate…….8.0g

Disolve in 900 ml distilled water and autoclave at 121 lbs for 15 minutes.

Solution II

Dextrose…… ….0.5g

Distilled water……..….100.0ml

Autoclave at 121 lbs for 5 minutes.

1. Working solution

Solution I…….9 parts

Solution II…….1 part

C. Hanks balanced salt solution

1. Solution A stock

Sodium chloride……40.0g

Potassium chloride…….....2.0g

Magnesium sulphate $MgSO_4.7H_2O$……..... 0.5g

Magnesium chloride……..0.5g

Dissolve in 200 ml distilled water. Dissolve 0.7g $Cacl_2$ in 30ml distilled water. Mix and make up to 250 ml with distilled water. Add 0.5ml chloroform. Store at +4^oC. The solution is stable for at least 1 year.

2. Solution B stock

Disodium hydrogen phosphate NaH2PO4.12H2O……...0.76g

Potassium dihydrogen phosphate ……..0.3g

Dextrose……..5.6g

Dissolve in 200ml distilled water. Make up to 250ml with distilled water.

Add 0.5ml of chloroform and store at +4^oC. The solution is stable for at least one year.

3. Working solution

Solution A……..…50.0 ml

Solution B…….…..…….……..…50.0 ml

Distilled water……..……..…….….....870.0 ml

0.4%phenolred…….....2.0 ml

Mix solution A and B 870mlof distilled water. Distribute into 500ml volumes and store at +4^{o}C and autoclave at 10 lbs for 15minutes.Before use adjust PH as Desired with 7.5% sodium bicarbonate.

D. Buffered neutral formalin (PH 7.0, 10%)

40% formalin…….....…….....90.0ml

Sodium dihydrogen phosphate NaH2PO4.2H2O…….....3.6 g

Disodium hydrogen phosphate Na2HPO4…….....5.75 g

Distilled water……..……810.0ml.

E. Trypsin 0.25%

Trypsin1:250 Difco ……2.5g

Calcium and Magnesium free phosphate buffered saline……...1000.0ml

Stir in conical flask with a magnetic stirrer for 2 hours, and then add 2.0 ml of phenol red. Filter through 0.2µm Millipore membranes into sterile screw capped bottles and at +4^{o}C.

F. Physiological saline (0.85% Nacl)

Sodium chloride AR…….....8.5g

Distilled water…….……..…….…….……1000ml

Autoclave at 121 lb for 15 minutes and store at +4^{o}C.