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Challenge studies in chickens to evaluate the efficacy of commercial Newcastle disease vaccines against the strains of Newcastle disease virus prevalent in South Africa since 2002

by

Dauda Garba BWALA

Submitted in partial fulfilment of the requirements for the degree
***Magister Scientiae* (Master of Science)**

Department of Production Animal Studies
Faculty of Veterinary Science
University of Pretoria

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SUMMARY

Challenge studies in chickens to evaluate the efficacy of commercial Newcastle disease vaccines against the strains of Newcastle disease virus prevalent in South Africa since 2002.

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Supervisor: Dr. Shahn P.R. Bisschop

Co-supervisor: Prof. Neil M. Duncan

Since 2002, the South African poultry industry has experienced outbreaks of Newcastle disease (ND) caused by a newly introduced virus (NDV) strain belonging to lineage 5d/VIId ("goose paramyxovirus" - GPMV). Control of the disease has proved difficult with commercially available vaccines appearing ineffective. In the first of two studies, broilers chicks were vaccinated with VG-GA vaccine (lineage II), then challenged with both GPMV and a "classic" challenge virus (RCV) of lineage 3d/VIII to compare the efficacy of the vaccine against both strains. In the second study, commercial and SPF hens in lay were vaccinated with La Sota vaccine and challenged with GPMV isolate, and

immunohistochemistry staining used to determine the distribution pattern of viral antigen in the oviduct of the hens. The second study also compared the efficacy of cloacal and ocular routes of vaccination.

The first study did not detect any statistically significant difference in protection offered by the vaccine against the GPMV strain in comparison to the RCV strain. The protection offered by the vaccine against challenge with both viruses was found to be dose-dependant with $10^{6.0}$ EID_{50} producing a 100% protection and 94.44% and 13.89% for $10^{4.5}$ EID_{50} and $10^{3.0}$ EID_{50} vaccination doses respectively. Protected birds did not manifest clinical signs, but still had macropathological lesions in some organs at necropsy. The computed protective doses (PD_{50} and PD_{90}) for the VG-GA vaccine were $10^{3.51}$ and $10^{4.38}$ for GPMV and $10^{3.79}$ and $10^{4.43}$ for RCV.

Results from the second study showed no clear difference in the protection of the oviduct from challenge with GPMV by either the cloacal and ocular routes of vaccination. Vaccinated birds were fully protected (100%) against challenge by La Sota vaccine, but not against infection and replication of the virus, as birds showed varying degrees of macropathology with numerous stained viral antigens in the oviducts demonstrated by immunohistochemistry. The susceptibility and colonisation of the oviduct of laying hens by both the lentogenic La Sota and the virulent NDV isolates was confirmed, with the uterus being more susceptible than magnum and isthmus. Necrosis and apoptosis of cells of the oviduct were not detected but cellular infiltration, gland dilatation and interstitial oedema were observed.

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Above all, I thank God Almighty for granting me the opportunity to do this work and for sustaining me both physically and spiritually.

To God, be the glory,

> *I will praise you, O Lord, among the nations;*
> *I will sing of you among the people.*
>
> *For great is your love, higher than the heavens;*
> *Your faithfulness reaches to the skies.*

The Lord God is a sun and shield; He bestows favour and honour;
no good thing does He withhold from those whose walk is blameless.

Psalm 108:3-4; 84:11

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABC	:	avidin biotin complex
APAAP	:	alkaline phosphatise-anti-alkaline phosphatise
APHIS	:	Animal and Plant Health Inspection Services
APMV	:	avian paramyxovirus serotype
APR	:	Acute Phase Reaction
AUCC	:	Animal Use and Care Committee
BSL 2+	:	biosafety laboratory class 2
BSA	:	bovine serum albumin
Ca^{2+}	:	Calcium ions
CAM	:	Chorioallantoic membrane
CIDRAP	:	Centre for Infectious Disease Research & Policy
CPE	:	cytopathic effect or cytopathogenic effect
CX	:	control
CV	:	cloacal vaccination
E_2	:	oestrogen
EDS	:	Egg Drop Syndrome
EEC	:	European Economic Community
EID_{50}/ml	:	embryo infective dose (50%)
ELISA	:	enzyme-linked immunosorbent assay
END	:	exotic Newcastle disease
ER	:	endoplasmic reticulum
EV	:	eyedrop vaccination
F	:	fusion protein
F_0	:	fusion protein precursor
FAO	:	Food and Agricultural Organization
FSH	:	Follicular stimulating hormone
GAIN	:	Global Agriculture Information Network
GFP	:	green fluorescence protein marker
GnRH	:	Gonadotropin releasing hormone
GPMV	:	goose paramyxovirus
HA	:	haemagglutination
H&E	:	haematoxylin and Eosin
HI	:	haemagglutinin-inhibition test
HIER	:	heat-induced epitope retrieval
HN	:	haemagglutinin protein
HN_{0616}	:	haemagglutinin protein precursor
HPAI	:	highly pathogenic avian influenza
HRP	:	horseradish peroxidase
ICPI	:	intracerebral pathogenic index
ICTVdB	:	Int. Comm. on Taxonomy of viruses, Database
Ig	:	immunoglobulin
IgA	:	immunoglobulin A
IgG	:	immunoglobulin G

IgM	:	immunoglobulin M
IHC	:	immunohistochemistry
IHS	:	immunohistochemical staining
ILT	:	infectious laryngotracheitis
ISH	:	*in situ* hybridization
IVPI	:	intravenous pathogenicity index
kDa	:	kilodaltons
KZN	:	KwaZulu Natal
L	:	large protein
LH	:	luteinizing hormone
M	:	matrix protein
mAbs	:	monoclonal antibodies
MDGs	:	Millennium Development Goals
MDT	:	mean death time
MIS	:	Müllerian-inhibiting hormone
mRNA	:	messenger RNA
n	:	number
NA	:	neuraminidase protein
NABC	:	National Agricultural Biosecurity Center
ND	:	Newcastle disease
NDV	:	Newcastle disease virus
nm	:	nanometer
NP	:	nucleoprotein
nt	:	nucleotides
OIE	:	Office International des Epizooties
OOCs	:	Oviduct organ cultures
ORF	:	open reading frame
P	:	phosphoprotein
P_4	:	progesterone
pAbs	:	polyclonal antibodies
PAP	:	peroxidase-anti-peroxidase
PBS	:	phosphate-buffered saline
PC	:	post-challenge
PCR	:	polymerase chain reaction
PD_{50}	:	protective dose (50%)
PD_{90}	:	protective dose (90%)
PRC	:	Poultry Reference Centre
PV	:	post-vaccination
RBC	:	red blood cell
RCV	:	Rainbow challenge virus
RdRd	:	RNA-dependant-RNA-polymerase
rER	:	rough endoplasmic reticulum
RNA	:	ribonucleic acids
RNP	:	ribonucleoprotein
RT-PCR	:	reverse-transcriptase polymerase chain reaction
SAPA	:	South African Poultry Association

SOP	:	Standard operating procedure
SPF	:	specific pathogen-free
UK	:	United Kingdom
µm	:	micrometer
US/USA	:	United State of America
USDA	:	United State Department of Agriculture
UTR	:	un-translated region
VG-GA	:	Villegas-Glisson/University of Georgia
vND	:	virulent Newcastle disease
WBC	:	White blood cells
WHO	:	World Health Organization

LIST OF FIGURES

LIST OF TABLES

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Newcastle Disease

Synonyms: Avian pneumoencephalitis disease; Ranikhet disease
Viscerotropic velogenic Newcastle disease (VVND)
Neurotropic velogenic Newcastle disease (NVND)
Fowl pest

Sources: Poultry diseases and meat hygiene; virology (directory & dictionary)

CHAPTER ONE – INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Newcastle disease (ND) is an infectious, highly contagious and pathogenic avian viral disease caused by a paramyxovirus. Defined as a List A disease by the Office International des Epizooties (OIE), it causes severe economic losses in domestic poultry. Newcastle disease is endemic in many parts of both the developing and the developed world (reviewed in Permin & Madsen, 2002; Alexander, 2001; Alders & Spradbrow, 2001a). Since its first official report in poultry in Java, Indonesia (Kranevald, 1926) and Newcastle-upon-Tyne (from where the disease and the virus got its name) (Doyle, 1927), ND has continued to re-emerge in both epidemic and endemic form throughout the world (Brown *et al*., 1999). The highly pathogenic form of Newcastle disease virus (NDV) is known to cause a devastating disease of poultry (Alexander, 1988c). The disease has greatly affected the poultry industry in one form or the other and represents a major limiting factor to the growth of the industry in many countries (Alexander, 2001). The greatest impact of ND is felt or seen in the village or backyard poultry production (Spradbrow, 1993). This could be attributed to the unavailability of vaccines for prevention as well as the difficulty associated with vaccinating chickens even where the vaccines are available due to the free-range and scavenging nature of the village poultry production system. This is however, not the case with commercial poultry where routine vaccination is practiced.

The Office International des Epizootics (OIE) in 1991, following the adoption of the definition put forward by the Member States of the European Economic Community (EEC) defined Newcastle disease as follows:

"Newcastle disease is an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a). the virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater,

b). multiple basic amino acid have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residue 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residue as described above would require characterization of the isolated virus by an ICPI test" (OIE, 2004).

According to the OIE definition "amino acid residue are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F_0 gene, 113-116 corresponds to residues -4 to -1 from the cleavage site" (OIE, 2004).

The highly pathogenic form of ND is and will continue to be a serious problem in Africa, Asia, Central America and parts of South America (Copland, 1987; Spradbrow, 1988; Rweyemamu *et al*., 1991; Alders & Spradbrow, 2001a). With this endemic nature of ND either in commercial or in village or backyard poultry and the little possibility of enforcing efficient biosecurity measures to prevent the spread from village to commercial poultry, vaccination remains the only alternative for controlling and minimizing the effects of the disease (Alexander, 2001). Despite the plethora of vaccines and the aggressive vaccination programme being practiced by the poultry industry all over the world and specifically in South Africa to control ND, ND has defied all logic and

continues to rear its ugly head in both epidemic and endemic forms causing monumental economic losses to the industry. Several reports on the use of various vaccines to control ND (Asplin, 1952; Utterback & Schwartz, 1973; Allan *et al*., 1978; Hamid *et al*., 1990; Parede & Young, 1990; Guittet *et al*., 1993; Alexander *et al*., 1999; Alexander, 2001; Senne *et al*., 2004; Kapczynski & King, 2005; Miller *et al*., 2007; Perozo *et al*., 2008) have shown that most of the commercially available ND vaccines do protect against the more serious clinical consequence of infection which includes clinical disease and mortalities. But there are also several reports that go to show that most of the commercially available ND vaccines are not performing optimally (Spalatin & Hanson, 1972; Utterback & Schwartz, 1973; Burridge *et al*., 1975; APHIS, 1978; Adu *et al*., 1990; Capua *et al*., 1993; Kapczynski & King, 2005; Miller *et al*., 2007) and there seems to be a lack of agreement on the level of protection offered by the commercially available vaccines against the newly emerging ND viruses (principally members of the genotype VII).

Following repeated outbreaks of ND in poultry in South Africa characterized by increased mortalities in broilers, drops in egg production and production of poor quality eggs even in vaccinated poultry, a pathogenicity study of goose paramyxovirus (GPMV) in vaccinated chickens and laying pullets was carried out. During the study, two separate experiments were carried out. In the first experiment, VG-GA (Avinew) ND vaccine was used against challenge with lineage 5d/VIId (GPMV) and “Rainbow” Newcastle disease virus (lineage 3d/VIII), while in the second experiment, La Sota ND vaccine was used against challenge with GPMV only, and intraocular and intracloacal routes of vaccination

were compared. The decision to compare intraocular and intracloacal routes of vaccination was based on reports emerging from the field which suggested that birds vaccinated intracloacally showed better protection than those vaccinated by spray. This assertion was arrived at following the result of several informal trials conducted by a large egg producer in South Africa (Dr Makwiti personal communication). The motivation for the use of this method of vaccination according to Dr Makwiti was the need to find a lasting solution to the consistent suboptimal protection offered by commercially available vaccines against Newcastle disease infection that was being experienced in the field. The mechanism and theory behind the intracloacal vaccination is yet to be elucidated, but the swabbing of the cloacae of 6 to 8-week old chickens with infective allantoic fluid has long been used in the United State of America to assess tropism and distinguish between viscerotropic velogenic NDV and other strains and their virulence (Hanson, 1980). Data collated from the first trial were statistically analysed while tissues of the oviduct from the second experiment were sampled for histopathological and immunohistochemical studies and the GPMV tropism/distribution in the tissues of the oviduct was assessed.

1.2.1 Poultry

Poultry are birds domesticated by man and include chickens, turkeys, ducks, geese, quails, ostriches, guinea fowl and certain other birds. They are kept by households as a source of protein (eggs and meat) and income to meet everyday family needs (Law & Payne, 1996). The economic significance of poultry varies significantly from country to country. However, in developing countries and sub-Saharan Africa, there is an increasing

demand for livestock product for the ever growing and more affluent population (Delgado, 2003; Gulati *et al*., 2005). Chicken is the livestock most commonly owned by women and families in the developing world, and increasing their productivity will contribute significantly toward increasing their food security and livelihood of the rural families (Alders & Spradbrow, 2001a).

In an address at the World Food Day Ceremony in Rome, 2007, the Director General, Jacques Diouf said "our planet produces enough food to feed its entire population. Yet, tonight, 854 million women, men and children will be going to sleep on an empty stomach". This statement of Jacques Diouf is true especially for sub-Saharan Africa, which has the highest prevalence of under nourishment, with one in three people deprived of access to sufficient food (FAO, 2006). Poultry production is generally acknowledged as the most efficient and cost-effective way of increasing the availability of high protein food (FAO, 1987) Eggs have long been presented as the standard reference food that is perfectly balanced, containing most essential amino acids, minerals and vitamins. Approximately 11.5% of daily protein requirement and 5% of daily energy requirement is provided by one egg (Branckaert *et al*., 2000). Poultry production as a socio-economic activity has been turned to a commercial entity from the mere subsistence form of agriculture (FAO, 2005). With the increasing population pressure challenging developing economies and the need to provide food security and meet the global Millennium Development Goals (MDGs), the need for every country to build up their poultry production capacity cannot be overemphasized. Unfortunately, the growth of the livestock industries or enterprises in developing countries has been severely constrained by animal diseases (Perry & Sones, 2007). Poultry production systems, especially in

developing countries, are faced with a myriad of challenges, key amongst which is disease. De Haan & Bekure (1991) reported an estimated annual loss of about US $2 billion in direct losses (mortality) and another US $2 billion in indirect losses (slow growth, lower productivity, increase morbidity and lower fertility, etc) in sub-Saharan Africa due to livestock diseases. The effect of these diseases is most severe in the developing economies where modern vaccines and medicaments, modern technologies for quick diagnosis and sound management practices are in short supply. Many of these developing economies are stacked with outdated service delivery systems that are incompatible with and do not meet the needs or requirement of their poor clients and are compounded by inadequate funding (Perry & Sones, 2007). In view of this, disease entities especially viral diseases have continued to pose a threat to the poultry industry despite efforts made at controlling them. Some of the viral diseases of poultry among others include Infectious Bursal disease (Gumboro), Egg Drop Syndrome (EDS), Highly Pathogenic Avian Influenza (HPAI), Infectious Laryngotracheitis (ILT), Mareks disease and Newcastle disease (ND).

1.2.2 South Africa and its poultry industry

The South African Republic population estimate in 2007 is around 49 660 502 people, distributed into nine provinces with a total land area of 1 219 090square kilometres (World Gazetteer, 2007). People living in the rural areas form 46% of the total population and five out of the nine provinces have a high rural to urban ratio of between 56-90% to 44-10% (Tsibane, 2001). According to Tsibane (2001), not much work has been done on

village or backyard poultry in South Africa for many reasons, among which was the country's apartheid laws. In view of this, there seems to be no accurate data on the actual population of village or backyard poultry and its contribution to the total poultry population of the country but it is estimated that village fowl make up more than 80% of the total domestic fowl population in Africa estimated at 1,068 million in 1995 (Guèye, 1998). Records showed South Africa to have a poultry population of 145 818 000 in 2004 (FAO, 2006). Out of this population, 32 million are laying chickens (FAO, 2006). The South African Poultry Association estimates broiler meat production in 2006 at 1 225 000 tonnes and 350 000 tonnes of eggs (SAPA, 2006). The South African poultry market has grown by 11% to R13.5 billion as at 31 March 2007, producing an average of 13.8 million broilers per week (GAIN Report, 2007). The Department of Agriculture however puts the poultry industry turnover at R15.73 billion, comprising R11.92 billion for broilers and R3.81 billion for eggs. At retail level, these turnovers as recorded by the Department of Agriculture increase to a total of R22.69 billion (reviewed in SAPA, 2006). During 2008, the breeder flock increased by 333 000 hens (5.68%) to an average of 6 199 000 hens, while 979 million broiler chicks placed in 2008 was 4.26% higher than the number placed in 2007 (SAPA stats, 2008). Approximate broiler production figures for the year 2008 was put at 936 million while that of egg production was put at 10.7 million which is higher than the 2007 production by 1.77% (SAPA stats, 2008). But generally there has been a negative growth in weekly egg production since August, 2008. Production in February, 2008 was 4.9% down compared to February, 2007 (SAPA stats, 2008). The industry alone is said to consume between 15 to 25% of the total maize crop production of South Africa and nearly 70% of the feed industry's production (GAIN

Report, 2006) and provides about 58% of all animal-product protein consumed in South Africa (SAPA, 2006)..

Despite this large quantity of broiler meat and eggs being produced by the poultry industry in South Africa, the meat requirement of the population is barely met and has to be supplemented by imports. In 2004 alone, a total of 181 997 tonnes was imported into South Africa, principally from Brazil and some other countries (GAIN Report, 2006). Imports in 2006 amounted to R1.2 billion or 293 000 tonnes, while the same report estimates 2007 import figures are to exceed 300 000 tonnes (GAIN Report, 2007). Import figures for eggs into South Africa in 2005 put the value at 722 tonnes (SAPA, 2006). About 72% of the meat import comes from Brazil and it represents more than 20% of South African total production. Part of the reason why the Brazilian import to South African is thriving apart from the demand side factors in South Africa, is because the production cost is so manageable and disease control is strictly adhered to, thus making the industry highly profitable and also making Brazil the world biggest exporter of broiler meat, with a revenue income of US $2.6 billion in 2004 (Sesti, 2005). What this means is that much more still need to done in South Africa especially as regards management and disease control with a view to reducing the overall contribution of these two items to the cost of production in the drive to achieving self sufficiency in poultry and poultry products. One of the diseases that needed to be tackled, especially in South Africa, is Newcastle disease whose sporadic occurrence has been associated with massive death, production losses and other costs associated with its control during outbreaks.

1.3.1 History of Newcastle disease

The first description of a disease outbreak in poultry that resembled and was termed Newcastle disease (ND) occurred in 1926 in Java, Indonesia (Kraneveld, 1926) and Newcastle-upon-Tyne, England (Doyle, 1927). However, there were reports of outbreaks of a similar disease in Central Europe that predates the Java and Newcastle reports (Macpherson, 1956; Halasz, 1912). Macpherson (1956) was of the view that the death of all chickens in the Western Isles of Scotland in 1896 was due to ND. Doyle (1935) named the disease "Newcastle disease" after the outbreak in Newcastle-upon-Tyne in Great Britain. This was to avoid giving a scientific name that may be confused with other disease entities. The name or nomenclature "avian paramyxovirus serotype-1" (APMV-1) as suggested by Tumova *et al* (1979a) is frequently used in place or together with the name ND in an effort to conform with the rules of naming individual isolates as recommended by the World Health Organisation (WHO, 1980). Citing Ochi and Hashimoto, Levine (1964) was of the view that ND might have been present in Korea as early as 1924. ND outbreaks also occurred in Ranikhet, India in 1927 (Edwards, 1928). In the United States, a disease of poultry characterized by mild respiratory and neurological signs was reported and termed *pneumoencephalitis* (Beach, 1942), but was later discovered to be ND. Since then numerous Newcastle disease viruses (NDV) that produce mild or no disease in chickens were isolated and reported around the world (Hitchner & Johnson, 1948; Asplin, 1952; Simmons, 1967; McFerran & Nelson, 1971). It is however possible that several outbreaks of ND may have occurred earlier and gone unrecognised in other places possibly due to the lack of expertise to recognise the new disease (Alexander, 1988b).

Alexander *et al* (2004), looking at the pattern of outbreaks all over the world, was of the suggestion that several panzootics of ND in poultry might have occurred since 1926. According to them, the first panzootic which started in the Far East spread very slowly across the globe and might have taken up to 20 years, but never reached poultry in the USA. The second panzootic which started at the end of 1960s was able to spread to all corners of the earth within the span of 4years (Hanson, 1972). The reason for the fast spread of the second panzootic was attributed to the development of the poultry industry, the commercialization of feed production and the enhanced trade in captive birds due to improved air transportation - all of which lead to greater contact between individual farms and birds of different regions of the world. Imported cage birds were known to be responsible for the introduction of panzootic virus into California poultry (Hanson, 1972; Francis, 1973). Outbreaks of ND in 1970 to 1972 in USA were linked to the importation of exotic birds (Walker *et al*., 1973). The third panzootic started in late 1970s going by antigenic and genetic evidence (Alexander *et al*., 1997; Lomniczi *et al*., 1998; Herczeg *et al*., 2001). Its spread was masked by the global use of vaccines since the mid-1970s. The fourth panzootic occurred in the 1980s in racing and show pigeons (*Columba livia*) and not in poultry, but the latter was said to be responsible for the spread of the virus into these categories of birds (Alexander, *et al*., 2004).

The first official diagnosis of Newcastle disease in South Africa was made in the UK (Weybridge) on a serum sample collected from poultry in Natal (KwaZulu Natal) province following high mortality in chickens after an outbreak of a disease characterised by severe respiratory, nervous and intestinal signs (Kaschula, *et al*., 1945). Kaschula

believed that ND might have been in Durban as early as 1944 as it is a port town and could have been the point of disease entry. This assumption of Kaschula may have some truth in it, because the signs and necropsy findings of the Natal outbreak closely resemble what was described by Hudson in Mombasa, Kenya in 1935. In fact, Kaschula *et al* (1945) made mention of a rumour suggesting that the entire African East Coast has been affected. The 1944/45 outbreak was however confined entirely to the sugar-cane belt of the Natal with the Indian free-ranging flocks suffering the heaviest as 100 000 fowls were lost before the disease was eventually stamped out. Abolnik (2007) corroborated Kaschula's suspicion that the ND might have been in South Africa before the first official report. In an 1892 South African agricultural journal authored by Greenlees (1892), she came across a letter in which a farmer complained of a fowl sickness that attacked his birds from time to time and defied all known treatments at that time and the signs described by the farmer are consistent with Newcastle disease. A letter by another farmer of Hamans Kraal (Hammanskraal) near Pretoria in 1903 reported the outbreak of a disease in a mixed breed, free-ranging fowl typical of ND (Rosenbloom, 1903).

Since then, ND outbreaks in poultry in South Africa have been on and off. Reports by Martinaglia (1926), De Kock (1954), Kluge (1964), Coetzee and Abrams (1965) confirm this assertion. The 1970 to 1972 outbreak was one of the most severe ND epidemics in South Africa as the growth in commercial poultry from 1960 had created a large concentration of susceptible poultry. Because of this and the rapid spread of the infection, the stamping out control measure used earlier became impractical (Coetzee, 1980). Since the outbreaks of 1970s, ND outbreaks have been sporadic and cyclical with each cycle

lasting for about 2-4 years. The 1993/1994 outbreak that caused the loss of millions of broilers in its peak (Coetzee, 1994) was followed by the 1998 to 2000 epidemic which killed about 469 056 poultry in only 25 outbreaks (Anonymous, 2005). Several other outbreaks of ND have been reported in South Africa (Verwoerd, 1995b; Abolnik, *et al*., 2004b). Around 1999 to 2000, a velogenic viscerotropic NDV strain termed as "goose paramyxovirus" (GPMV), of the lineage 5d was introduced into South Africa from the Middle and Far East and was responsible for the outbreak in KwaZulu/Natal (KZN) province (Abolnik, *et al*., 2004a) and most of the other subsequent ND outbreaks in South Africa (Abolnik, 2007).

1.4.1 Aetiology - The Newcastle disease virus (NDV)

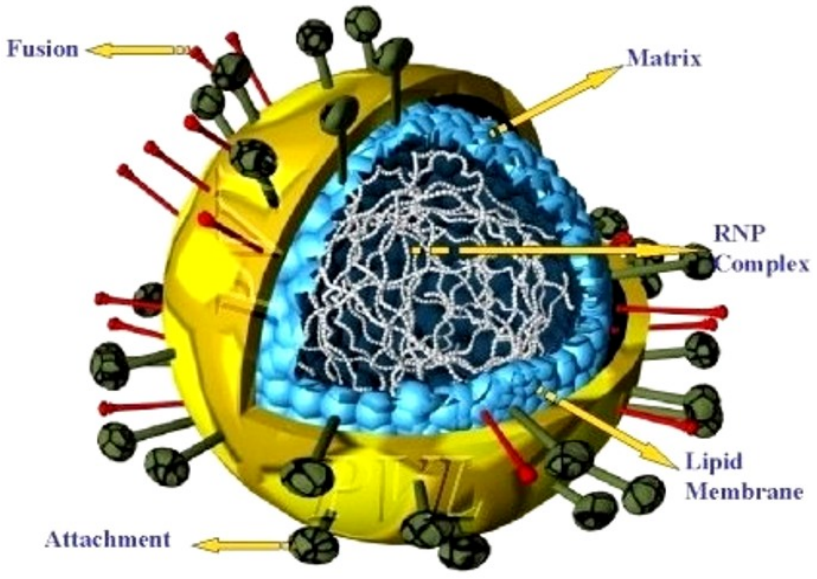


Fig 1.1. *The Newcastle Disease Virus* (**Source***: Poultry.com* & *www.isracast.com)*

The aetiological agent of Newcastle disease, Newcastle disease virus is of the order *Menonegavirales* of the family *Paramyxoviridae* and *Avulavirus* genus (Lamb *et al*., 2000; Mayo, 2002a; 2002b). Also referred to as avian paramyxovirus-1 (APMV-1), the current taxonomy has nine APMV serotypes (APMV-1 to APMV-9) as contained in Alexander (1988c), with APMV-1 known to infect numerous avian species (Lamb *et al*., 2000). Most of these serotypes appear to be present in natural reservoirs of specific feral avian species although other host species are also susceptible (reviewed in Lewis, 2005). APMV-2 and APMV-3 viruses are the only serotype apart from APMV-1 that have made significant disease and economic impact on poultry production (Alexander, 1993; reviewed in Lewis, 2005) although APMV-6 and APMV-7 are also known to cause disease in poultry (Alexander, 2003). APMV-1 cross-reacts with other serotypes especially APMV-3 (reviewed in Alexander, 1993). Because of the severity of the disease produced/induced even in a given host such as chickens by the different isolates and strains of NDV, Beard & Hanson (1984) summarized and divided NDV into forms or pathotypes based on the clinical signs of the disease produced in infected chickens.

Viscerotropic Velogenic virus	Acute lethal infection characterized by high mortality, usually with haemorrhagic lesions in the intestines of dead birds.
Neurotropic Velogenic virus	High mortality following respiratory and neurological signs, but gut lesions are usually absent.
Mesogenic virus	Low mortality with respiratory and neurological signs. Death usually seen in young birds.
Lentogenic	Causes mild or inapparent respiratory infection with no mortality.
Asymptomatic enteric	Avirulent infection with the virus replicating primarily in the gut.

These groupings, as put forwards by Beard and Hanson, are by no means clear-cut, as considerable overlapping occurs even in experimental infections of specific pathogen-free (SPF) chickens (Alexander & Allan, 1974). Also in field cases so many other factors come into play that may entirely alter the presentation of the disease.

1.4.2 Morphology and genomic structure of NDV

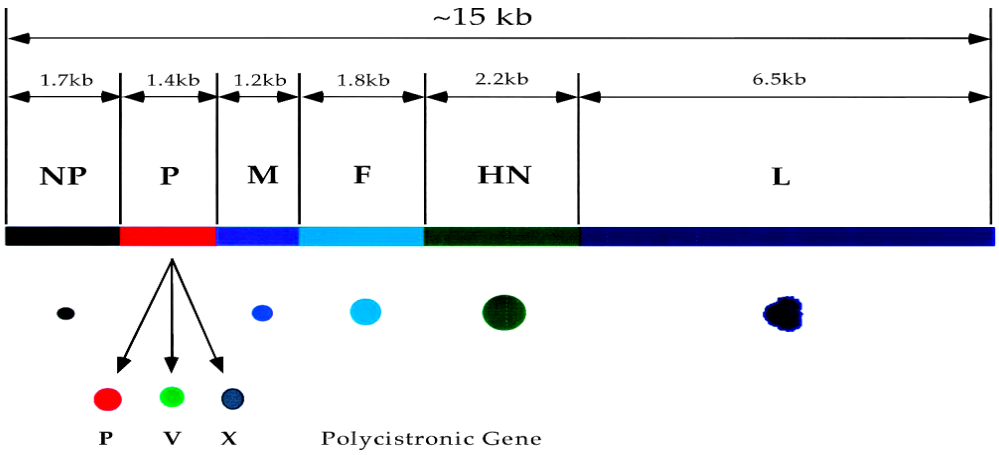


Fig 1.2. *Genomic organization of NDV with relative gene sizes (Seal et al., 2000b)*

Paramyxovirion are pleomorphic, enveloped, and roughly spherical ranging between 150 to 400nm in size (Dubois-Dalcq *et al*., 1984). The envelope membrane contains 8-12nm glycoprotein spikes (Lamb & Kolakofsky, 2001). The NDV genome consists of a negative-sense; single-stranded RNA molecule of 5.2 to 5.7 x 10^6 Daltons molecular weight (Kolakofsky *et al*., 1974) and replicates entirely in the cytoplasm of host cells (Lamb & Kolakofsky, 2001). The genome has 15,186; 15,192 or 15,198 nucleotides (Phillips *et al*., 1998; Huang *et al*., 2004a; Czeglédi *et al*., 2006). The genome codes six

proteins namely: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase protein (HN) and the large polymerase protein (L) in the 3'-NP-P-M-F-HN-L-5' order (Lamb & Kolakofsky, 1996). *Paramyxoviridae* accessory genes V and W occur mostly as ORFs (open reading frames) that overlap within the P gene transcriptional unit (Lamb and Kolakofsky, 2001).

The nucleocapsid protein (NP) is the major structural unit of the nucleocapsid (Samson, 1988). It appears as a flexible helical structure with a diameter of about 18 nm and 1µm length in negative-staining electron microscopy and consists of a single polypeptide of 489 residues with a molecular weight of about 53 kDa (Yusoff & Tan, 2001). NP performs several functions in viral replication such as encapsidation of the genome RNA into an RNase-resistant nucleocapsid thereby acting as a transcription anti-terminator, preventing the polymerase from stopping subsequent transcription (Peeples, 1988). The action of NP in the transcription-replication process is in association with the P-L polymerase and the M-protein. The helical assembly of nucleocapsid emerging from the disruptive virus particle is known as 'herring bone' and is typical of *paramyxovirinae* family (Kolakofsky *et al*., 2005**).**

The phosphoprotein (P) has 395 amino acids with a calculated molecular weight of ~42 kDa (McGinnes *et al*., 1988; Steward *et al*., 1993). The precise role of P is not known but it plays numerous functions in the replication and transcription of virus. As part of the P:L complex, P is known to stabilize the L protein as they act as the viral RNA-dependent RNA polymerase (Smallwood *et al*., 1994). P also prevents the uncontrolled

encapsidation of non-viral RNA by the NP protein (Errington & Emmerson, 1997). P may also function in the enzymatic activities of the nucleocapsid like polyadenylation, capping, methylation or cleavage of mRNA, or even as a cofactor in transcription (Peeples, 1988). The overlapping V ORF is found on the P gene and the V protein has been associated with replication and pathogenesis of the virus by serving as a virulence factor (by inhibiting the activation of host interferon) (reviewed in Zou *et al*., 2005).

The largest NDV protein (L, approximately 250 kDa) comprises 2204 amino acids (Yusoff *et al*., 1987) and together with P, they constitute the RNA-directed RNA polymerase found in virions (Hightower *et al*., 1975; Samson, 1988). L is thought to be responsible for the catalytic activity of the transcriptase complex (Samson, 1988). L may also be involved in transcript modification as well as capping, methylation and polyadenylation (Hunt *et al*., 1984).

The M gene contains 364 amino acids and has a calculated molecular weight of approximately 40 kDa (Chambers *et al*., 1986b; Seal *et al*., 2000a). The M protein is believed to play an important role in the assembly of virus during replication and to interact with the nucleocapsid, lipid bilayer and the exposed surface glycoproteins of membranes to perform its function (Yusoff & Tan, 2001).

The HN glycoprotein is made up of about 2000 nucleotides (nt) that carry an ORF encoding 571, 577, 581 or 616 amino acids (Sakaguchi *et al*., 1989; Tan *et al*., 1995). HN_{0616} which is the largest of all is convertible to a biological active HN protein through

proteolytic cleavage of residues from the C-terminus of the HN_0 precursor (Sakaguchi *et al*., 1989) while the other three amino acids are already in their active forms and are usually found in virulent strains (Yusoff *et al*., 1997). HN protein possesses haemagglutinin (HA) and neuraminidase (NA) activities (Scheid and Choppin, 1974). HN is responsible for viral attachment to its host cell and act as a pathogenic determinant of NDV (Lamb, 1993; Lamb & Kolakofsky, 2001; Yusoff & Tan, 2001). The ability of the HN protein to bind to receptors on the surface of red blood cells (RBCs) provides NDV and other paramyxoviruses with the ability to agglutinate RBC. The ability to haemagglutinate RBCs and specific inhibition of agglutination by antisera (Burnet, 1942) has become a useful diagnostic tool. The thermostability of the HN protein has also become a useful tool for characterization and epizootiologic studies (Hanson & Spalatin, 1978) as well as a rapid method for distinguishing virulence between strains.

The F protein that mediates fusion of viral and host cellular membrane contains 553 amino acids with a calculated molecular weight of ~55 kDa (Chambers *et al*., 1986a; Salih *et al*., 2000). The earliest work showing the involvement of F protein in fusion activity was from the finding that the F protein precursor (F_0) is synthesized in an inactive form and has to be activated by cleavage with the host protease into a biologically active disulfide-linked heterodimer, F_1 and F_2 polypeptides (Homma & Oluchi, 1973; Scheid & Choppin, 1974; Scheid *et al*., 1978). The cleavability of the precursor F_0 to F_1 and F_2 is the major determinant for virulence (Peeters *et al*., 1999). The Office International des Epizooties (OIE, 2000) has proposed the use of the sequencing procedure of the F_1/ F_2 cleavage site as an alternative method for studying the

pathogenicity of NDV in vivo. This is because the pathogenicity of NDV is closely linked to the biological properties of the F glycoprotein (Rott & Klenk, 1988). In addition, the sequencing of the cleavage site of the F gene has provided researchers with a tool to differentiate between highly virulent and low-virulence NDV strains (Meulemans *et al*., 2002).

1.4.3 Newcastle disease virus replication/infection cycle

The knowledge of viral replication is essential for the understanding of viral pathogenesis, immunity, chemotherapy and viral oncogenesis (Sharma & Adlakha, 1995a). Viral replication according to Wise & Carter (2005) is a very complex and varied process and the mechanics of replication depends largely upon the type of nucleic acid and genome organization of each particular virus. Generally, virus replication can arbitrary be divided into phases viz: initiation (attachment, penetration and uncoating), replication (genome synthesis, RNA production and protein synthesis) and release (assembly, maturation and exit from cell) (Heaphy, 2007). The NDV replication cycle is the most rapid of all paramyxoviruses, replacing host protein synthesis with viral protein synthesis within 6 hours while producing maximal yields of viruses within 12 hours post infection (Hightower & Bratt, 1974). NDV replication strategy is generally the same with most negative (-) stranded RNA viruses as put forwards by Peeples (1988). A comprehensive review on the replication of *Paramyxoviridae* has been written by Lamb & Kolakofsky (1996).

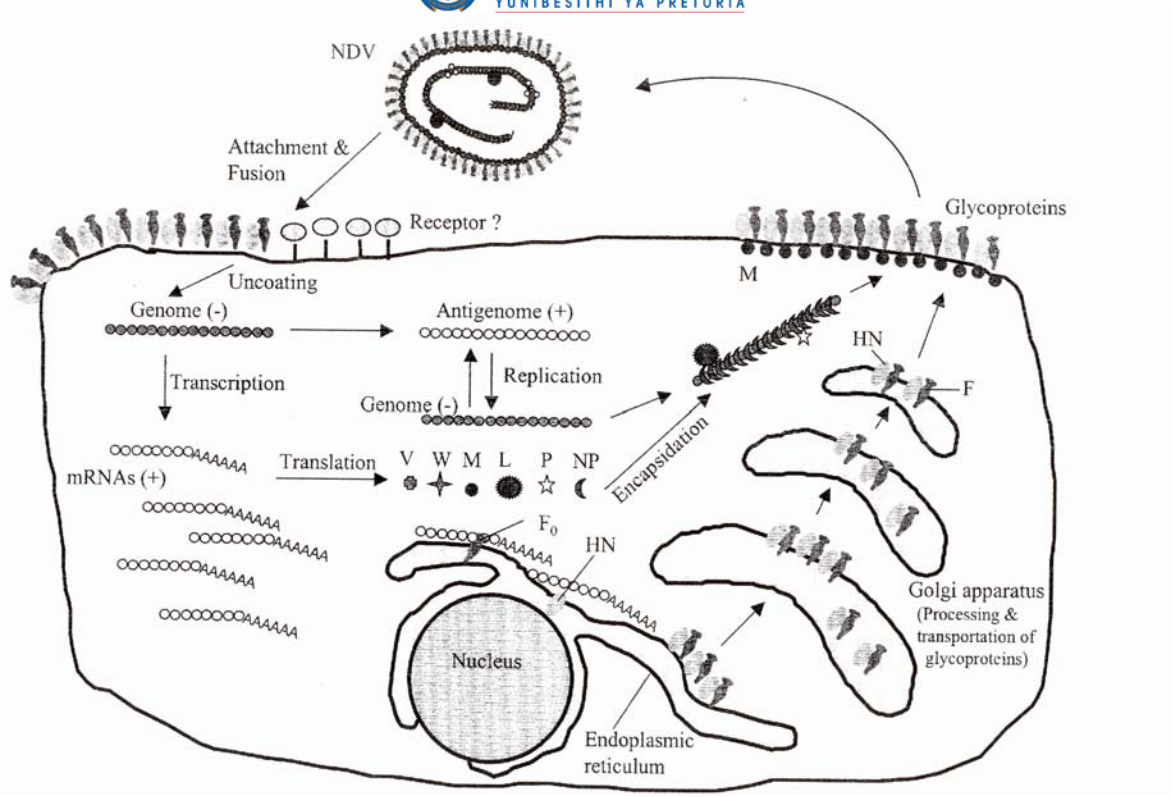


Fig. 1.3. The replication cycle of NDV *(Adapted from Yusoff & Tan, 2001)*

1.4.3.1 Attachment, fusion and penetration

The first step in the replication of NDV is the attachment of the virus to cell receptors, and is mediated by HN polypeptide (reviewed in Alexander, 2003, Alexander & Senne, 2008). The lipid envelope of paramyxovirus contains surface glycoproteins: haemagglutinin-neuraminidase (HN) and the fusion (F) protein which mediates early interaction with target cells (Scheid & Choppin, 1974). The HN binds to the sialic acid receptors on the surface of the target cells (reviewed in Lamb, 1993) and is also known to have receptor-destroying (neuraminidase) activity while the F protein assists in the penetration of host cell by the invading virus by mediating fusion of the virion envelope with the cellular plasma membrane (reviewed in Horvath *et al*., 1992). The second stage

in viral replication following attachment is penetration into the host cell. What triggers and controls the mechanism, timing and site of viral entry remains to be elucidated (Lamb *et al*., 2006). The cytopathic effect of cell infection by paramyxovirus is characterized by the formation of syncytia (giant cell formation) which is seen later in the infection when proteolytically-cleaved F protein has been expressed. Such cytopathic effect (CPE) can lead to tissue necrosis in vivo and might also be a mechanism of virus spread (Choppin & Scheid, 1980).

1.4.3.2 Transcription and replication

Following penetration, the virion undergoes uncoating, a process which involves the loss of the protective envelope or capsid upon entry into the cytoplasm of the host cell, thus allowing the virus to express its genome. The process is poorly understood (Heaphy, 2007). The events involved in intracellular genome synthesis, RNA production and protein synthesis (transcription, replication and assembly) of paramyxovirus occurs entirely in the cytoplasm (Alexander, 2003; Kolakofsky *et al*., 2005; Wise & Carter, 2005; reviewed in Abolnik, 2007). Viral replication involves genomic expression in which most viruses must produce all or most of the components of a template-specific RNA-dependent RNA polymerase (RdRp), and other proteins in order to transcribe full-length complementary RNA molecules from RNA templates (reviewed in Rybicki, 2000). The NDV RNA synthetic (transcriptase) activity is activated by the removal of the viral envelope which occurs when the virus penetrates into a host cell (Huang *et al*., 1971). The HN_0 and F_0 glycoproteins are synthesized in the rough endoplasmic reticulum (rER) whereas the viral structural proteins (NP, P, L and M) and the non-structural

proteins of V and W are produced in the cytoplasm after which they undergo a number of post-translational modifications and finally get transported across the endoplasmic reticulum (ER) to the Golgi apparatus (Yusoff & Tan, 2001). The viral structural proteins together with the newly synthesized RNP complexes are assembled and transported to the plasma membrane (Lamb & Kolakofsky, 2001).

1.4.3.3 Viral assembly and exit

The plasma membrane of infected cells is where the NDV assembles its components to produce infective viruses by budding (Bang, 1953; Feller *et al*., 1969; ICTVdB Management, 2006). The assembly of viral nucleoproteins either as helices or as isometric particles have some commonality between them (Harrison *et al*., 1996). Though, the process of viral assembly is simple and specific, it is driven by increasing concentration of genomic or pre-genomic RNA and of structural proteins (reviewed in Rybicki, 2000).

1.4.4 Genotypes/lineages of Newcastle disease viruses

NDV isolates have been grouped antigenically through the analysis of the binding patterns against a panel of monoclonal antibodies (mAbs) (Alexander *et al*., 1986; 1997; Russell & Alexander, 1983; reviewed in Alexander, 2003; Alexander & Senne, 2008). The system provided a method for rapidly sorting viruses into broad groups but failed to differentiate antigenically similar but genetically non-identical viruses (Alexander *et al*., 1999; Aldous *et al*., 2003). Restriction site mapping and sequence analysis of the F gene saw NDV strains being divided into 8 genotypes or lineages (Ballagi-Pordany *et al*.,

1996; Lomniczi *et al*., 1998; Herczeg *et al*., 1999). Several sub-lineages have been described within these eight lineages (reviewed in Abolnik, 2007). Despite the considerable genetic diversities detected in NDV, viruses sharing temporal, geographic, antigenic or epidemiological parameters tend to fall into specific lineages. This has been useful in assessing the global epidemiology and local spread of ND (Alexander, 2000; Aldous *et al*., 2003), resulting in the definition of three main panzootics of ND (Alexander, 1997; Lomniczi *et al*., 1998; Sakaguchi *et al*., 1989; Toyoda *et al*., 1989; Ballagi-Pordany *et al*., 1996). Ballagi-Pordany *et al* (1996), using restriction enzyme analysis in a preliminary characterization study, grouped NDV into six lineages (I to VI). Aldous *et al* (2003), following a recent revision of genetic grouping of NDV, confirmed these groupings (lineages1-6), and two further lineages (VII and VIII) and several sublineages. Lineages 3 and 4 were further subdivided into four sublineages (a to d) while lineage 5 was subdivided into five sublineages (a to e). Thus, lineage 3 has sublineages 3a, 3b, 3c and 3d which correspond to former genogroups III, IV, V and VIII. Lineage 5 has 5a to 5d sublineages and corresponds directly to previously determined lineages VII a to d, and a further branch 5e (Aldous *et al*., 2003).

Genotype 1 contains avirulent viruses that were often isolated primarily from waterfowls but also from chickens (Aldous *et al*., 2003). Genotype II- IV viruses were responsible for the first panzootic that took place between the mid-1920s to the 1950s. Genotype V and VI caused the second panzootic that occurred during the 1960s and 1970s (Alexander, 1988b; Lomniczi *et al*., 1998; reviewed in Herczeg *et al*., 1999 and Yu *et al*., 2001 and Abolnik *et al*., 2004a), while the subtype VIb virus caused the third panzootic

of pigeons (Alexander, 1988b; Collins *et al*., 1993; reviewed in Herczeg *et al*., 1999 and Abolnik *et al*., 2004b) which originated from the Middle East. Genotype VII/lineage 5 which has been causing outbreaks of ND around the world since the mid 1980s and has been described as causing the fourth panzootic (Lomniczi *et al*., 1998; Herczeg *et al*., 1999; Yang *et al*., 1999; Alexander *et al*., 1999; Liang *et al*., 2002). Genotype VII comprises of VIIa, VIIb, VIIc (lineages 5a, 5b, 5c), and the newly emerging NDV strain from China and Taiwan VIId/5d (Yu *et al*., 2001) which has been described as "goose paramyxovirus" (GPMV) in earlier reports (Xin *et al*., 1997; Wang *et al*., 1998). Genotypes VIII and X has also been described (Huang *et al*., 2004b; Zou *et al*., 2005).

1.4.5 Genotype VIId/lineage 5d and the fourth NDV panzootic

Newcastle disease virus (APMV-1) is known to infect about 241 different species of birds both naturally and experimentally (Kaleta & Baldauf, 1988). However, waterfowl such as geese and ducks are known to be asymptomatic carriers (Yin & Liu, 1997; Takakuwa *et al*., 1998; Alexander, 1997; Wan *et al*., 2004). Since the 1990s, infections with velogenic ND in both chickens and goose flocks have been reported in some parts of China (Liu *et al*., 1999; Ni *et al*., 2001; Ren *et al*., 1997; Xin *et al*., 1997; Liu *et al*., 2003).

In 1999, a disease outbreak in goose farms in Shanghai with a mortality of 10-20% in adult geese was reported. It caused 100% mortality in young geese less than 15 days of age. The novel virus that caused the outbreak was designated as SF02 and named as "Goose paramyxovirus" (GPMV) (Zou *et al*., 2002; Zou *et al*., 2005). GPMV has been

phenogenetically analyzed and found to be an avian paramyxovirus serotype-1 (Zou *et al*., 2005) and all but one strain of viruses of goose origin sequenced by Liu and his colleagues fell into lineage 5d (Liu *et al*., 1999; 2003). An earlier paramyxovirus infection in geese was also described as "goose paramyxovirus infection" in reports of Chinese literature (Xin *et al*., 1997; Wang *et al*., 1998; Liu *et al*., 1999). Liu *et al* (2007) characterized 64 isolates and reported most of the virulent isolates (45 of them) including all the isolates that are of goose origin to belong to the lineage 5d.

GPMV has been reported as highly pathogenic to chickens, pigeons, partridges and ducks (Zou & Gong, 2003), with (Mean Death Time) MDTs of 45.6 and 60 hours and ICPIs of 1.80-1.94 (Liu *et al*., 2003). A GPMV isolate with an MDT of 38.8 hours, an ICPI of 2.0 and producing cytopathogenic effects (CPE) in chicken embryo fibroblast cells within 40 hours has been reported (YuWen *et al*., 2004). Live and attenuated vaccines are known to fully protect chickens from clinical disease and death against challenge with VIId/5d (Liu *et al*., 2003). GPMV is reported to have some unique features which differentiate it with other APMV-1 viruses. It has 15,192nt (6nt extra fragment) ACACTC, in UTR between the NP and P genes as well as an additional anti-sense ORF containing the same 6nt fragment (Huang *et al*., 2002). While this insertion also occurs in NDVs of genotypes VI, VII, VIII, and IX, genotypes I, II, III, IV, and V do not have it (Huang *et al*., 2004b; Zou *et al*., 2005). However, the anti-sense ORF containing 6nt fragment is only found in SF02 genome and is absent in any other APMV-1 strains (Zou *et al*., 2005). It has been suggested that the difference in the intragenic regions of HN and P genes of NDV and SF02 affects the efficiency of RNA editing of the P gene and may be responsible for the

difference in the GPMVs pathogenicities for fowl and waterfowls (Zou *et al*., 2005). In addition, the 3′ leader of GPMV genome shares high identity with APMV-6 and other APMV-1 viruses, but its 5′ trailer is more variable (Zou *et al*., 2005).

Several outbreaks of velogenic ND between 1999-2006 affecting poultry in Kwa Zulu-Natal and other provinces in South Africa have been reported. The outbreaks were characterized by heavy morbidity and mortality involving both commercial and ornamental birds such as chickens, geese, ostriches, pheasants, peacocks, Hadeda Ibis (*Bostrychia hagedash*) chicks and doves, and were attributed mostly to genotype VIId (Abolnik *et al*., 2004a; Abolnik, 2007). Genetic similarities were established between the South African isolates of 1999 onwards with viruses from the Far and Middle East, supporting the theory of introduction from the Far East (Abolnik, 2007). Liu *et al* (2007), pointed out that the close genetic similarities between the China outbreaks and outbreaks of 1990s in other parts of the world have provided the epidemiological link which constituted the fourth ND panzootic.

1.5.1 Newcastle disease (ND)

It is true that the vast majority of birds are susceptible to infection with ND viruses of varied virulence for chickens. However, the disease caused by any virus may vary from species to species and many other factors come into play to alter or modify the course of the disease (Alexander *et al*., 2004). Mostly the variation is seen around the two extremes of high and low virulence but some virus may show intermediate virulence (mesogenic).

Even the virulent viruses may sometimes infect and replicate in vaccinated birds without causing clinical signs (Parede & Young, 1990; Guittet *et al*., 1993; Capua *et al*., 1993).

The clinical signs seen in birds infected with NDV vary widely and are dependent on viral factors like pathogenicity (which depends on the virulence and tropism of the virus), host factors (specie, age and immune status), concurrent infections, route of exposure, duration and magnitude of the infection dose and external factors such as social and environmental stress (McFerran & McCracken, 1988).

Signs of ND may generally consist of ruffled feathers, depression, diarrhoea, prostration, oedema of the head and wattles, nervous signs such as paralysis and torticollis and respiratory signs such as gasping and coughing (McFerran & McCracken, 1988). Egg production may drop or completely cease and the eggs are often misshapen, rough and thin-shelled with watery albumen. The cessation of egg-laying may precede more overt signs of the disease and death in egg-laying birds. Egg production in most ND cases returns to normal levels after 3-4 weeks but in some it never returns and birds may go into moult. Sudden death may be seen without the development of clinical signs (McFerran & McCracken, 1988).

Lesions are varied but generally include haemorrhagic lesions of the intestinal tract. They include petechial and small ecchymotic haemorrhages on the mucosa of the proventriculus, multifocal necrosis of the intestinal mucosa and haemorrhage and necrosis of the Payer's patches and ceacal tonsils. Oedematous, haemorrhagic and degenerative ovaries may also be seen (McFerran & McCracken, 1988; Beard, 1998).

Microscopic lesions generally consist of scattered cell necrosis in the parenchymal organs and perivascular cuffing, neuronal degeneration, gliosis and lymphoid aggregation in the central nervous system. Follicular atresia and infiltration of inflammatory cells and formation of lymphoid aggregates are seen in both the ovary and oviduct (Alexander & Senne, 2008). Just as with the clinical signs, no gross or microscopic lesion can be termed pathognomonic (McFerran & McCracken, 1988) and microscopic lesions are of limited diagnostic significance (Alexander *et al*., 2004).

Although clinicians may strongly suspect ND in a flock of domestic chickens, a definitive diagnosis cannot be made based on clinical signs or gross lesions. This is due to the similarities with many other poultry diseases. A final diagnosis is based on isolation and identification of the virus through pathotyping which includes MDT, IVPI (intravenous pathogenicity index), and ICPI, Reverse-transcription polymerase chain reaction (RT-PCR) and sequencing of the cleavage site may be used to determine the pathogenicity of NDV isolates (Alexander, 1997). Monoclonal antibodies can also be used to establish the antigenic profile of NDV isolates (Alexander *et al*., 1997). Haemagglutination inhibition test (HI) and other serological tests like enzyme-linked immunosorbent assay (ELISA) are commonly used for routine serological monitoring.

There is no known effective treatment for ND. The commonly used method for ND control includes vaccination, biosecurity, constant surveillance, and eradication or stamping-out depending on the disease status of the country. Stamping-out is only

practical in disease-free countries but is not practical in countries where the disease is enzootic.

1.5.2 Epizootiology of Newcastle disease

Assessment of the epizootiology of ND in the world is extremely difficult and complicated. This is because in some many countries, the disease is not reported at all or only reported when outbreaks occur in commercial poultry, while that of village chickens and backyard flocks is ignored. Secondly, the activities of man have lead to the spread of the virus through the transportation of infected birds around the world (Alexander, 1993). Alexander (2003) pointed out that estimating the geographical distribution of NDV is further being complicated by the use of live vaccines in most of the countries of the world. In some countries, live vaccine viruses which are sufficiently virulent to be considered as Newcastle disease when infecting poultry are still being used (Alexander *et al*., 2004). Thirdly, despite the internationalization of the monitoring of virulent Newcastle disease (vND) by agencies such as Food and Agricultural Organisation (FAO) of the United Nations and the Office International des Epizooties (OIE- also known as World Health for Animal Diseases), the data being generated may not present a true picture on the distribution of vND, because of the preference being given to outbreaks in commercial over village or backyard poultry (FAO, 1985; Alexander, 2003; Alexander & Senne, 2008).

Newcastle disease's greatest impact is on the backyard chicken production (Spradbrow, 1993). Despite the importance of village chickens in most developing countries as the

source of the much needed protein in the form of meat and eggs as well as provision of petty cash and other varied important uses (Alders & Spradbrow, 2001a), ND remains or is responsible for most of the devastating losses in village poultry (Alexander, 2001). It was estimated that 90% of village chickens in Nepal die from ND each year (Spradbrow, 1992).

Newcastle disease virus infects animals ranging from reptiles to man (Lancaster, 1966). Kaleta & Baldauf (1988) concluded that both natural and experimental infection with NDV has been demonstrated in 241 species of birds representing 27 of the 50 orders. This represents about 236 species of pet birds and free-living birds in addition to domestic avian species which include chicken, turkey, goose, duck and pigeon (Kaleta & Baldauf, 1988). The occurrence and the severity of the disease in all birds may however vary from species to species (Alexander, 2000) as other varied factors come into play that may modify the presentation of the disease. ND is most severe in chickens, peafowl, guinea fowls, pheasants, quails and pigeons but more mild in turkeys while canaries and finches may not show clinical disease (Beard, 1998). Psittacine and other wild birds may act as carriers (reviewed in CIDRAP, 2003).

Transmission of ND occurs by inhalation or ingestion through direct contact between healthy birds and bodily discharges of infected birds specifically faeces and secretions from the nose, eye and mouth (NABC, 2007). Mechanical spread through contaminated farm implements, shoes, clothing and premises also occurs. Environmental factors such as temperature, humidity and stocking density affects the success of the inhalation route

of infection (Alexander, 1995). Vaccination and debeaking crews, feed-delivery personnel, manure haulers, poultry egg buyers as well as poultry farm owners and workers are mostly responsible for mechanical transmission (USDA/APHIS, 2003).

Lancaster (1966), Lancaster & Alexander (1975), Alexander (1988d) and Alexander (1995) reviewed the modes of NDV spread and suggested up to eight sources or methods as being implicated in the various panzootics. The involvement of exotic pet, game and show birds, racing pigeon and waterfowl who act as inapparently-infected carriers is of very high significance. Many species of caged birds are known to harbour very virulent ND without showing clinical signs (Alexander, 1988d). Wild cormorants were suggested as the source of 1992 North Dakota outbreak of velogenic ND among range turkeys (Grow, 1992; Wobeser *et al*., 1993). The outbreak of ND in the United Kingdom among commercial poultry in 1997 (Alexander *et al*., 1998) and an earlier outbreak in Scandinavian countries by similar viruses in 1996 (Alexander *et al*., 1999) was linked to the unusual pattern of movement of migratory birds. Virulent NDV have also been isolated from captive caged birds (Senne *et al*., 1983) and racing and show pigeons were responsible for the late 1970s panzootic that spread NDV to all parts of the world (Alexander, 1997).

1.5.3 Pathogenesis and pathophysiology of NDV

The pathogenesis of NDV varies greatly and depends on a number of factors principal amongst which includes the virulence of the infecting virus and tropism of the virus (reviewed in Lewis, 2005). The host to a large extent plays an important role in the

virulence of NDV strains. Chickens are highly susceptible while ducks and geese may not show clinical signs when infected even by strains lethal to chickens (Higgins, 1971). Other factors which may influence the appearance of the disease in chickens includes the age and immune status of the bird, dose of the virus and route of exposure, concurrent infection and social and environmental stress. All these factors go a long way to determine the course of the disease or infection.

In chickens, the virus enters the body via the respiratory and the intestinal tract and is mostly caught either in the nose or the trachea. Once in the trachea, the virus spread by ciliary action and cell-to-cell infection (reviewed in APHIS, 2005). From the initial multiplication at the point of introduction, virus is carried by viraemia to the liver, spleen, kidney and lungs and virus can be found in practically all tissues within 22-44 hours of infection. Virus invades the brain after multiplication in non-nervous tissues has ceased (from 60 hours post-infection) whereupon birds start dying (Kouwenhoven, 1993).

Paramyxoviruses produce intracytoplasmic inclusion bodies which are accumulations of viral structural components and thus distort the cell and cellular activities during infection. NDV infection also leads to the production of syncytia due to the changes produced in the cell membrane which results from the fusion of infected with uninfected cells (Sharma & Adlakha, 1995b). Other cell changes due to the specific effects of viral replication includes cloudy swelling of cells due to an increased permeability of the plasma membrane and results in leakage of lysosomal ezymes into the cytoplasm of affected cell and the eventual destruction of the cells. The budding of viruses from the

plasma membrane also results in the production of persistent infection and as the cells yield the viruses that grows and divides for long periods inside such infected cells, it leads to slow and progressive changes in the cells due to biochemical changes and the eventual cell death and the resultant pathologic effects (Sharma & Adlakha, 1995b). Also, the persistently infected cells lose their capacity to perform specialised function and antigenic changes are produced in the cell membrane of such infected cells. NDV also causes ciliostasis and destruction of cilia and the subsequent lowering of resistance of mucosal surfaces to secondary bacterial infections (Sharma & Adlakha, 1995b). The cell death due to the replicating viruses leads to secondary effects such as:

- inflammation from complement activation and/or liberation of endogenous permeability factors from damaged cells,
- haemostasic response which lowers blood volumes and diarrhoea causing fluid imbalance and,
- fever, resulting from the release of endogenous pyrogens by the virus (Sharma & Adlakha, 1995b).

The ability to replicate in a wide range of tissues and organs results in fatal systemic infections (Rott, 1979). Studies have also shown that the HN protein can contribute significantly to viral spread in the host and thus its virulence (Huang, *et al*., 2004a). The V ORF protein found on the P gene protein is an interferon antagonist (Park *et al*., 2003; Huang *et al*., 2003) and therefore likely to be involved in pathogenesis and host-range restrictions (Mebatsion *et al*., 2001; Park *et al*., 2003).

The initial immune response to NDV infection is cell- mediated and can occur in 2-3days post infection, especially with live vaccine strains (Ghumman & Bankowski, 1975; Timms & Alexander, 1977). Reynolds & Maraqa (2000) however concluded from their study that the cell mediated response to NDV by itself is not protective against challenge with virulent NDV when compared to humoral immunity. Serum antibody can be detected in chicken 6-10 days post-infection. Titres reach their peak within 3-4 weeks and declines to undetectable levels in 8-12 months (Animal Health Australia, 2006). Antibodies in secretions especially of the respiratory and intestinal tract are seen at about the time humoral antibodies are first detected. IgA and IgG are immunoglobulins chiefly found in the respiratory tract (Parry & Aitken, 1977). Similar immunoglobulins occur in the Harderian gland following ocular infections (Parry & Aitken, 1977; Powell *et al*., 1979).

1.6.1 Economy of Newcastle disease and sustainable livelihood

According to Alexander & Senne (2008) the global economic impact of vND is enormous and unsurpassed by any other poultry virus until the emergence of the highly pathogenic Asian (H5N1). ND probably represents a bigger drain on the world's economy than any other animal virus. In developed countries where the poultry industry is well developed and very important in terms of its contribution to the economy, outbreaks of vND are extremely costly. Also the cost associated with the control measures which may includes stamping out, vaccination and constant or repeated seromonitoring represents a continuing loss to the industry (Leslie, 2000). For example, the 1971 major outbreak of vND in California resulted in the destruction of 12 million

birds. The eradication efforts cost $56 million in a clean-up that took 4 years and costing $275 million (Hahn & Clark, 2002). It also resulted in an increased cost for poultry consumers. Adjusting for inflation, the control and clean-up costs a total of $1.16 billion in 2003 dollars (Clark, 2003). These costs did not take into consideration the costs of lost markets, trade embargos, and increased prices to consumers. The cost for controlling the END outbreak of 2002 to 2003 in California was $160million (reviewed in CIDRAP, 2003). Even countries that are free of vND face the cost of continuous and repeated testing to maintain that status for the purpose of trade (Alexander & Senne, 2003). The endemic nature of the disease in many developing countries represents an important limiting factor in the development and growth of the poultry industry or commercial poultry and the establishment of trade links. In the village setting, people depend on the village chickens to supply much needed dietary protein in the form of eggs and meat, as well as income for petty spending. But the constant losses from vND severely affects both the quality and quantity of food of people on marginal diet especially children and women (Spradbrow, 1992; Sen *et al*., 1998), as well as their income and thus perpetuate the twin evils of poverty and malnutrition.

The poultry industry in South Africa, as is the case with most African countries and developing economies all over the world, is made up of both scavenger birds and exotic breeds with the latter being managed in highly integrated commercial setting, while the former are kept by subsistence farmers on the other hand. Outbreaks in the village chicken usually spill over into commercial operations resulting in severe economic losses. This was exemplified by the 1993/94 outbreak that caused an estimated loss of a

million broilers in a week during the peak (Coetzee, 1994). Because ND is notifiable to the OIE, outbreaks usually leads to trade restriction and the consequent loss of revenue from the restriction in trade on poultry and poultry products. This makes ND a serious disease of high economic threat especially to the broiler industry, but of less importance in the Ostrich farming because of its slow spread (Verwoerd, 1995a). The village chicken productions have been credited with significant contribution towards increasing the food security and secure livelihoods for the villagers because of the multifunctional roles of local chickens (Alders, 2000). Village chicken provides meat and eggs, food for special festivals, offering for traditional ceremonies, pest control and petty cash for spending (Alders & Spradbrow, 2001a). The biggest challenge toward achieving this is the endemic presence of ND in developing countries and especially its effects on the village chicken production (Spradbrow, 1993; Awan *et al*., 1994). Poultry production is the most efficient and cost-effective way of increasing the availability of high-protein food (FAO, 1987), as eggs are known to provide the most perfectly balanced food containing all the essential amino acids, minerals and vitamins (Branckaert *et al*., 2000).

Most researchers that studied village poultry are of the agreement that ND is the single greatest constraint to village poultry production (Alexander, 1988a, Spradbrow, 1993, Kitalyi, 1988, Alexander, 2001; Alders & Spradbrow, 2001b). Kaschula in 1944/1945 recounted the experiences of the Indian workforce at the sugar mill in KZN, South Africa, at the “hands” of ND. He pointed out that the barrack nature of habitation and the free mixing of birds from different households of different ages with no barrier assist in the spread of ND in the face of outbreaks. A visit to a barrack settlement of the Indians

will only find ducks (who were resistant) walking around after the ND outbreak has wiped out the chickens population. This is in contrast to the flocks owned by the Europeans which were well housed and fed, and losses were limited. The Africans Kraal mode of settlement also restrict and limits the movement of fowls between kraals and therefore losses from ND were not heavy as those experienced by the Indians (Kaschula *et al*., 1945).

The extremely high mortalities associated with ND are the major discouragement for the poor villagers from investing much of their hard-earned and scarce resources and time in expanding their flock size. Alexander (2001) said "ND may represent a disaster to those relying on village chickens as a food or trading commodity but this reservoir of virulent NDV must be considered a continuing threat to poultry populations throughout the world". This is because village chickens have been speculated to be a reservoir of virulent NDVs from which spillover into the commercial poultry sector posses a serious threat (Verwoerd, 1995b). The economic impact of vND will be very difficult to assess as it involves not just the direct commercial losses, but also the effect on human nutrition and health and loss of potential socioeconomic gain in some countries, if such food was plentiful (Alexander, 2003). The effect on egg production together with the mortalities caused by ND affects the quality and quantity of dietary protein and significantly affects human health (Steneroden *et al*., 2004).

1.7.1 Reproductive anatomy and physiology of the hen

The avian female reproductive system performs two main functions: a) production of steroid hormones which influence sexual activity in general, and b) egg production.

1.7.2 Anatomic structure of the hen's reproductive system

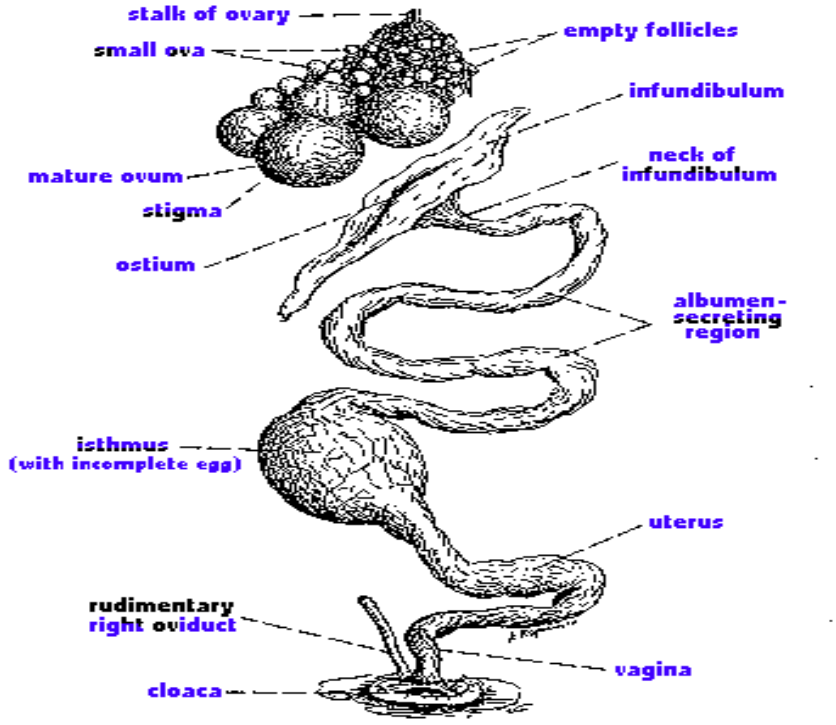


Fig. 1.4 The Hen's reproductive system showing the ovary and the various sections of the oviduct ***(Magnum is labelled as the albumen secreting region)***

Adapted from: http://www.iacuc.arizona.edu/training/poultry/images/female.gif

The reproductive system of a mature bird (Galliformes) is made up of a single functional left ovary and its oviduct although occasional cases of a functional right ovary and

oviduct may be present (Johnson, 1986). The right ovary and oviduct that are present during embryonic stages regresses before hatching (Joyner, 1994) under the influence of Müllerian-inhibiting hormone (MIS) whose action on the left ovary is inhibited by the greater amount of oestrogen secreted by the left ovary (Johnson, 2004). The left ovary, attached by the mesovarian ligament to the abdominal wall and the cephalic end of the kidney is made up of a mass of small ova (about 4 000) out of which 200-500 reach maturity (Johnson, 1986; Joyner, 1994). The ovary appears as a small piece of fat in young birds and assumes a cobblestone appearance as the primary oocyte or ovum develops under the influence of gonadotropin in mature hens into a hierarchy of follicles of different sizes, some of which are yellow yolk-filled with the majority being small white follicles (Johnson, 1986; Joyner, 1994; Johnson, 2004). The hierarchical follicles are highly vascularised and innervated with the highest blood flow to the largest preovulatory follicles (reviewed in Johnson, 1986; 2004). The ovary produces the ova (yolk) as well as the hormones which regulates and modulates the reproductive activity.

The oviduct, derived from the left Müllerian duct during oogenesis is a coiled or folded tube of about 80cm in length with five distinguishable regions viz: infundibulum, magnum (albumen-secreting region), isthmus, shell gland (uterus) and the vagina (Johnson, 1986; Austic & Nesheim, 1990; Joyner, 1994; Johnson, 2004; The State of Queensland, 2007). The oviduct has a muscular wall with a good blood supply and is basically a conduit from the ovary to the cloaca with each of the five different regions specialized for a particular function (Austic & Nesheim, 1990; Johnson, 2004). It is attached to the peritoneal cavity by dorsal and ventral ligaments (Johnson, 1986). The

oviduct of an immature fowl is small while the size varies in different species and at different stages of reproduction and age (Austic & Nesheim, 1990).

Table 1.1 Sections of the hen's oviduct and their respective functions

Organ/Section	Length in lay (cms)	Approx. time egg spends in this section	Functions
1.Infundibulum	9cms	15-30minutes	-Receives yolk from ovary -Fertilization occurs if sperm is present -First layer of albumen is produced
2. Magnum	33cms	2-3hours	-Secretion of albumen (majority) -Water & minerals are added to albumen
3.Isthmus	10cms	1-2hours	-Formation of the inner and outer shell membrane
4.Uterus (shell gland)	10-12cms	20-26hours	-Addition of vitamins, salts and water to the egg -Laying of cuticle & calcification of the shell membrane -Pigmentation of the shell (where applicable)
5.Vagina	12cms	Few minutes (1-5mins)	-Rotation of the egg -Expulsion of the egg (oviposition)

A table showing the various sections of the hen's oviduct with their length during lay, their functions and the approximate time an egg spends in each section (Johnson, 1986; North & Bell, 1990; Austic & Nesheim, 1990; Johnson, 2004).

1.7.3 Reproductive physiology and the effects of diseases

1.7.3.1 Reproductive physiology

The unilateral avian oviduct is divisible into five functional regions with each of the sections performing a variety of functions, which through their interaction give rise to fully-formed eggs (Solomon, 2002). The reproductive cycle of the hen, characterized by a well-ordered ovulation and oviposition cycle and by a strict hierarchical growth of follicles is coordinated by the neuro-endocrine system (Decuypere *et al*., 1999; Austic & Nesheim, 1990). The physiological and endocrine control of reproduction is subject to influence and variations due to environmental or selection factors (Decuypere *et al.*, 1999).

The neuronal aspect of reproductive control is coordinated by the hypothalamus. Three sections of the hypothalamus are involved and include the infundibular nuclear complex, preoptic regions and the supraoptic region (Austic & Nesheim, 1990). The maturity of the immature bird's reproductive organs (ovary and oviduct) is under both neural and hormonal control. Both the ovary and the oviduct rely on the neuroendocrine information relayed to it from the hypothalamus, pituitary gland, adrenal glands, the ovarian tissues themselves, and a host of other small glands to perform their function (Austic & Nesheim, 1990). Both the ovary and the oviduct are highly vascularised and innervated by both sympathetic and parasympathetic nerves (Johnson, 1986; Austic & Nesheim, 1990). The neuroendocrine control of the avian reproduction is represented graphically below.

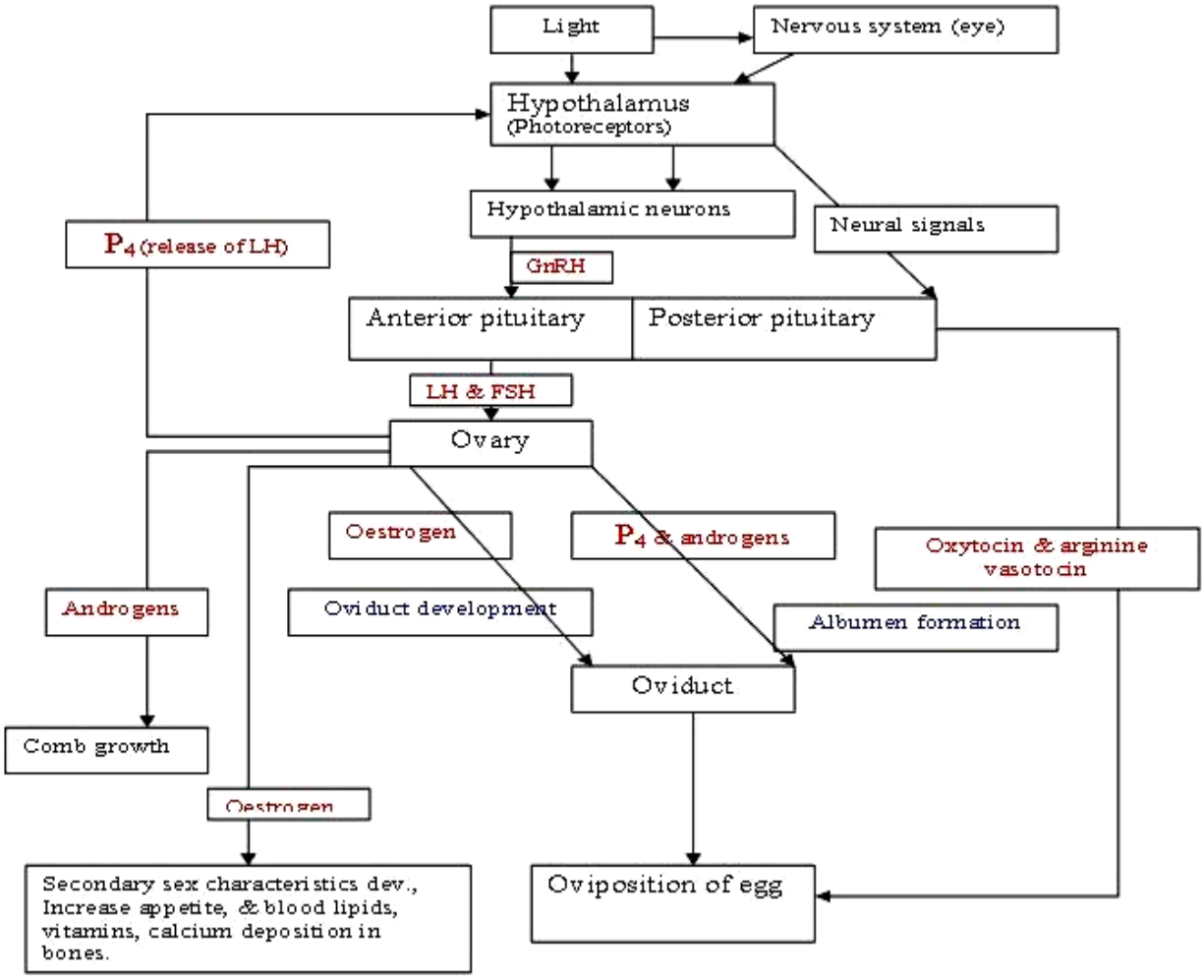


Fig. 1.5 Relationship between the nervous system, endocrine glands and the reproductive system of avian female species (Austic & Nesheim, 1990).

1.7.3.2 Effects of disease on the reproductive functions of the hen

The reproductive efforts of birds can be influenced by disease processes either by acting directly and altering the ability of the lining cells to perform their specialized functions or by generally compromising the health of the bird. Notable among those that affect the cells of the oviduct are Infectious Bronchitis, Newcastle disease and Egg Drop Syndrome

(Solomon, 2002). All these diseases produce some form of pathology in the reproductive organs of the affected bird (Biswal & Morrill, 1954; Dhinakar Raj *et al*., 2001; Rao *et al*., 2002; Chousalkar & Roberts, 2007). The net effect of these diseases is either a change in quality of the egg produced in terms of the shape and/or texture of the shell or a complete drop in the quantity produced. Solomon (2002) described the travail of the eggs from the ovary to cloacae as a "chaos" – a "great disorder", as many events can conspire to alter the morphology of the reproductive tract and hence its capability to produce those fractions essential for "normal" egg formation. *Escherichia coli* (reviewed in Keymer, 1980), *Mycoplasma* spp (reviewed in Branton & Deaton), *Salmonella enteritidis* (Craig *et al*., 2002), Newcastle disease (Riddell, 1996) and many other diseases have been reported to either reduce feed and egg production efficiency or temporarily damage the shell-producing mechanism of infected fowl.

Diseases are reported to cause stress in infected animals (reviewed in Gallili & Ben-Nathan, 1998; Johnston & Gous, 2006). The environment is a composite of interacting stressors and the bird's success in coping with the severity of the stressor(s) as well as the bird's physiological ability to respond properly (Siegel, 1980). Such environmental stressors induce a stereotyped neuroendocrine response which results in physiological adaptation (reviewed in Borghetti *et al*., 2009). Stress imposed on birds either through external (temperature, feed deprivation, etc) or internal (diseases) sources are reported to cause apoptosis and subsequent cell proliferation in the tissue of the anterior pituitary, and have the ability to reduce or prevent the secretion of LH (Chowdhury & Yoshimura, 2002; Johnston & Gous, 2006). In addition, viral infections have been reported to cause

stress reactions with the elevated production of endogenous glucocorticoids among which is corticosterone (Blalock, 1987; Dunn *et al*., 1989) causing involution of lymphoid organs and generalized immunosuppression (Ben-Nathan & Feuerstein, 1990). The stress adaptive response has a substantial effect on both inflammatory and immune response by activating a neuroendocrine response based on:

(a) activation of the hypothalamic-pituitary-adrenal axis with subsequent peripheral secretion of cortisol from adrenal glands;

(b) somatotropic axis activity;

(c) activation of the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-thyroid systems (reviewed in Borghetti *et al*., 2009).

Cortisol, another glucocorticoids produced as a result of stress during disease conditions, decreases Ca^{2+} absorption from the intestines and increase renal excretion of Ca^{2+} from the body (Eiler, 2004). The adrenal gland via the secretion of corticosterone is reported to regulate the timing of the pre-ovulatory LH surge which is necessary for ovulation (Wilson & Cunningham, 1980). Corticosterone also modulates the responsiveness of the hypothalamus to tropic stimuli and that the exposure of corticosterone can alter the responsiveness of some ovarian tissues (Etches *et al*., 1984). Chronic and acute stress causes an increase in neurogenic amines and corticosterone which is reported to inhibit skeletal calcification in growing birds and induce osteoporosis in adult birds (Siegel & Latimer, 1970, Urist & Deutsch, 1960). It also causes losses in weight and reduced reproductive capability in adult birds, often despite increased food consumption

(reviewed in Siegel, 1980). Stressors have been reported to changes in oviduct structure and function (reviewed in Solomon, 2002; Bain, 2005).

Biochemical changes purportedly occur in viral-infected cells, inducing pathologies that are not visible to the naked eye or with the use of light or electron microscope. Cytokines and chemokines produced at inflammatory sites and/or locally in the central nervous system can modulate brain function and hormonal secretion by the endocrine glands. They can also induce the Acute Phase Reaction (APR) characterized by fever, anorexia, hormonal changes and metabolic modifications such as protein catabolism, lypolysis and gluconeogenesis (reviewed in Borghetti *et al*., 2009). Reproductive performance has been reported to correlate significantly with plasma protein concentration, White blood cell count (WBC counts) and parasite intensity. Therefore, according to Sharma & Adlakha (1995); it would be unwise to ignore any cell showing evidence of viral replication, even if microscopic lesions are not evident. In addition, regardless of the pathogenic mechanism involved, disease results in decreased efficiency of feed utilization and a lower general health status of the individual, both of which are important sources of economic loss.

1.7.4 Effects of ND on the reproductive functions of hens

1.7.4.1 Egg production and quality

A marked effect on egg production is reported as one of the signs of ND affecting the reproductive system. The changes could include partial to complete drop in production, accompanied by production of smaller eggs, misshapen and rough-shelled eggs and shell-

less to thin-shelled eggs containing watery albumin (McFerran & McCracken, 1988). Some of the eggs produced may have little or no pigment (Animal Health Australia, 2006). In some birds surviving infection with ND, egg production may never reach its former capacity, while some of the birds may go into moult (McFerran & McCracken, 1988; NABC, 2007).

1.7.4.2 Macropathology of the reproductive system of ND-infected hens

Depending on the virulence of the infecting pathotypes and a host of other factors, the virus infects and replicates in almost all organs causing varying degrees of lesions (Peeples, 1988). The use of the Oviduct Organ Cultures (OOCs) confirmed the susceptibility of the reproductive tract of oestrogen-treated chicks to NDV (Rao *et al*., 2002). Chickens and turkeys infected in lay with velogenic viruses usually have egg yolk in the abdominal cavity, flaccid and degenerative ovarian follicles, and variable haemorrhage and discoloration of the other reproductive organs (Alexander, 2003; Alexander & Senne, 2008). Other changes reported by McFerran & McCracken (1988) include resorption of yolk, roughness of external follicular outline and a shrunken oviduct. Congestion and oedema of the oviduct has also been reported (Rao *et al*., 2002).

1.7.4.3 Histopathology of the reproductive system in ND-infected hens

Microscopically, the reproductive tract of an infected bird may contain variable changes. The greatest functional damage is purported to occur in the uterus (Biswal & Morrill, 1954). Atresia of follicles and an infiltration of inflammatory cells (lymphocytes, macrophages and plasma) and the formation of lymphoid aggregates are also seen in the

oviduct (Alexander, 2003). The Rao et al. (2002) study shows that the severity of the lesions matches the virulence of the infecting ND virus in either *in vitro* (OOCs) or *in vivo* trials. Virulent viruses cause ciliostasis in OOC(uterus) a day post-inoculation compared to 2 days, 2-3 days and 4-5 days taken by Avian Pneumovirus (Khehra & Jones, 1998), Infectious Bronchitis virus (Dhinakar Raj & Jones, 1996) and Egg Drop Syndrome 76 virus (Dhinakar Raj *et al*., 2001) respectively. The ciliostasis is a result of the NDV replicating in the ciliated lining epithelium of the uterus. In an *in vivo* trial where velogenic isolates were inoculated, a more severely degenerative and diffuse pathology of the reproductive tract reveals necrotic changes in the glandular epithelial cells and the accumulation of cellular debris and fibrin between folds within the lumen of the uterus respectively. The surface epithelial cells in the magnum showed extensive foci of necrosis, with subsequent desquamation and atrophy of tubular glands (Rao *et al*., 2002). Rapid and complete damage of the ciliated epithelium of the uterus and magnum affects the bird's ability to produce a good quality egg (Rao *et al*., 2002).

1.8.1 Immunohistochemistry as a tool for disease diagnosis

1.8.1.1 Definition of immunohistochemistry

Immunohistochemistry (IHC) refers to the process whereby cell-associated proteins/antigens in tissue section are localized via the binding of antibodies specifically to these antigens (Ramos-Vara, 2005). The word immunohistochemistry is derived from the words "immuno" which refers to antibodies and "histo" to tissue (Wikipedia, 2007) and is often used interchangeably with immunohistochemical staining (IHS). Immunohistochemistry relies on the ability of antibodies to bind to specific antigens in

tissue sections (Haines & Chelack, 1991; Ramos-Vara, 2005) and the commonly used immunoglobulin (Ig) is IgG and less of IgM (Ramos-Vara, 2005). Since the identification of tissue antigens via a direct fluorescence method was revolutionized by Coons and his colleagues in 1941 (Coons *et al*., 1941), IHC has become an important tool of investigation in both diagnostic histopathology and research of infectious diseases (Kämmerer *et al*., 2001; Ramos-Vara, 2005; Wikipedia, 2007).

IHC combines the scientific fields of immunology, histology and chemistry (Ramos-Vara, 2005). The principle of IHC which was hitherto simple has become increasingly complex as more emphasis is placed on improved sensitivity and specificity (Mighell *et al*., 1988). Until recently, IHC made use of flourescein dye-labelled antibodies on fresh or frozen tissue samples (Coons *et al*., 1941), a method that produced labile stains that were visible only with an ultraviolet microscope. Methods that use enzyme-labelled antibodies suitable for tissues fixed in standard fixatives such as formalin have been widely reported in the literature. Immunodetection systems include direct horseradish peroxidase (HRP) (Nakane & Pierce, 1966), peroxidase-anti-peroxidase (PAP) (Sternberger, 1969), avidin-biotin complex (ABC) (Hsu *et al*., 1981) and the alkaline phosphatise-anti-alkaline phosphatise (APAAP) (Cordell *et al*., 1984).

The use of monoclonal antibodies (mAbs) has been emphasized in IHC over polyclonal antibodies (pAbs) because of their higher specificity and the fact that they reduce the chances of cross-reactivity and thus false positive results (Haines & Chelack, 1991). Monoclonal antibodies are derived from a single B-cell clone and produced by

hybridoma technique. They provide excellent specificity because the antibody binds to a single epitope on one antigen (Ramos-Vara, 2005). Polyclonal antibodies (pAb) contains antibodies to a range of antigens and thus may cause greater nonspecific background staining and to be less specific than mAb (Ramos-Vara *et al*., 2008).

For IHC, the maintenance of the morphology of tissues and cells and accessibility of antigenic sites is quintessential. For this reason tissue blocks are immersed into fixative solution to prevent artifactual diffusion of soluble tissue components, arrest enzymatic activity and therefore autolysis, and protect tissues against the deleterious effects associated with various stages of the IHC process (Hayat, 2002; Pierce, 2007). Fixatives used in histopathology can broadly be classified into cross-linking (non-coagulative) fixatives and coagulative fixatives (Ramos-Vara, 2005). Ramos-Vara (2005) described formaldehyde as the gold standard fixative for routine histology and IHC because it preserves mainly peptides as well as the general structure of cellular organelles, and interacts with nucleic acids with no effects on carbohydrates (Eltoum *et al*., 2001). The application of IHC on formalin-fixed tissues has greatly improved the diagnostic capability of anatomic pathologists. Advantages include:

- The same tissue sample can be used for both routine histology and IHC,
- Retrospective diagnosis can be performed in cases where fresh tissue samples are no longer available.
- IHC is one of the most important ancillary techniques in the characterization of neoplastic diseases in humans and animals.

1.8.1.2 Direct versus indirect immunohistochemical techniques

Immunohistochemical techniques are either direct or indirect. The direct method is a one step staining method and is the simplest of the immunocytochemical methods (Ramos-Vara, 2005; ihcworld, 2008). It involves a labelled antibody reacting directly with the antigen in tissue sections (Coons & Kaplan, 1950). Labels such as fluorochromes, enzymes, colloidal gold, and biotin have been used (Polak & Noorden, 2003). The technique utilizes one antibody and the procedure is short and quick but lacks sufficient sensitivity for the detection of most antigens in routinely processed tissues due to little signal amplification (Ramos-Vara, 2005; ihcworld, 2008). It is rarely used since the introduction of the indirect method.

The indirect method involves the use of an unlabelled primary antibody (first layer) which reacts with tissue antigen, and a labelled secondary antibody (second layer) that reacts with the primary antibody (ihcworld, 2008). The secondary antibody must be against the IgG of the animal species in which the primary antibody has been raised. The method was developed by Coons *et al* (1955) in response to the need for more sensitive antigen detection. The indirect method is preferred due to the enhanced sensitivity of antigen detection. Secondary antibodies bind to the primary antibody, therefore intensifying the visible signal produced. Also no conjugation of the primary antisera is required. This is because the procedure utilizes an enzyme-conjugated anti-immunoglobulin secondary antibody (Haines & Chelack, 1991). This method is also economic, since one labelled second layer antibody can be used with many first layer antibodies (raised from the same animal species) to different antigens (ihcworld, 2008).

The second layer antibody may be labelled with an enzyme such as peroxidise, alkaline phosphatase or glucose oxidase, and the method is called immunoenzyme method.

Basic steps in the indirect method after tissue processing and the adherence of paraffin-embedded tissue sections to microscopic slides include:

Step 1: Pre-immunologic procedures – includes all procedures done before incubation with the primary antibody:

- Deparaffinization in two-step xylol and rehydration of tissue slides in graded alcohol of 100%, 96% and 70%.
- Blocking endogenous peroxidise with 3% Hydrogen peroxidase.
- Proteolytic digestion and antigen retrieval (e.g., HIER - heat-induced epitope retrieval) in target retrieval solution with protease.
- Background blocking non-specific bindings

Step 2: Immunologic reactions – all reaction between the primary and tissue antigens:

- Incubation of section with primary antiserum
- Incubation with secondary reagent
- Incubate slides with ABC complex

Step 3: Procedures necessary to visualize the antigen-antibody binding

- Chemical reaction with substrate and a chromogen to produce a coloured reaction product.
- Counterstain with Mayer's haematoxylin

- Dehydration and coverslip

Step 4: Interpretation and reporting of IHC results (Ramos-Vara *et al*., 2008; Manzer, 2008).

1.8.1.3 Avidin-Biotin Complex (ABC) method

ABC method is a standard IHC method and is the most widely used detection methods (Rhodes, 2001). Central to the wide use of this method is the remarkable affinity that exists between avidin and biotin; which is the highest known affinity in nature between a ligand and a protein (Livnah *et al*., 1993). IHC methods based on avidin-biotin binding have been popular because of their reliability and exquisite sensitivity (reviewed in Dodson, 2002). The ABC method ensures greater amplification of the visible signal produced by the binding of primary antibodies to specific antigen in tissue sections (Hsu *et al*., 1981). The increased sensitivity of the ABC immunodetection method is due to the high affinity of the egg-white glycoprotein avidin for the B-vitamin called biotin which has a binding site for avidin and can be attached via other sites to an antibody (biotinylated) or other macromolecules such as enzymes fluorochromes and other labels (Hsu *et al*., 1981).

Generally, the avidin-biotin system is known to produce superior results compared to PAP, ABC alkaline phosphatise and ABC glucose oxidase methods (Haines & Chelack, 1991). The ABC method has numerous advantages which include: Increased enzymatic label at the tissue antigen site; increased detection efficiency, less primary antibody is

required and assay time is reduced when compared to the PAP method (Pierce, 2007). Despite their reliability and high sensitivity, most avidin-biotin systems produce abundant non-specific background staining especially when harsh antigen retrieval methods are used (Ramos-Vara, 2005) and also due the high presence of endogenous biotin in a large numbers of tissues (reviewed in Dodson, 2002).

1.8.1.4 Polymer-based system

Developed in the early 1990s (Dodson, 2002), the polymer-based systems are based on dextran polymer technology which allows for the binding of a large number of enzyme molecules (horseradish peroxidise or alkaline phosphatase) to secondary antibodies via an inert dextran/polymer backbone (Dodson, 2002; Ramos-Vara, 2005). The development of the polymeric labelling is principally to increase the sensitivity of IHC over earlier techniques and overcome the shortcomings of non-avidin-biotin polymer-based system/method (Novocastra Laboratories, 2005; Ramos-Vara & Miller, 2006). The technology has been applied to both primary antibodies and the detection of a variety of antigens (reviewed in Ramos-Vara & Miller, 2006) The polymer-based technology is reported to have the advantages of increased sensitivity, reduced background non-specific staining due to endogenous biotin or avidin, and a reduction in the number of assay steps as compared to 3-step avidin-biotin methods (Dodson, 2002; reviewed in Ramos-Vara, 2005; Ramos-Vara & Miller, 2006).

1.8.1.5 NovoLink™ polymer-based detection system

The NovoLink™ Polymer Detection System utilises a novel controlled polymerization technology to prepare polymeric HRP-linker antibody conjugates. The NovoLink™ products are used in an IHC procedure, to allow the qualitative and semi-quantitative identification by light microscope of antigens in sections of formalin-fixed, paraffin-embedded tissues, via sequential steps with interposed washing steps. Endogenous peroxidase activity is quelled using the Novocastra™ peroxidase block. The application of the Novocastra™ Protein Block also reduces the non-specific binding of primary antibody and polymer; while the Novocastra™ Post Primary Block is used to enhance penetration of the subsequent polymer reagent. The Novocastra™ Polymer recognises mouse and rabbit primary immunoglobulins (Novocastra Laboratories, 2005).

1.8.2 Immunohistochemistry in the study and diagnosis of ND

Immunohistochemistry, as a tool of diagnosis have been used to study the pathogenesis and pathology of Newcastle disease in various avian species and tissues (Lockaby *et al*., 1993; Ojok & Brown, 1996; Brown *et al*., 1999; Rao *et al*., 2002; Al-Garib *et al*., 2003b, Oldoni *et al*., 2005; Wakamatsu *et al*., 2006; Piacenti *et al*., 2006). Generally regarded as an alternative to virus isolation or serology, IHC offers a rapid means of identifying various antigens, including viruses. The advantage of its use on formalin-fixed, paraffin-embedded tissues means diagnosis can be obtained even in cases where fresh sera or fresh tissues are unavailable (Lockaby *et al*., 1993). Lockaby and his colleagues (Lockaby *et al*., 1993) used immunoperoxidase histochemistry to develop an IHC method or staining technique for the diagnosis of ND in experimentally-infected SPF chickens

and commercial broilers presented for diagnostic evaluation of spontaneous respiratory disease. Using a monoclonal antibody to NDV and specific against the phosphoprotein (P), they demonstrated positive staining of tissues from both the NDV-inoculated and the commercial broilers. Positive staining was seen as cytoplasmic inclusions in the tracheal and bronchial epithelial cells. Positive staining were even demonstrated early in the infections when the SPF birds were still serologically negative as a period of 10 days was shown to be necessary for the development of a significant serum ELISA titre to NDV in experimentally infected chickens (Lockaby *et al*., 1993).

Investigating the relationship of viral antigen distribution to lesions (i.e. pathology and pathogenesis) using a mixture of mAbs in experimentally infected SPF cockerels, Ojok & Brown (1996), demonstrated the greatest amount of viral in the proventriculus, small intestine, spleen, thymus and eyelids with most of the immunohistochemical labelling confined to large mononuclear cells and occasionally in lymphocytes. They concluded that there is variability in organ predilection and antigen expression among strains of NDV, even among the most virulent.

Several other researchers using either mAbs (Rao *et al*., 2002; Oldoni *et al*., 2005; Wakamatsu *et al*., 2006; Piacenti *et al*., 2006), a cocktail of mAbs (Brown *et al*., 1999; Kommers *et al*., 2001), and green fluorescence protein (GFP) markers (Al-Garib *et al*., 2003b) looked at the distribution and the pathogenesis of various strains of NDV in various avian species and organs. Rao *et al* (2002) reported positive immunoperoxidase staining of both the vaccine viruses and the virulent field isolate in all the sections of the

oviduct with the uterus as being more susceptible than the other parts of the oviduct. Using *in situ* hybridization and IHC to study the pathogenesis of various NDV strains and recombinants in embryonated chicken eggs, extensive positive staining of viral antigens in mesenchymal cells throughout the chorioallantoic membrane (CAM) and the embryo of infected eggs was reported.

Wakamatsu and his colleagues (Wakamatsu *et al*., 2006) reported viral antigens predominantly in lymphoid tissues, respiratory tissues, heart, brain and the tissues of the gastrointestinal tract. Piacenti *et al* (2006) reported viral antigens in a variety of tissues with most of the staining signals restricted to the cytoplasm of epithelial cells, macrophage-type cells, lymphocytes, lymphoid aggregates and in glial cells and neurons of the cerebrum and brainstem. Kommers *et al* (2001) reported a very strong positive staining by IHC and ISH in all the affected lymphoid aggregates and organs of the infected birds. Using a cocktail of anti-NDV monoclonal antibodies, viral antigens were detected in the same tissues that were positive by ISH in chickens infected with velogenic viscerotropic NDV while Al-Garib *et al* (2003b) demonstrated viral antigen in epithelial cells of alveoli, macrophages and fibroblast and lymphocytes in other organs and myocardial cells of the heart.

Despite the amount of work done on the pathogenesis and immunohistochemical study of NDV in different tissues of poultry (Parede & Young, 1990; Lockaby *et al*., 1993; Ojok & Brown, 1996; Brown *et al*., 1999; Al-Garib *et al*., 2003b, Oldoni *et al*., 2005; Wakamatsu *et al*., 2006; Kapczynski & King, 2005; Piacenti *et al*., 2006; Miller *et al*.,

2007; Perozo *et al*., 2008), only two of these studies involved the reproductive tract of poultry (Biswal & Morrill, 1954; Rao *et al*., 2002). While Biswal & Morrill, (1954) looked at the pathology of the reproductive tract of laying pullets infected with ND, Rao *et al.* (2002) did an *in vitro* and *in vivo* evaluation of the virulence of Newcastle disease virus and vaccines for the chicken reproductive tract, using viral isolation and IHC studies in oviduct organ culture from precocious oviducts induced by oestrogen.

1.9 Problem statement and objectives of the study

The South African poultry industry is the most vibrant and viable in sub-Saharan Africa and continues to dominate the South Africa agricultural sector. As recorded by the Department of Agriculture, and quoted by SAPA (2008), turnover from the industry was R19.9 billion for the financial year of 31 March 2008. Poultry meat production was put at 1 077 million tons and egg production at 360 566 tons (SAPA, 2008).

Newcastle disease is probably the most important poultry disease in South Africa and remains as the major threat to the growth, sustainability as well as profitability of both the village and the commercial poultry industry. Recently (since 2002), outbreaks of ND in South Africa have been caused by a recently introduced strain of the virus (lineages 5d) known as “goose paramyxovirus” (GPMV). As the outbreak has progressed, the virus has proved to be more persistent than previous strains and is known to cause disease even in waterfowls which previous strains of NDV were not known to do (Abolnik, 2007).

The GPMV is in the group of the newly emerging virulent NDV strains that are of great concern as they may be able to overcome vaccination barriers (Panshin *et al*., 2002) making the control of the disease more difficult with vaccines appearing to be less effective in controlling the disease in the field. Most recently workers have reported that current vaccination programmes against ND are either ineffective or may not be optimized (Senne *et al*., 2004; Kapczynski & King, 2005; Miller *et al*., 2007). Others pointed out that antigenic differences between the infecting virus and the vaccine virus may influence the level of protection achieved by a vaccine (Liu *et al*., 2003; Czegledi *et al*., 2006; Miller *et al*., 2007). In South Africa, drops in egg production of between 30-40% are being reported even in apparently healthy and well vaccinated pullets that are feeding well with no cases of mortality following infection (Bisschop, personal communication). Sometimes mortalities were even reported in well-vaccinated flocks (Horner's personal communication reported in Abolnik, 2007). The mechanism by which the lineage 5d (which represents most of the emerging strains of NDV) overcomes the vaccination barrier is unknown. Probable causes for these observations or problems were:

1. Problems associated with poor vaccine application or other concomitant infections and stresses causing poor immune response to vaccine in the field. This is because the administration techniques of a vaccine may cause considerable variation in the individual antibody immune response and affect the level of protection of a vaccinated population (Senne *et al*., 2004). Several other factors that affect the efficacy of vaccines and vaccination in poultry have been reported (Hudson *et al*., 1974; McMullin, 1984; Pattison & Cook, 1996; Gallili & Ben-

Nathan, 1998) to include the vaccine itself, factors regarding vaccine delivery and other factors endogenous to the birds such genetic, age, immune status, concurrent diseases and the administration of immunosuppressive vaccines. Other factors which include stress factors (both social and environmental) imposed upon the vaccinated animal prior to or at the time of vaccination and the general level of management of the flock may also affect vaccination efficacy.

2. The GPMV strain may be more virulent than previous strains and therefore able to overwhelm the protection offered by the conventional vaccines. The GPMV has been reported to infect even waterfowl and other ornamental birds, and is a member of the newly emerging NDVs of the genotype VIId/5d (Liu, *et al*., 2003; Abolnik, 2007). It has an ICPI value of between 1.80 to 1.94 and a MDT of 45.6-60 hours. GPMV is also reported to have a sequence difference of 6 nt fragment in the intragenic region of HN and P and an additional anti-sense ORF in its gene which are reported to cause differences of RNA-editing efficiency of P gene and the expression of V protein, which may increase the virulence of the virus by inhibiting the activation of host interferon (reviewed in Zou, *et al*., 2005).

3. The virus may have undergone mutation over the years which enabled it to "avoid" the immune system of infected birds. Cases of viruses of low pathogenicity mutating to that of high pathogenicity have been reported (Alexander *et al*., 1997; Collins *et al*., 1998; Kirkland, 2000; Westbury, 2001). In addition records show that the newly emerging NDV strains which GPMV

represents (Liu, *et al*., 2007) are causing concern that they can overcome vaccination barrier (Yu, *et al*., 2001; Panshin, *et al*., 2002; Kapczynski & King, 2005).

In order to try and limit egg production losses, certain poultry producers in South Africa have resorted to cloacal application of live Newcastle disease vaccines before and during the laying period based on the assumption that superior immunity might be achieved in the oviduct through more direct application of the vaccine.

The need to assess the efficacy of some of the commercially available ND vaccines against GPMV in comparison to other NDV strains as well as the desire to determine if cloacal vaccination really offers a better protection against challenge in comparison to oronasal route (eye-drop vaccination) motivated this study. The study also sought and assessed the tropism and/or distribution of the NDV for the various sections of the oviduct of challenged hens considering the importance of the oviduct to egg production.

The aims and objectives of this study were to:

- Determine if any difference could be detected in the level of protection achieved by the use of Avinew ND vaccine against a recently isolated lineage 5d strain (GPMV) versus that achieved against a “classical” South Africa isolate lineage 3d (“Rainbow challenge virus”)

- Assess the pathogenesis of GPMV in birds with different immune status by evaluating the clinical signs, mortalities and macroscopic lesions produced by the infecting virus.

- Assess and determine whether intracloacal route of vaccination using La Sota ND vaccine offered better protection than ocular/spray routes of vaccination as being speculated in the field.

- Determine the pattern of tissue tropism of NDV for the reproductive tract of pullets following introduction into the host by vaccination and challenge.

CHAPTER 2. MATERIALS AND METHODS

2.1 Experiment 1 - Avinew vaccine trial study

This experiment was performed in a containment unit under BLS 2 + conditions at the research facilities of the Poultry Reference Centre of the Faculty of Veterinary Science, University of Pretoria. Approval for this research was obtained from the Animal Use and Care Committee (AUCC) of the University of Pretoria (*appendix 1a*).

2.1.1 Vaccine

Avinew® Newcastle disease vaccine used was a freeze-dried live vaccine against Newcastle disease produced by Merial of France. The vaccine contained live NDV, VG/GA strain, a lentogenic NDV strain. Each dose was found to contain $10^{6.5}$ EID_{50}/ml following titration of the vaccine.

2.1.2 Challenge viruses

The first Newcastle disease virus strain used as a challenge virus in this study was a velogenic NDV that was isolated from chicken tracheas, identified by the number PRC 171/06 with a mean death time (MDT) of 48 hours and intracerebral pathogenicity index (ICPI) of 1.85. It was identified by PCR and molecular sequencing as highly pathogenic and of the lineage 5d/VIId ("goose paramyxovirus" GPMV) (Genbank Ref. # FJ985978).

The second challenge virus was an NDV strain termed "Rainbow challenge virus" (RCV) and was isolated in 1993 by Rainbow Farms laboratory at Hammarsdale and identified by

molecular sequencing as belonging to a group of NDV unrelated to GPMV. It was of lineage 3d/VIII (Genbank Reference # FJ985977). It had an ICPI of 2.0 and MDT of 48 hours making it comparable to the Hertz strain (the standard challenge strain used in the UK) in terms of these pathogenicity parameters. Previous trial work, accepted for the registration purposes for Avinew vaccine indicated that the expected PD_{50} against Hertz was about $10^{4.0}$ EID_{50} (Vanmarcke, Johan - Personal communication). The virus challenge dose was 0.2ml, allowing for a total dose of $10^{5.3}$ EID_{50} per bird, for both the challenge strains used. The route of challenge was by intramuscular injection.

2.1.3 Experimental model, design and procedures

SPF White Leghorn chickens (n=126) were hatched and raised in isolation until 9-days of age. Birds were then individually identified with numbered wing tags after being randomly assigned into six treatment groups of 18 birds each and two control groups of nine birds each (Table 2.1). At 10-days of age, birds in the treatment groups were vaccinated with Avinew ND vaccine using 3 different doses viz: $10^{3.0}$ EID_{50} (groups 4 & 8), $10^{4.5}$ EID_{50} (groups 3 & 7) and $10^{6.0}$ EID_{50} (groups 2 & 6). The two control groups (1 & 5) were not vaccinated. At 27-days of age, all the chickens were challenged by intramuscular route with one of the two different NDV challenge strains at a dose of $10^{5.3}$ EID_{50}. GPMV was used to challenge groups 1, 2, 3 and 4 while groups 5, 6, 7 and 8 were challenged with RCV (Table 2.1). Birds were observed for 10 days post-challenge and chicks were scored at the two daily observations as either: 0 = normal, 1 = sick and 2 = dead. All the birds were watered and fed *ad-libitum*. All birds that died from the

challenge were necropsied and organs examined for the presence of gross lesions. All birds were individually weighed before vaccination as well as before challenge.

The challenge model used was a combination of the OIE protocols for testing the potency of ND live and ND inactivated oil vaccines (OIE, 2004) and had been used many times by the Poultry Reference Centre, University of Pretoria for evaluating the efficacy of vaccines. This particular challenge model was chosen to maximise the ability of the challenge trial to perceive subtle differences in the efficacy of Avinew vaccine against the different strains of Newcastle disease virus. The effects of the challenge on the different groups were assessed by evaluating clinical signs, mortality rates, gross pathology in organs and mortality scores.

Table 2.1 Newcastle Disease Vaccination and Challenge Schedule

Group No.	Chicks per group	Day 10- Vaccination	Day 27- Challenge
1	9 Chicks	NONE	GPMV
2	18 Chicks	$10^{6.0}$ EID_{50}	GPMV
3	18 Chicks	$10^{4.5}$ EID_{50}	GPMV
4	18 Chicks	$10^{3.0}$ EID_{50}	GPMV
5	9 Chicks	NONE	RAINBOW
6	18 Chicks	$10^{6.0}$ EID_{50}	RAINBOW
7	18 Chicks	$10^{4.5}$ EID_{50}	RAINBOW
8	18 Chicks	$10^{3.0}$ EID_{50}	RAINBOW

2.1.4 Data analysis

The clinical and mortality scores were analysed statistically to check for the level of significance of the protection achieved by each dose. Data on the weight of chickens at 14 days of age was also assessed to determine if any effect could be found related to body weight or the gender of the challenged birds. Statistical analysis of the data was carried out using multiple linear regression, adjusting for dose, sex and day 14 weights using Stat 10.0 (StataCorp, College Station, TX). The protective dose (PD_{50} and PD_{90}) were calculated using the method recommended by Reed and Muench (1938) for calculating the 50% end point of virus titration.

2.2 Experiment 2 - Immunohistochemical studies

2.2.1 Experimental design

Specific pathogen-free (SPF) White Leghorn and commercial Hyline Brown hens were procured and assigned separately into eight groups (four groups each for SPF and commercial hens) of 10 hens each per isolator. The birds were allowed to acclimatize for two days after which they were vaccinated with NEW VAC-LS® Newcastle disease vaccine (B1 type, LaSota strain, live virus – Forte Dodge®, Brazil; FD6033A; Batch No: 002/07; Expiry: May/2009) at the manufacturer's recommended dose. Birds in groups 1, 3, 5 and 7 were vaccinated via the cloacal route while birds in groups 2, 4, 6 and 8 were vaccinated via eyedrop (Table 2.2). Vaccine was diluted using the commercial eye-drop diluent supplied by the manufacturer. Birds were then monitored daily and one bird from

each group was euthanased days 2, 4, 6, 8 and 10 post-vaccination (PV) as outlined in Table 2.2.

On day-14 post-placement in the isolators and day 12 PV, the remaining birds were challenged via eye-drop with the same GPMV challenge virus used in Experiment 1. The eye-drop route of challenge was preferred to intramuscular injection, to reduce the severity of the effect of the challenge on the birds and to avoid peracute deaths. A bird from each group was euthanazed on days 2, 4, 6, 8 and 10 post-challenge (PC) (Table 2.3).

Tissues were collected in 10% buffered formalin at necropsy and processed for routine haematoxylin and eosin (H&E) staining as well as immunohistochemical staining (IHC) for ND viral antigen. H&E and IHC-stained slides were then examined using a light microscope for lesions and viral staining respectively in order to determine the severity of lesions and viral cell tropism in the different tissues of the oviduct.

Table 2.2 Newcastle disease Vaccination and Challenge Schedule of Hens

	Day 0		Day 2		Day 4		Day 6		Day 8		Day 10		Day 12		Day 14	
Iso.	**No**	**Tmt**	**No**	**Vac**	**Tmt**	**No**	**Tmt**	**No**	**Tmt**	**No**	**Tmt**	**No**	**Tmt**	**No**	**Tmt**	**No**
1	10 SPF	Place Birds	10	CV	Rem. 1	9	Rem. 1	8	Rem. 1	7	Rem. 1	6	Rem. 1	5	Chall.	5
2	10 SPF	Place Birds	10	EV	Rem. 1	9	Rem. 1	8	Rem. 1	7	Rem. 1	6	Rem. 1	5	Chall.	5
3	10 SPF	Place Birds	10	CV	Rem. 1	9	Rem. 1	8	Rem. 1	7	Rem. 1	6	Rem. 1	5	Chall.	5
4	10 SPF	Place Birds	10	EV	Rem. 1	9	Rem. 1	8	Rem. 1	7	Rem. 1	6	Rem. 1	5	Chall.	5
5	10 COM	Place Birds	10	CV	Rem. 1	9	Rem. 1	8	Rem. 1	7	Rem. 1	6	Rem. 1	5	Chall.	5
6	10 COM	Place Birds	10	EV	Rem. 1	9	Rem. 1	8	Rem. 1	7	Rem. 1	6	Rem. 1	5	Chall.	5
7	10 COM	Place Birds	10	CV	Rem. 1	9	Rem. 1	8	Rem. 1	7	Rem. 1	6	Rem. 1	5	Chall.	5
8	10 COM	Place birds	10	EV	Rem. 1	9	Rem. 1	8	Rem. 1	7	Rem. 1	6	Rem. 1	5	Chall.	5

Key: *SPF - Specific Pathogen-free hens; COM– Commercial hens; Tmt– Treatment; No. – Number of hens/group; Vac. – Vaccination; CV – Cloacal vaccination; EV – Eyedrop vaccination; Rem. – Removed; Chall. - Challenged*

Table 2.3 Newcastle disease Challenge Schedule of hens (follows on from Table 2.2)

Day 14			Day 16		Day 18		Day 20		Day 22		Day 24	
Iso.	**No.**	**Tmt**	**Tmt**	**No.**	**Tmt**	**No.**	**Tmt**	**No.**	**Tmt**	**No.**	**Tmt**	**No.**
1	5 SPF	Chall.	Remove 1	4	Remove 1	3	Remove 1	2	Remove 1	1	Remove 1	0
2	5 SPF	Chall.	Remove1	4	Remove 1	3	Remove 1	2	Remove 1	1	Remove 1	0
3	5 SPF	Chall.	Remove1	4	Remove 1	3	Remove 1	2	Remove 1	1	Remove 1	0
4	5 SPF	Chall.	Remove1	4	Remove 1	3	Remove 1	2	Remove 1	1	Remove 1	0
5	5 COM	Chall.	Remove1	4	Remove 1	3	Remove 1	2	Remove 1	1	Remove 1	0
6	5 COM	Chall.	Remove1	4	Remove 1	3	Remove 1	2	Remove 1	1	Remove 1	0
7	5 COM	Chall.	Remove1	4	Remove 1	3	Remove 1	2	Remove 1	1	Remove 1	0
8	5 COM	Chall.	Remove1	4	Remove 1	3	Remove 1	2	Remove 1	1	Remove 1	0

Key: *SPF - Specific Pathogen-free hens; COM – Commercial hens; Tmt – Treatment; No. – Number of hens/group; Chall. - Challenged*

2.2.2 Positive and negative control birds

Ten White Leghorn SPF birds were used as the positive control. The positive control birds were not vaccinated but challenged with the same GPMV at the same dose and by the same route. Oviduct tissues were sampled on days 2PC and 6PC after euthanasia and on days 3, 4 and 5 post-challenge following deaths from NDV.

Two SPF birds were kept as negative control birds. They were neither vaccinated nor challenged. Both birds were euthanased with the last post-vaccination euthanasia and the oviduct was sampled from both birds.

2.2.3 Virus challenge, dose and inoculation route

The challenge virus used was a local velogenic Newcastle disease virus (NDV) strain with a Mean Death Time (MDT) of 48hrs and Intracerebral Pathogenicity Index (ICPI) of 1.85. Based on Polymerase chain reaction (PCR) and molecular sequencing, it was classified as highly pathogenic and of the genotype 5d/VIId (GPMV; Genbank Ref. # FJ985978). The virus challenge dose was $10^{7.0}$ EID_{50}/0.1ml/bird. Birds were challenged by eye-drop as against the intramuscular injection used in the first trial. This is because the oronasal route is possibly the most common route of infection. The dose was increased, one because of the route used for infecting the birds, and secondly because the birds were matured laying hens that are less susceptible than younger chickens.

2.2.4 Bleeding and identification of birds

Birds were assigned randomly to treatment groups. Both the SPF and the commercial birds (n=10 each) were randomly bled on arrival to check their Newcastle disease (ND) antibody status. Following vaccination and before challenge, 10 birds each from both the SPF and the commercial hens were bled to assess the level of sero-conversion to the vaccination. All birds to be euthanazed were bled before euthanasia. Antibody titres to ND were determined using a commercial NDV ELISA Kit (Newcastle Disease Virus Antibody Test Kit - FlockChek*; IDEXX Laboratories Inc, Maine, USA). Results were presented as titre groups which range from 1 to 18 and directly correspond to titre values of 397 to 32,000. The titre groups used with their corresponding titre values are: 1 (397), 2(1000), 3(2000), 4(3000), 5(4000), 6(5000), 7(6000), 8(8000), 9(10,000), 10(12,000), 11(14,000), 12(16,000), 13(18,000), 14(20,000), 15(22,000), 16(24,000), 17(28,000) and 18(32,000). The titre groups were automatically calculated based on the formula below:

Calculations

1. Negative Control mean (NCx) $\frac{\text{Well A1 A(650) + Well A2 A(650)}}{2} = \text{NCx}$

2. Positive Control mean (PCx) $\frac{\text{Well A3 A(650) + Well A4 A(650)}}{2} = \text{PCx}$

3. Sample to Positive (S/P) Ratio $\frac{\text{Sample Mean – NCx}}{\text{PCx – NCx}} = \text{S/P}$

4. Titre – Relates S/P at a 1:500 dilution to an endpoint titre:

$$\textbf{Log}_{10}\textbf{Titre} = \textbf{1.09 (Log}_{10}\textbf{ S/P) + 3.36}$$

Serum samples with S/P ratio of:

≤ 0.20 = Negative, and
> 0.20 (titres greater than 396) = Positive

A(650) = Measure and record absorbance values at 650nm (IDEXX Laboratories, 2007).

2.2.5 Clinical observations and euthanasia

All birds were observed twice daily throughout the trial, at eight hours interval. One bird from each of the eight groups were humanely euthanazed on days 2, 4, 6, 8 and 10 post-vaccination (PV) and post-challenge (PC) (Table 2.2 and Table 2.3) and the various parts of the oviduct sampled. Birds were monitored according to the Poultry Reference Centre's Standard Operating Procedure (PAS/PRC/035) (*Appendix 2a).*

Euthanasia was done by asphyxiation with carbon dioxide (CO_2) according to the Poultry Reference Centre's Standard Operating Procedure (SOP 0104) which was previously approved by the Animal Use and Care Committee (AUCC) (*Appendix 2b).*

2.2.6 Necropsy techniques and tissue sampling

All the euthanazed birds were necropsied immediately after euthanasia and the oviducts sampled aseptically. Birds were opened according to routine post-mortem procedure and the cut heel and chest reflected toward the head to expose the abdominal content. The abdominal viscera were removed to expose the reproductive organs, the different sections of the oviduct were identified according to a figure in North and Bell (1990) and the various portions of the oviduct (magnum, isthmus and uterus) were collected in pre-labelled containers containing buffered 10% formalin identifying the bird, method of vaccination, date euthanazed and the tissue. Samples were identified as follows:

- CV (1, 3, 5, and 7) = Cloacal vaccination (isolators 1, 3, 5, and 7)
- EV (2, 4, 6, and 8) = Eye (ocular) vaccination (isolators 2, 4, 6, and 8)

- Date of euthanasia were identified as: 2, 4, 6, 8 and 10 PV (post-vaccination) and 2, 4, 6, 8, and 10 PC (post-challenge) and organs are identified as uterus, magnum and isthmus.

One to two centimetres (1-2cm) of each tissue were section and stored in the pre-labelled container containing formalin for further processing.

2.2.7 Tissue processing for H & E and IHC

Tissue samples were fixed by immersion in 10% neutral buffered formalin for 24 hours. At the pathology laboratory sampled were further identified and given a laboratory number as either CV (cloacal vaccination) or EV (eye vaccination), sample number (S) e.g. S1947 -08; EV4 S1992-08. Tissues were trimmed and embedded in paraffin wax. Sections were cut at 4 µm and 3µm for H&E and IHC respectively.

2.2.8 Immunohistochemistry

Paraffin-embedded tissue sections were mounted on positively charged Superfrost® Plus glass slides, dried overnight in a 58^{o}C oven, and then stained using a standard IHC protocol (California Animal Health & Food Safety uncontrolled protocol, Paramyxovirus Type-1 IPX Stain #DHIS-02-716 of July 17, 2008; by Mike Manzer) (*Appendix 3)*. Some minor modifications were incorporated into the protocol in order to suit the standard operating procedure (SOP) of the IHC laboratory, Pathology Section, University of Pretoria.

Step1. Tissue deparaffinization, rehydration and quenching of endogenous peroxidases

Tissues were deparaffinised through immersion of the slides for five minutes each in Xylol Solution 1 and 2, followed by rehydration through immersion of the slides for three minutes each in graded alcohols of 100%, 96% and 70%. Slides were subsequently immersed in 3% Hydrogen Peroxide in methanol for 15 minutes to neutralize endogenous peroxidase activity (Vander Lugt *et al*., 1995). Slides were then rinsed four times with distilled water.

Step 2. Heat-Induced Epitope Retrieval (HIER)

Slides were microwave-heated in a container with 350mls of 1X Dako Target Retrieval Solution (10X concentration product #S1699 – *see appendix 4 for reconstitution*) with 3.5mg Protease, Type XIV (Sigma product #P5147-5G) for 40 minutes at 96^{o}C. Slides were then allowed to cool to room temperature, rinsed four times in distilled water and then immersed in a mixture of 0.1Molar (pH 7.6) Phosphate Buffered Saline (PBS), containing 0.1% Bovine Serum Albumin (BSA) Fraction V (Ref: 10 735 094 001, Roche Diagnostic GmbH, Germany) for five minutes. *See appendix 5 for preparation*.

Step 3. Protein Block

This was done in order to reduce non-specific binding of primary antibody and polymer. NovoLink™ Protein Block (# RE7102) from Novocastra Laboratories Ltd was applied to sections for five minutes in a humidified reaction chamber. Slides were rinsed in distilled water and incubated/immersed in PBS/BSA buffer for five minutes in a staining cup.

Step 4. Milk Powder blocking

A 2% milk powder solution was prepared (Elite Fat-Free Instant Milk Powder, Clover S.A Pty, South Africa, H2038-3-2-500G; *see appendix 6 for preparation*), filtered and kept refrigerated. It was used as a general tissue block and also as a diluent for the primary antibody. After decanting the PBS/BSA buffer, the cup was filled with the 2% milk powder solution for five minutes. Slides were subsequently covered with the 2% milk powder solution for 30 minutes in a humidified chamber (in order to block all ongoing reactions). The humidified chamber minimized moisture loss and thus prevented the slides from drying out.

Step 5. Primary Antibody

A monoclonal antibody to NDV (Anti-NDV P pure ascites 10-5E6, 1985), raised in mice, specific for the phosphoprotein (P) genome of NDV, and obtained from Mark Peeples of Rush-Presbyterian-St. Luke's Medical Centre Chicago, III, USA was used. The NDV mAb was diluted in the 2% milk powder solution to produce a working dilution of 1:500 (30µl of the Anti-NDV mAb in 15mls 2% milk powder solution). The mAb was reconstituted for daily use.

Without washing the slides, the excess milk solution was shaken off and the perimeter of the tissues dabbed with soft toilet paper (making sure not to touch the tissue sections). All slides were then flooded with the reconstituted primary antibody ensuring that the entire sections on each slide were fully covered with the antibody. Slides were incubated in

humidified chambers for one hour, followed by rinsing in distilled water and immersion in 2% milk solution for 10 minutes in a reaction cup.

Reagent (antibody) Control

For antibody control purposes, the NDV mAb was replaced with Wesselsbron mAb. In each of the staining batch of 40 slides per day, two positive control slides were stained with NDV mAb and two slides with the negative control reagent (keeping all other staining variables constant including the concentration and incubation time of the NDV mAb). This was done in order to better assess the specificity of the NDV mAb.

Step 6. Post-Primary Block

Slides were incubated with the Post-Primary Block (# RE7111) in the reaction chamber for one hour, after which slides were rinsed four times in distilled water followed by immersion in 2% milk solution for 10 minutes.

Step 7. NovoLink™ Polymer Detection System Kit

Excess milk solution was shaken off; the slides dabbed and incubated in NovoLink™ Polymer solution (# RE7112) for one hour in a humidified chamber. Slides were subsequently rinsed four times in distilled water and immersed in 2% milk solution for 10 minutes. Slides were then rinsed in distilled water four times and kept in PBS/BSA buffer for five minutes and stained with the NovaRed® substrate solution.

A NovoLink™ Polymer Detection System Kit (RE7150-K;) from Novocastra Laboratories Limited, Balliol Business Park, Benton Lane, Newcastle upon Tyne, UK, supplied by Southern Cross Biotechnology (PTY) Ltd, Cape Town was used for the IHC staining procedure.

Step 8. Substrate development

Slides were incubated with the Vector Nova Red Substrate Kit (SK 4800; Vector Laboratories) for 1-2 minutes until suitable staining developed. This was based on examination of the positive-tissue control sections (using the same substrate) at 100X magnification under light microscope in the IHC laboratory. Vector® Nova Red® produces a red reaction product. The Nova Red® substrate solution was reconstituted according to the manufacturer's instructions. Slides were then washed four times in distilled water.

Step 9. Haematoxylin counterstaining

Slides were stained lightly in haematoxylin for about 30 seconds after which they were washed in running tap water for approximately 10 minutes until the desired colour was obtained.

Step 10. Dehydration and mounting

Slides were routinely dehydrated through increasing alcohol concentration (70%, 96% and 100%), after which sections were kept in Xylol solution until mounted. Balsam oil premount was applied and slides were coverslipped for permanent record.

2.2.9 Nature of positive staining, target cells in the oviduct and scoring system for positive and negative immunostaining

Stained slides were examined at 400X magnification for the presence of a) clustered granular/stippled positive staining in the epithelial cells of the oviduct and occasional monocyte-macrophages and lymphocytes within the connective tissue stroma and /or b) extracellular foci/clusters of positive staining (that were not clearly cell-associated). In examining the slides, the whole section was examined and one "hot spot" defined as the field with most immunostaining in a section, was counted and graded according to Brown *et al* (1999), as follows:

- NA = tissue or slide not available
- Negative (-) = when no positive staining was seen.
- Positive (+) = when 1 positively-staining cell or focal extracellular cluster of positive staining was seen in the selected 400X high power field.
- Positive (++) = when 2≤5 positively-staining cells or focal extracellular clusters of positive staining were seen per selected 400X high power field.
- Positive (+++) = when 6 or more positively-staining cells or focal extracellular clusters of positive staining were seen per selected 400X high power field.

2.2.10 Statistical analysis

The graded results (scores) of the stained tissue slides of either -, +, ++, or +++ were replaced with figures for the purpose of statistical analysis as follows:

- Tissue not available = NA
- Negative staining (-) = 0

- Positive staining (+) = 1
- Positive staining (++) = 2
- Positive staining (+++) = 3.

The scores were imported into Excel Spreadsheets and subjected to simple statistical manipulations to produce barcharts and line graphs for interpretation.

CHAPTER 3: RESULTS

3.1 Experiment 1 – The Avinew vaccine trial

3.1.1 Clinical signs and mortality

Birds appeared clinically normal for the first 48 hours post-challenge in all the treatment groups, except in the Rainbow virus-challenged control group (group 5). In this group clinical signs of ruffled feathers and depression were observed on day 2 post challenge (PC). Most of the other groups started showing clinical signs on day 3 PC. The time of onset of clinical signs for all the groups, the number of birds that developed clinical signs and the number of mortalities are presented in Table 3.1. Some of the sick birds progressed to complete depression, passage of greenish watery diarrhoea, sternal recumbency with drooling salivation, complete paralysis and then death. By the evening observation on day 3 PC, there were three, four and one death in group 1 (goose paramyxovirus - GPMV challenged control), group 4 (lowest vaccine dose treatment group challenged with GPMV) and group 8 (lowest vaccine dose treatment group challenged with RCV), respectively, while on day 4 PC there were six, one, eight, nine, and 15 deaths in groups 1, 3, 4, 5, and 8 respectively (data not shown). Both control groups had 100% (n=18) mortality by day 4 PC. On day 5 PC, group 4 had one death while the remaining two chickens in group 8 died resulting in 100% (n=18) mortality for group 8 as well. Group 7 ($10^{4.5}$ EID_{50} vaccine dose challenged with RCV) had its first and only death on day 7 PC, which was also the end of mortalities until day 10 PC, when all the surviving birds were humanely euthanazed and the experiment terminated. The summary of mortalities of the test and the control birds is presented graphically in Fig. 3.1.

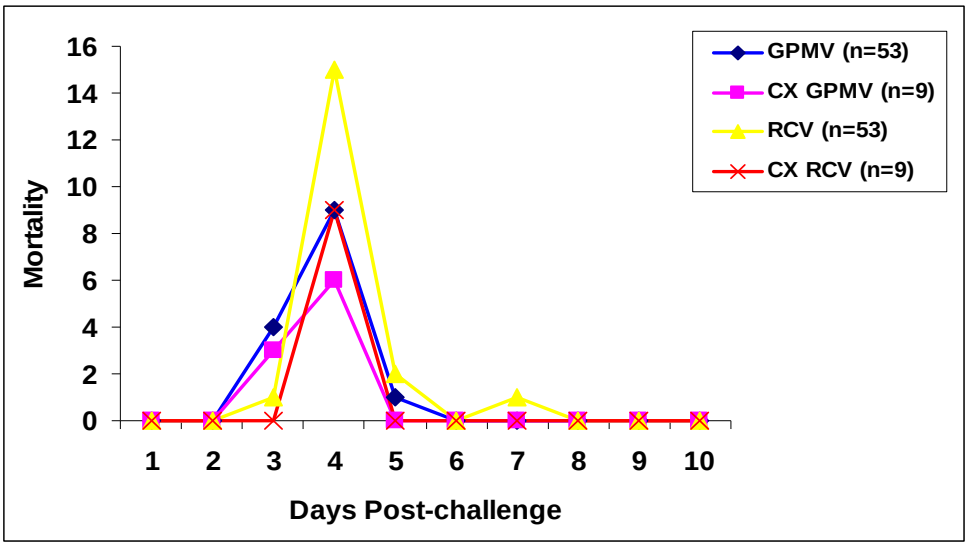


Fig. 3.1 Graph showing the daily mortality figures for all the treatment groups after challenge.

GPMV = Goose paramyxovirus (combined treatment groups); CX GPMV = Control group challenged with GPMV; RCV = Rainbow challenge virus (combined treatment groups); CX RCV = Control birds/group challenged with RCV.

Valid results were obtained from 124 of the 126 chickens originally used for the trial. Only two deaths were encountered from causes not related to the trial and these were therefore excluded from the study. During the challenge trial, 51(41.13%) chickens died while 73(58.87%) chickens survived the challenge (i.e. they were still alive up to day 10 PC when the trial was terminated, but with some of the birds showing clinical signs). The $10^{3.0}$ EID_{50} treatment groups (groups 4 and 8) had 4 birds (11.11%) surviving without any clinical signs while the $10^{4.5}$ EID_{50} (groups 3 and 7) and $10^{6.0}$ EID_{50} (groups 2 and 6) treatment groups had 34 birds (88.89%) and 34 birds (91.12%) surviving, respectively without clinical signs (Table 3.1).

Table 3.1 Clinical disease and occurrence of mortality in SPF chickens vaccinated with varied doses of Avinew Newcastle disease vaccine and challenged intramuscularly with GPMV and RCV strains of Newcastle disease virus.

Groups	Vaccine Dose	Challenge Virus	Clin. Signs (first evident)	Number sick/Total[A]	Number dead/Total[A]
Group 1	None	GPMV	3 DPC	9/9	9/9
Group 2	$10^{6.0}$ EID_{50}	GPMV	3 DPC	1/17	0/17[B]
Group 3	$10^{4.5}$ EID_{50}	GPMV	3 DPC	2/18	1/18
Group 4	$10^{3.0}$ EID_{50}	GPMV	3 DPC	14/18	13/18
Group 5	None	RCV	2 DPC	9/9	9/9
Group 6	$10^{6.0}$ EID_{50}	RCV	7 DPC	2/17	0/17[B]
Group 7	$10^{4.5}$ EID_{50}	RCV	7 DPC	2/18	1/18
Group 8	$10^{3.0}$ EID_{50}	RCV	3 DPC	18/18	18/18

Total[A] = the number of 4-wk-old chicks per group/isolator that were used for the trial. The figures under the number sick/Total[A] and number dead/Total[A] refer to the number of birds that fell sick and died respectively during the course of the whole trial (i.e. up to 10-days post-challenge when the trial was terminated).

[B] These groups had 17 chickens each instead of the 18 chickens originally placed as a result of the death of one chicken from each group due to causes not related to the challenge.

DPC = Days post-challenge; EID_{50} = Embryo infective dose (50%); GPMV = Goose paramyxovirus.

Table 3.2 presents the average of the daily clinical and mortality scores (0 = normal, 1 = sick and 2 = dead) for all the birds. The control groups had an average score of above 1.5 while the treatment groups that received the highest dose of vaccine ($10^{6.0}$ EID_{50}) and challenged with GPMV and Rainbow virus had average scores of 0.047 and 0.011 respectively. The other treatment groups fell between the highest score of 1.517 and lowest score of 0.011. The average scores were plotted against the different vaccine doses into a line graph as presented in Fig. 3.2. The higher average scores as shown in both

Table 3.2 and Fig. 3.2 indicate little or no protection at all while lower scores indicate better protection and fewer clinical signs with low or no mortalities.

Table 3.2 Treatment groups and their computed average clinical scores, post-challenge

Tmt (Vac.)	Groups - Challenge Virus (Average Scores)	
Control	Group **1**-GPMV (1.517)	Group **5**-RCV(1.517)
$10^{3.0}$ EID_{50}	Group **4**-GPMV (1.103)	Group **8**-RCV(1.478)
$10^{4.5}$ EID_{50}	Group **3**-GPMV (0.122)	Group **7**-RCV (0.044)
$10^{6.0}$ EID_{50}	Group **2**-GPMV (0.047)	Group **6**-RCV (0.011)

Average scores were calculated from the twice daily scorings for 10 days post challenge for individual birds in each group.

Tmt (Vac.) = Treatment (vaccination); GPMV = Goose paramyxovirus; RCV = Rainbow challenge virus

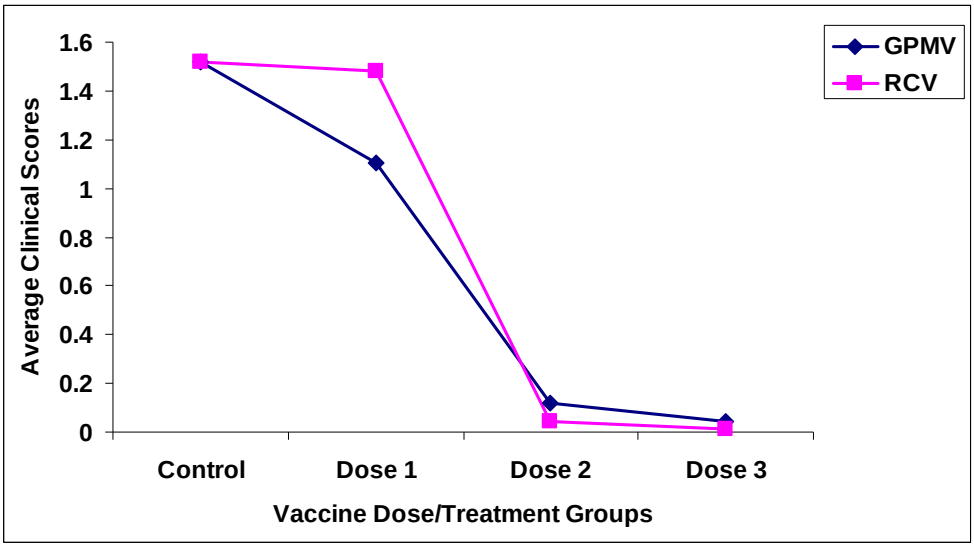


Fig. 3.2 Averages of the clinical scores of 4-wk-old chickens vaccinated and challenged with Goose paramyxovirus (GPMV) and Rainbow challenge virus (RCV).
The average scores were plotted against the control as well as the different doses of vaccines administered to the different treatment groups. Dose 1 = $10^{3.0}EID_{50;}$ Dose 2 = $10^{4.5}EID_{50;}$ Dose 3 = $10^{6.0}EID_{50}$

3.1.2 Gross Pathology

All the birds used in this trial were necropsied after death or after euthanasia, either during or at the termination of the trial. All the birds in the two control groups had variable macropathological lesions in the trachea, spleen, intestine, caecal tonsils, proventriculus and heart.

Gross pathology included haemorrhage and congestion of the trachea, necrohaemorrhagic foci in the caecal tonsils and proventriculi. Some of the control birds had haemorrhagic enteritis and pin-point haemorrhages on the serosal surface of the pericardium. The most severe macrocoscopic lesions were observed in the caecal tonsils and proventriculi and these particular lesions were consistent in all the birds in both control groups. Chickens in groups 4 and 8, which received $10^{3.0}$ EID_{50} doses of vaccine, had gross lesions similar to those of the control birds. Most of these birds had obvious haemorrhage and foci of necrosis in the caecal tonsils and proventriculi, with only a few birds having additional aforementioned lesions in the tracheas and intestines. Two birds among those euthanized at the termination of the study from group 4 had no macroscopically-obvious lesions. Despite the fact that the birds in groups 2 (n=16), 3 (n=16), 4 (n=4), 6 (n=15), and 7 (n=16) appeared “healthy”, 15 of these birds in group 3, and eight in group 8 had necro-haemorrhagic lesions in the caecal tonsils. Only three birds in group 3, and nine birds in group 7, had no visible lesions. Twelve birds from groups 2 and 6 respectively had haemorrhages in the caecal tonsils while five birds from groups 2, 3, 4, 6, 7, and 8 had no visible gross pathology.

Using the mathematical technique devised by Reed and Muench (1938) for determining the 50% end-point of virus titration, the 50% and 90% protective dose (PD_{50} and PD_{90}) for Avinew vaccine against challenge with GPMV and RCV was calculated. The PD_{50} and PD_{90} were $10^{3.51}$ and $10^{4.38}$ for GPMV and $10^{3.79}$ and $10^{4.43}$ for Rainbow virus, respectively. The PD_{50} and PD_{90} are measurements of the concentration of the test vaccine required to protect 50% and 90%, respectively of the test population from challenge with the respective viruses. The higher PD values for the Rainbow virus means that a higher concentration of the trial vaccine is needed to protect the trial birds against the viral challenge.

3.2. Experiment 2 – IHC study of the distribution of NDV in the oviduct of hens

3.2.1 Clinical Signs and mortality in the vaccinated and challenged hens

All vaccinated birds appeared healthy post-vaccination. However, there was a transitory drop in egg production from 31 eggs (38.6%) before vaccination to 14 eggs (17.5%) one day post vaccination (day 1 PV). Production then increased to 25 eggs (34.72%) on day 3 PV and 25 eggs (39.06%) on day 5 PV (Table 3.3). The drop in production was observed in both the commercial and the SPF birds, but was more marked on the SPF than the commercial birds.

Table 3.3 Days of euthanasia and the daily egg production of birds vaccinated with La Sota ND vaccine and challenged with NDV

DAY POST-EXPOSURE	SPF HENS	COMM HENS	CONTROL HENS (SPF)	
	% Eggs Laid (n= No. of birds)	*% Eggs Laid (n= No. of birds)*	*% Eggs Laid (n= No. of birds)*	*Mortality (n= No. of birds)*
Placed Day 1	20.00% (n= 40)	30.00% (n= 40)	50.00% (n= 10)	0
Placed Day 2	25.00% (n= 40)	35.00% (n= 40)	50.00% (n= 10)	0
Vaccination	***La Sota ND vac.***	***La Sota ND vac.***	***Not vaccinated***	-
Day 0	27.50% (n= 40)	50.00% (n= 40)	70.00% (n= 10)	0
Day 1PV	15.00% (n= 40)	20.00% (n=40)	60.00% (n= 10)	0
Day 2PV*	15.00% (n= 40)	32.50% (n= 40)	80.00% (n= 10)	0
Day 3PV	19.44% (n= 36)	50.00% (n= 36)	90.00% (n= 10)	0
Day 4PV*	19.44% (n= 36)	61.11% (n= 36)	80.00% (n= 10)	0
Day 5PV	18.8% (n= 32)	59.40% (n= 32)	90.00% (n= 10)	0
Day 6PV*	9.38% (n= 32)	40.63% (n= 32)	70.00% (n= 10)	0
Day 7PV	14.30% (n= 28)	50.00% (n= 28)	80.00% (n= 10)	0
Day 8PV*	14.29% (n= 28)	39.29% (n= 28)	90.00% (n= 10)	0
Day 9PV	8.30% (n= 24)	50.00% (n= 24)	90.00% (n= 10)	0
Day 10PV*	12.50% (n= 24)	50.00% (n= 24)	90.00% (n= 10)	0
Challenged (Day 12PV)	***Challenged*** 20.00% (n= 20)	***Challenged*** 70.00% (n= 20)	***Challenged*** 70.00% (n= 10)	- 0
Day 1PC	20.00% (n= 20)	60.00% (n= 20)	50.00% (n= 10)	0
Day 2PC*	15.00% (n= 20)	40.00% (n= 20)	50.00% (n= 8)	2a
Day 3PC	31.30% (n= 16)	100.0% (n= 16)	42.86% (n= 7)	1b
Day 4PC*	6.25% (n= 16)	75.00% (n= 16)	28.57% (n= 7)	5b
Day 5PC	25.00% (n= 12)	75.00% (n=12)	0.00% (n= 3)	1b
Day 6PC*	33.33% (n= 12)	58.33% (n= 12)	0.00% (n= 0)	1b
Day 7PC	37.50% (n= 8)	75.00% (n= 8)	0.00% (n= 0)	0
Day 8PC*	25.00% (n= 8)	75.00% (n= 8)	0.00% (n= 0)	0
Day 9PC	0.00% (n= 4)	25.00% (n= 4)	0.00% (n= 0)	0
Day10PC*	0.00% (n= 4)	0.00% (n= 4)	0.00% (n= 0)	0
TOTAL (eggs = 505)	**20.99%**	**55.25%**	**23.76%**	**10 (n= 10)**

PV = Post-vaccination; PC = Post-challenge; SPF = Specific Pathogen-free hens; COMM = Commercial hens; * = Days that 4 hens were euthanized/group (i.e. one hen per treatment group, giving a total of 8; 4 SPF and 4 commercial hens a day), control birds were euthanized only on days 10PV and 2PC while all the remaining control birds died from the challenge before they could be euthanized; n = number of birds per group; [a] = Control birds euthanized humanely according to the experimental design; [b] = Control birds that died due to the challenge before they could be euthanized.

Following challenge, all birds appeared clinically normal until day 3 PC, when two SPF birds in two of the groups vaccinated via the cloacal route (isolator 1 and 3) had ruffled feathers. One of these birds (isolator 3) died on day 4 PC from causes not associated with the trial, just before bleeding and euthanasia. The bird was removed from the trial. All the remaining birds appeared healthy for the entire study period. There were no deaths from ND-related causes. Egg production of the birds dropped from 18 eggs (45%) to 16 eggs (40%) on day 1 PC and increased again to 17 eggs (53.13%) on day 3 PC. Egg production finally plummeted to 1 egg (12.5%) on day 9 PC. Of the total 505 eggs produced by all the birds during the study, 106 eggs (20.99%), 279 eggs (55.25%) and 120 (23.76%) were produced by the SPF, the commercial and the control birds, respectively (Table 3.3).

The positive control birds (unvaccinated SPF but challenged with the same virus and dose and via the same route as the trial birds) started showing ND-related clinical signs on day 1 PC. Four of the birds started passing greenish faeces on day 1 PC, even though they appeared clinically normal. Two hens were euthanased according to the research design while the remaining eight (100%) control birds appeared depressed and sleepy at the evening observation on day 2 PC. Birds were also anorexic. By the evening observation on day 3 PC, one control bird was found dead. Five more birds died on day 4 PC, one on day 5 PC, and by the morning of day 6 PC, all the positive control birds were dead from viral challenge-associated causes. Only the two birds euthanized on day 2 PC, died according to the study protocol. Pre-challenge egg production of 9 eggs (90%) dropped to 7 eggs (70%) and 5 eggs (50%) on days 1 and 2 PC, respectively (Table 3.3).

From day 2 PC to day 6 PC, when the last bird was euthanized, 7 (18.42%) of the total 38 eggs laid were either soft-shelled or shell-less (data not shown). The two negative control birds (neither vaccinated nor challenged) did not manifest any signs of disease throughout the trial period and were laying at 100% (2 eggs per day) until they were euthanized on day 10PV (data not shown). The negative control birds did not lay any soft-shelled or shell-less eggs.

3.2.2 Serology (ELISA)

Pre-vaccination serology (ELISA) confirmed the SPF status of all the White Leghorn SPF birds used in this study. All 10 of the SPF birds that were bled tested negative for NDV antibodies. The 12 commercial Hyline Brown hens had NDV antibody titres expressed as titre groups of between 2^5 and 2^{17} on arrival (*See appendix 7 for result as provided by the serology laboratory*). The post-vaccination NDV antibody titres of all the birds bled and subsequently euthanized, expressed as a titre group are presented in Table 3.4.

Post-vaccination titres of the SPF birds that were bled on each of the euthanasia days remained at zero (0) for the first six days PV, with the exception of EV2 S1965-08 that had a titre of 2^1on day 6 PV. On day 8 PV, SPF birds CV1 S1987-08 and EV4 S1992-08 had an antibody titre of 2^1, while bird EV2 S1991-08 had a titre of 2^2. By day 10 PV, the euthanized SPF birds had titres of 2^5 (CV1 S1995-08); 2^7 (CV3 S1996-08) and 2^{12} (EV2 S2000-08 and EV4 S2001-08), signifying seroconversion to the vaccination.

Table 3.4 Table showing post-vaccination NDV antibody titres of SPF and commercial hens vaccinated by cloacal and eye-drop route with La Sota live NDV vaccine and expressed as a titre group (assayed by ELISA)

Day Euth.	COMMERCIAL HENS			SPF HENS		
	Bird No.	*Vac.*	*Titres (Log 2)*	*Bird No.*	*Vac.*	*Titres (Log 2)*
2PV	CV5 S1947-08	CV	10	CV1 S1946-08	CV	0
2PV	CV7 S1948-08	CV	16	EV2 S1949-08	EV	0
2PV	EV6 S1951-08	EV	17	EV4 S1950-08	EV	0
4PV	CV5 S1953-08	CV	9	CV1 S1952-08	CV	0
4PV	CV7 S1955-08	CV	18	CV3 S1954-08	CV	0
4PV	EV6 S1958-08	EV	7	EV2 S1956-08	EV	0
4PV	EV8 S1959-08	EV	17	EV4 S1957-08	EV	0
6PV	CV5 S1963-08	CV	16	CV1 S1961-08	CV	0
6PV	CV7 S1964-08	CV	14	CV3 S1962-08	CV	0
6PV	EV6 S1967-08	EV	16	EV2 S1965-08	EV	1
6PV	EV8 S1968-08	EV	12	EV4 S1966-08	EV	0
8PV	CV5 S1989-08	CV	16	CV1 S1987-08	CV	1
8PV	CV7 S1990-08	CV	18	CV3 S1988-08	CV	0
8PV	EV6 S1993-08	EV	14	EV2 S1991-08	EV	2
8PV	EV8 S1994-08	EV	15	EV4 S1992-08	EV	1
10PV	CV5 S1997-08	CV	11	CV1 S1995-08	CV	5
10PV	CV7 S1998-08	CV	15	CV3 S1996-08	CV	7
10PV	EV6 S2002-08	EV	11	EV2 S2000-08	EV	12
10PV	EV8 S2003-08	EV	8	EV4 S2001-08	EV	12

Day Euth. = Day of euthanasia; SPF = Specific pathogen-free hens; COMM = Commercial hens; Vac. = Vaccination route; CV = Cloacal vaccination; EV = Eyedrop vaccination; PV = Post-vaccination; S = histopathology sample registration number.

The antibody titres against NDV vaccine (La Sota) for both the SPF and the commercial hens as detected by ELISA and expressed as titre group was summarized, the group and daily average calculated and the data displayed graphically in Fig. 3.3.

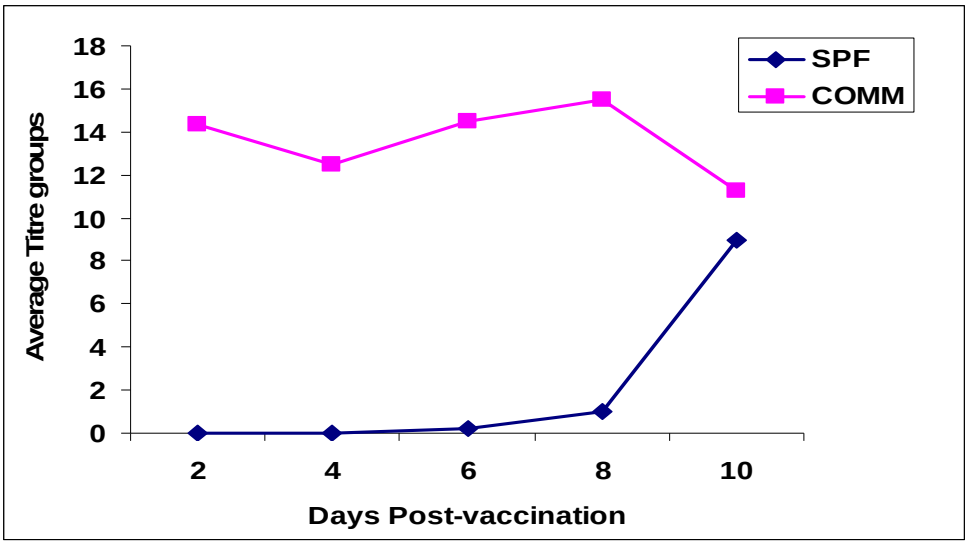


Fig. 3.3 Line graph showing the average ND antibody titre of both SPF and commercial hens vaccinated with La Sota vaccine, as assayed by ELISA, and expressed as log 2 titre groups *(data are from birds bled and euthanazed from day 2PV to day 10PV)*.

The average titres as displayed in Fig. 3.3 fluctuated between 2^{11} and 2^{16} for the commercial birds while those of the SPF birds picked up gradually from an average titre of 0.25 to the highest value of 9. The drop in the line graph for the commercial birds on day 4 PV was due to birds CV5 S1953-08 and EV6 S1958-08 that had antibody titre values of 2^{9} and 2^{7}, respectively, which were in stark contrast to the other members of the group that had titres of up to 2^{17}. In addition, the drop in the graph (again for the commercial birds) seen on day 10 PV was due to the generally low antibody titres of birds euthanized on day 10 PV as compared to those euthanazed on day 8 PV (Table 3.4). The SPF birds' average antibody titres picked up gradually from day 6 PV (0.25) to day 8 PV (1.0), before a steep climb to an average peak titre of 9 on day 10 PV.

Sera from the first post-challenge bleed (day 2 PC), had titres of between 2^{8} and 2^{18} for the commercial birds while the SPF birds had titres of between 2^{3} and 2^{16}. Second (day 4

PC), third (day 6 PC), fourth (day 8 PC) and fifth (day 10 PC) post-challenge sera had antibody titres of between 2^8 and 2^{18} for the commercial birds while those of the SPF birds varied from 2^2 to 2^{18}. The ND antibody titres of both the post-challenge SPF and commercial birds were as presented in Table 3.5. Fig. 3.5a compared the post-challenge average NDV antibody titres of the commercial birds vaccinated by cloacal and eye-drop routes.

Table 3.5 Table showing the NDV antibody titre of SPF and commercial hens vaccinated by cloacal and eye routes with La Sota live ND vaccine and challenged with GPMV, expressed as a titre group (assayed by ELISA)

Day	COMMERCIAL HENS			SPF HENS		
Euth	*Bird No.*	*Vac.*	*Titres*	*Bird No.*	*Vac.*	*Titres*
2PC	CV5 S2060-08	CV	13	CV1 S2058-08	CV	15
2PC	CV7 S2061-08	CV	17	CV3 S2059-08	CV	3
2PC	EV6 S2064-08	EV	8	EV2 S2062-08	EV	14
2PC	EV8 S2065-08	EV	18	EV4 S2063-08	EV	16
4PC	CV5 S2112-08	CV	13	CV1 S2110-08	CV	7
4PC	CV7 S2113-08	CV	16	CV3 S2111-08	Removed from study	
4PC	EV6 S2116-08	EV	11	EV2 S2114-08	EV	11
4PC	EV8 S2117-08	EV	15	EV4 S2115-08	EV	18
6PC	CV5 S2167-08	CV	13	CV1 S2165-08	CV	8
6PC	CV7 S2168-08	CV	14	CV3 S2166-08	CV	18
6PC	EV6 S2171-08	EV	18	EV2 S2169-08	EV	15
6PC	EV8 S2172-08	EV	9	EV4 S2170-08	EV	7
8PC	CV5 S2175-08	CV	10	CV1 S2173-08	CV	11
8PC	CV7 S2176-08	CV	12	CV3 S2174-08	CV	7
8PC	EV6 S2179-08	EV	18	EV2 S2177-08	EV	16
8PC	EV8 S2180-08	EV	11	EV4 S2178-08	Removed from study	
10PC	CV5 S2199-08	CV	11	CV1 S2197-08	CV	18
10PC	CV7 S2200-08	CV	9	CV3 S2198-08	CV	8
10PC	EV6 S2203-08	EV	8	EV2 S2201-08	EV	11
10PC	EV8 S2204-08	EV	13	EV4 S2202-08	EV	12

Euth = Day of euthanasia; PC = Post-challenge; Vac. = Vaccination routes; CV = Cloacal vaccination; EV = Eyedrop vaccination; S = Histopathology sample registration number. Birds CV3 S2111-08 and EV S2178-08 were removed from the trial because they died of nonviral challenge-associated causes.

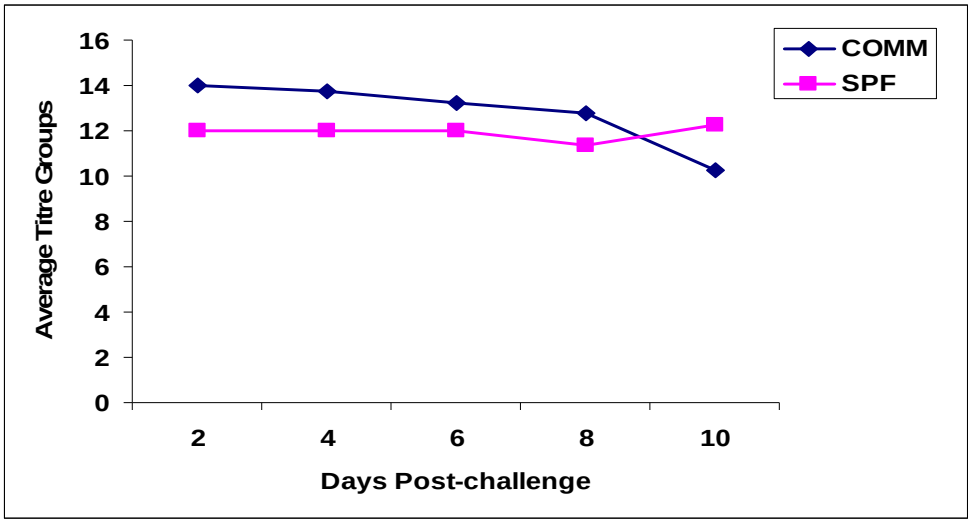


Fig. 3.4 Line graph showing the NDV antibody titre of both SPF and commercial hens vaccinated with La Sota vaccine and challenged with GPMV, as assayed by ELISA and expressed as titre groups (*data captured here are from birds that were bled during euthanasia from day 2PC to day 10PC*); SPF (n= 18), Commercial (n= 20).
COMM = Commercial hens; SPF = Specific pathogen-free hens; PC = Post-challenge

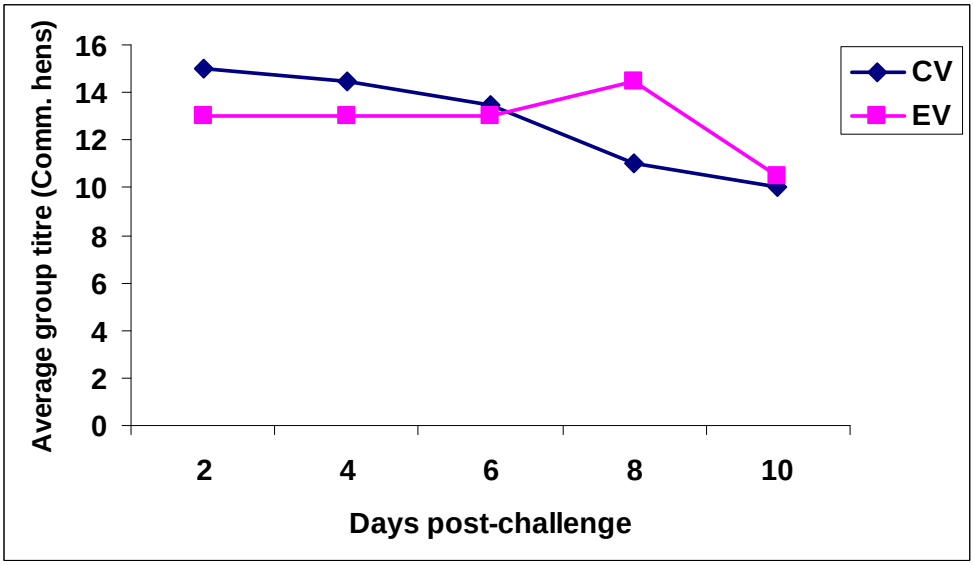


Fig.3.5a Graph comparing the post-challenge average NDV antibody titre of cloacally and eye-drop vaccinated commercial hens.
PC = Post-challenge; CV = Cloacal vaccination; EV = Eye-drop vaccination

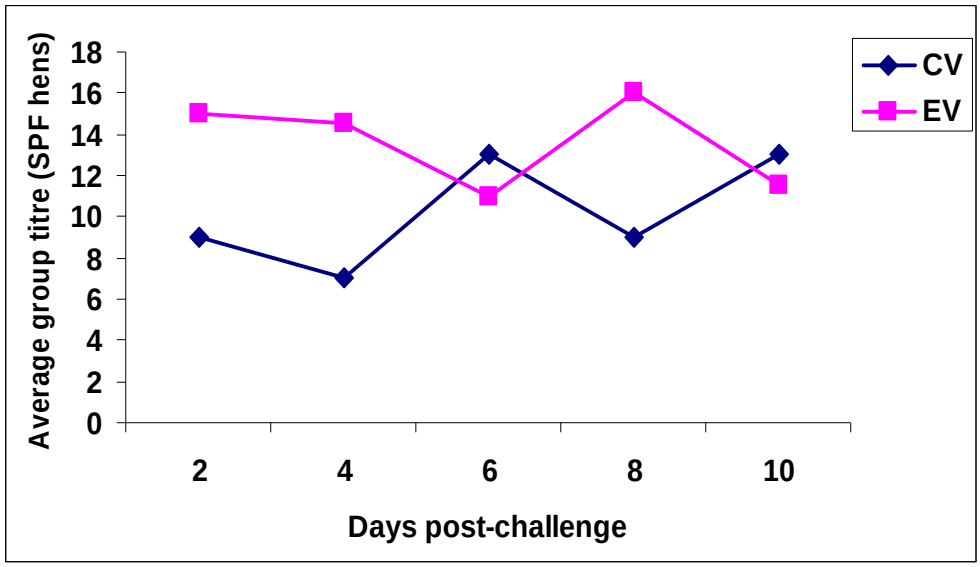


Fig. 3.5b Graph comparing the post-challenge average NDV antibody titre of cloacally and eye-drop vaccinated SPF hens.
PC = Post-challenge; CV = Cloacal vaccination; EV = Eye-drop vaccination

3.2.3 Gross Pathology

Birds were necropsied immediately after they were euthanazed (or after they died naturally as in the case of the positive control birds) and the different parts of the oviduct sampled. All birds euthanazed on days 2, 4, 6, 8, and 10 post-vaccination (PV) had no grossly visible pathology. Bird CV1 S1946-08, one of the SPF hens euthanazed on day 2PV was found to have a metastatic uterine adenocarcinoma that had infiltrated almost all of the abdominal organs. The oviduct of this bird was therefore not sampled and the bird was removed from the trial. Birds EV2 S1991-08, CV3 S1996-08 and EV4 S2001-08, all of which were SPF hens, had small, flaccid, and inactive oviducts. These birds were probably not in active egg production.

SPF hens CV1 S2058-08, CV1 S2110-08, EV2 S2169-08 and EV2 S2177-08 had small, flaccid and inactive oviducts indicating that they were not in lay. Birds CV3 S2111-08

and EV4 S2178-08 died from causes unrelated to the viral challenge and were therefore removed from the study. Bird EV8 S2180-08, euthanazed on day 8 PC, and birds CV5 S2199-08, CV7 S2200-08, EV2 S2201-08, EV6 S2203-08 and EV8 S2204-08, euthanazed on day 10 PC, had necrohaemorrhagic foci in their caecal tonsils. Most of the birds with the necrohaemorrhagic lesions in their caecal tonsils were vaccinated by ocular route except for birds CV5 S2199-08 and CV7 S2200-08 that were vaccinated through the cloacal route. In addition, all of the birds with necrohaemorrhagic lesions in their caecal tonsils were commercial hens that had been previously vaccinated, except bird EV2 S2201-08, which was an SPF bird.

The macropathology observed in the positive control birds was variable, depending on the duration of the infection. Birds euthanazed on day 2 PC (CX 1A 2PC and CX 1B 2PC) has no gross lesions. Bird CX 1A 3PC, that died on day 3 PC, had pin-point haemorrhages on the serosal surface of the spleen, slight haemorrhaging in the lumen of the proventriculus, congested ovarian follicles, and a segmentally congested duodenum filled with greenish watery content. Five birds died on day 4 PC, four of which had matted vents stained with greenish or whitish faecal material. Macroscopic lesions included congested tracheas, kidneys and lungs, pericardial haemorrhages as well as haemorrhages in the caecal tonsils. Only one bird had haemorrhagic lesions in the proventriculus. However, all the positive control birds had marked degeneration of the ovarian follicles. This was characterized by resorption of the yolk, ill-defined external follicle outlines, and congested follicles, with some having yolk material lying free in the abdominal cavity. The bird that died on day 5 PC had a diffusely congested trachea, pin-

point white spots throughout the spleen, a congested heart and haemorrhages in the proventriculus and ceacal tonsils. The duodenum of this bird was haemorrhagic and the ovarian follicles were also severely haemorrhagic and degenerated. Similar lesions were seen in the last bird that died on day 6 PC. In addition, the spleen in this bird was markedly enlarged and diffusely haemorrhagic.

3.2.4 Histopathology of the oviduct

The observed histopathology in all three sections of oviduct was variable but generally mild in nature. Most lesions were observed in the post-challenge birds, namely: Mild interstitial oedema; focal to scattered lymphocytic and plasmacytic infiltration in the interstitium (Fig. 3.11 and Fig. 3.12); occasional dilated glands, some of which contained granular pink material that appeared to be mineralized (Fig. 3.12); mild interstitial fibrosis and moderate loss of glands. These changes were observed throughout the oviduct. However, lesions were most pronounced in the uterus, with only a few lesions seen in the magnum and the isthmus. The positive control birds had similar lesions.

3.2.5. Immunohistochemistry

Immunohistochemistry (IHC) results were obtained from 38 birds post-vaccination, 38 birds post-challenge (*see appendix 7 for sample numbering*), two negative control birds and five positive control birds. The two negative birds were neither vaccinated nor challenged and were euthanazed together with birds euthanazed on day 10 PV. The use of the mAb (Anti-NDV P pure ascites 10-5E6, 1985) to investigate viral distribution/tropism in the reproductive tract resulted in the following observations:

- Both intracellular and extracellular (or not clearly cell-associated) positive staining was observed (Fig. 3.13 to Fig. 3.21)
- Positive staining was red-orange or tan-coloured, finely granular/stippled and tended to occur in focal clusters (Fig. 3.13 to Fig. 3.21).
- Intracellular staining was obviously intracytoplasmic (Fig. 3.17).
- Most of the cell-associated positive staining occurred within epithelial cells (surface and glandular) throughout the oviduct (Fig. 3.13, Fig. 3.14 and Fig. 3.17).
- Positive staining was also frequently associated with lymphocytes within lymphocytic infiltrates throughout the interstitium of the oviducts (Fig. 3.15 and Fig. 3.16).
- Occasional fibroblasts in the interstitium were also seen to contain NDV-specific positive staining (Fig. 3.19 and Fig. 3.20).
- In addition, occasional mononuclear cells (monocyte, macrophages and/or lymphocytes) in the interstitium contained intracytoplasmic positive staining.

The distribution of positive staining (and therefore apparent cell tropism) within the oviduct was similar for both the vaccinated and challenged birds. However, NDV-specific positive staining was more severe/widely distributed throughout the oviduct of the challenged hens compared to the hens that were only vaccinated. The IHC results for the post-vaccinated and post-challenged birds are presented in Table 3.6 and Table 3.7, respectively.

Negative control birds and irrelevant reagent control

Positive staining was not observed in any sections of the oviduct of negative tissue control hens. In addition, no NDV-specific positive staining was demonstrated in the oviducts of positive hens when the NDV mAb was replaced with an irrelevant Wesselsbron mAb in the immunodetection process (Fig. 3.22). These results confirm the specificity and sensitivity of the NDV mAb.

3.2.5.1. Post-vaccination IHC results

The quantity of positive IHC staining in the oviduct (uterus, magnum and isthmus) of both the commercial and SPF hens vaccinated with La Sota vaccine gradually increased from a total average of 1 on day 2 PV to reach its highest total average of 2.13 on day 10 PV. The quantity of IHC staining from day 2PV to day 10PV, increased from an average of 0.6 to 1, 0.2 to 0.63 and 0.2 to 0.5 for the uterus, magnum and isthmus respectively (Fig. 3.6a). Furthermore, within the 10-day trial period, positive staining was observed in all three sections of oviduct (uterus, magnum and isthmus). However, the greatest amount of viral antigen was consistently seen in the uterus (Fig. 3.13 and Fig. 3.14), followed by the magnum, and then the isthmus (Table 3.6; Fig. 3.6a). Staining scores in the various sections of oviduct were summated and the averages calculated to facilitate the interpretation of staining results (minus the birds in each group that died of non challenge-associated causes). ND viral antigen-associated positive staining was seen in the oviduct of commercial hens as early as day 2 PV. Staining increased on day 4 PV, and then decreased gradually until day 8 PV, before increasing again on day 10 PV. The presence of positive staining as early as day 2 PV have been associated with viral antigen deriving from previous vaccinations. The SPF birds first showed NDV-specific positive

staining in oviductal tissues on day 4 PV. The amount of positive IHC staining in the oviduct of SPF hen increased gradually from an average of 0.25 on day 4PV to 3.0 on day 8 PV before it declined to an average of 2.25 by 10 PV (Fig. 3.6b and Table 3.6).

There was no significant/obvious difference between cloacal and eye vaccination routes regarding tissue tropism or distribution of the virus as identified by IHC either in commercial or SPF birds. Nonetheless, slightly more positive staining was observed in all three sections of the oviduct from SPF hens, compared to commercial hens, except for the first 4 days post-vaccination (Fig. 3.6b). Cloacally-vaccinated SPF birds showed slightly more positive IHC staining in the oviduct compared to SPF birds vaccinated via eyedrop. The same pattern was observed in the commercial birds. However, the uterus of the commercial birds vaccinated via eyedrop (EV COMM) had a slightly more positive IHC staining than the uterus of the cloacally-vaccinated commercial (CV COMM) hens (Fig. 3.7). This was due to the combined positive IHC staining of three birds (EV6 S1958-08, EV6 S2002-08 and EV8 S2003-08) with an average of 0.78 as against the 0.5 average scores of the four cloacally vaccinated commercial (CV COMM) hens (CV5 S1947-08, CV7 S1948-08, CV5 S1997-08 and CV7 S1998-08) (Table 3.6).

Table 3.6 Distribution of live La Sota vaccine virus in various sections of the oviduct of SPF and commercial hens as detected by immunohistochemistry (IHC), post-vaccination

COMMERCIAL HENS					SPF HENS				
Day Euth.	*Sample No.*	*Uterus*	*Magnum*	*Isthmus*	*Day Euth.*	*Sample No.*	*Uterus*	*Magnum*	*Isthmus*
2PV(1)	CV5 S1947-08	+	+	+	2PV(1)	CV1 S1946-08	-	-	-
2PV(1)	CV7 S1948-08	++	-	-	2PV(1)	EV2 S1949-08	-	-	-
2PV(1)	EV6 S1951-08	-	-	-	2PV(1)	EV4 S1950-08	-	-	-
4PV(2)	CV5 S1953-08	-	-	++	4PV(2)	CV1 S1952-08	+	+	-
4PV(2)	CV7 S1955-08	-	-	-	4PV(2)	CV3 S1954-08	-	-	-
4PV(2)	EV6 S1958-08	+++	++	-	4PV(2)	EV2 S1956-08	-	-	-
4PV(2)	EV8 S1959-08	-	-	+	4PV(2)	EV4 S1957-08	-	-	-
6PV(3)	CV5 S1963-08	+	++	+	6PV(3)	CV1 S1961-08	+++	-	-
6PV(3)	CV7 S1964-08	-	-	-	6PV(3)	CV3 S1962-08	++	+	++
6PV(3)	EV6 S1967-08	-	-	-	6PV(3)	EV2 S1965-08	-	-	-
6PV(3)	EV8 S1968-08	-	-	-	6PV(3)	EV4 S1966-08	-	-	-
8PV(4)	CV5 S1989-08	-	-	-	8PV(4)	CV1 S1987-08	-	-	+
8PV(4)	CV7 S1990-08	+	+	-	8PV(4)	CV3 S1988-08	-	-	-
8PV(4)	EV6 S1993-08	-	-	-	8PV(4)	EV2 S1991-08	+++	+++	+++
8PV(4)	EV8 S1994-08	-	-	-	8PV(4)	EV4 S1992-08	++	+	-
10PV(5)	CV5 S1997-08	+	-	-	10PV(5)	CV1 S1995-08	+	-	-
10PV(5)	CV7 S1998-08	+	+	+	10PV(5)	CV3 S1996-08	+++	+++	+++
10PV(5)	EV6 S2002-08	++	+	-	10PV(5)	EV2 S2000-08	+	+	-
10PV(5)	EV8 S2003-08	++	-	-	10PV(5)	EV4 S2001-08	-	-	-

- = IHC negative for NDV; + = 1 IHC positive cell or focal extracellular cluster of positive staining per 400× magnification; ++ = 2-5 IHC positive cells or focal extracellular clusters of positive staining per 400× magnification; +++ = >5 IHC positive cells or focal extracellular clusters of positive staining per 400× magnification; NA = tissue or slide not available.

Day Euth. = Days Euthanized; PV = Post-vaccination; CV = Cloacal vaccination; EV = Eyedrop vaccination

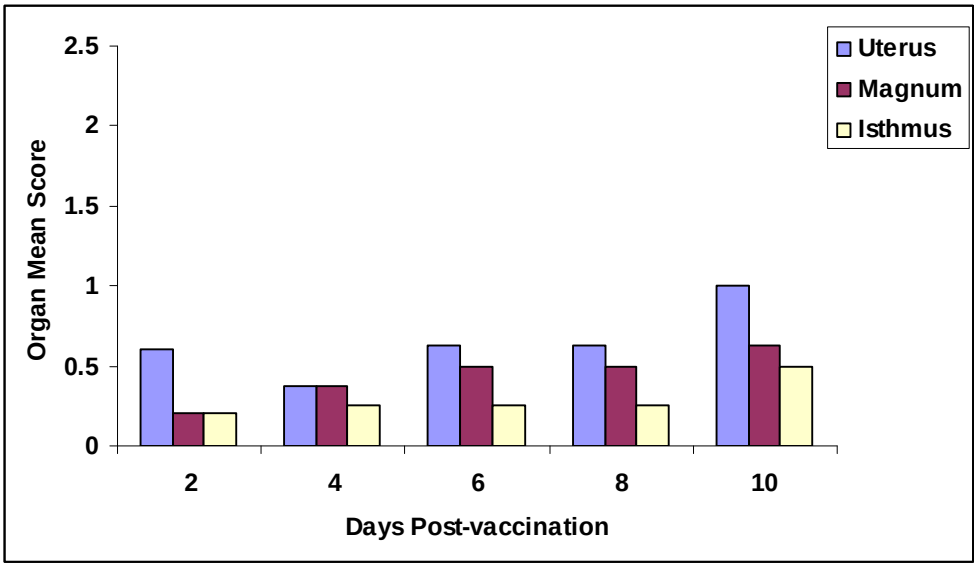


Fig. 3.6a Distribution of viral antigens in the tissues of the oviduct of both SPF and commercial hens vaccinated with LaSota NDV vaccine.

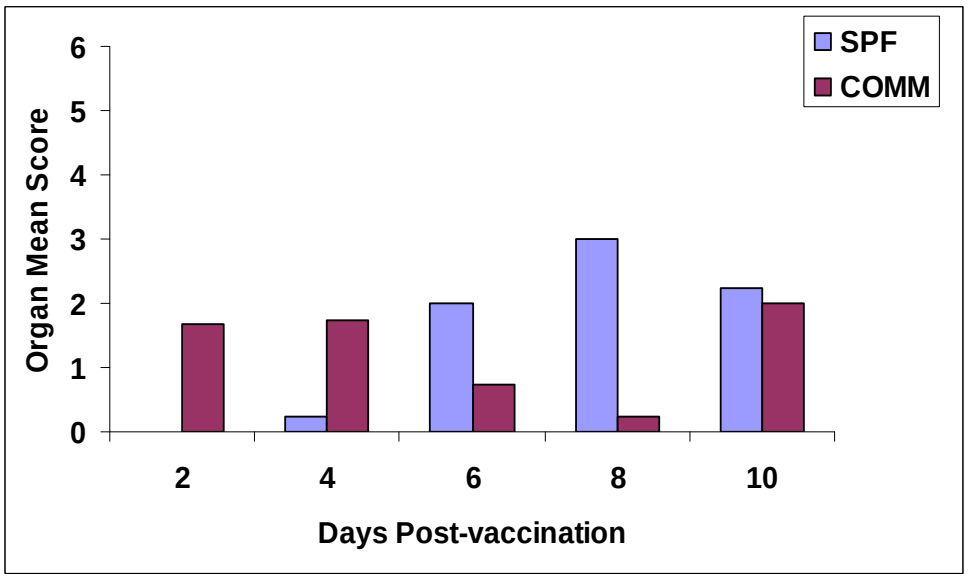


Fig. 3.6b Post-vaccination distribution of NDV in the oviducts of SPF and commercial hens vaccinated with Live La Sota vaccine **(*mean scores captured here consist of the scores of all three sections of oviduct*)**.
PV = Post-vaccination; SPF = Specific Pathogen-free hens; COMM = Commercial hens

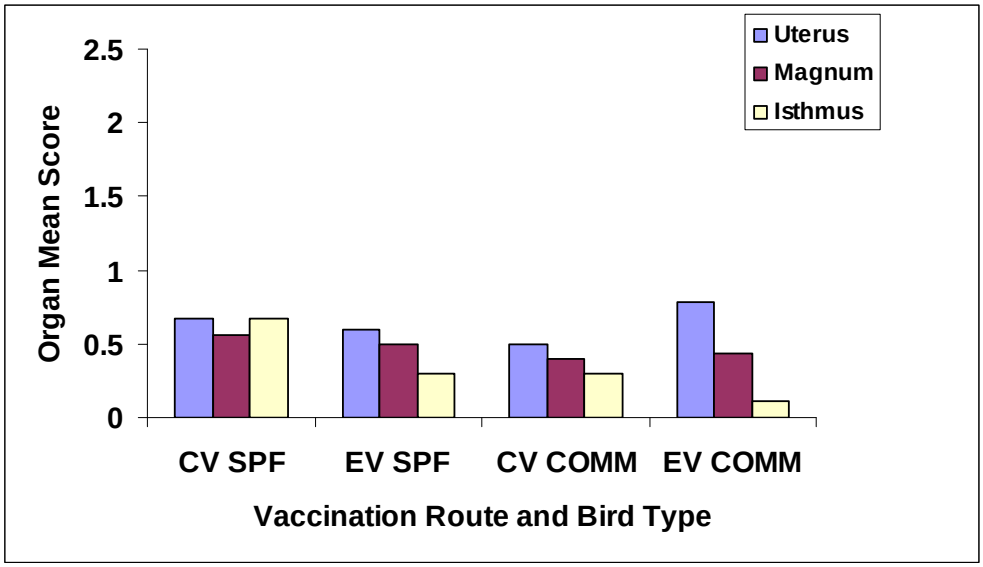


Fig. 3.7 Post-vaccination distribution of La Sota vaccine virus in tissues of the oviduct of SPF and commercial hens vaccinated via cloacal and eyedrop routes.
CV = Cloacal vaccination; EV = Eyedrop-vaccination; SPF = Specific pathogen-free birds; COMM = Commercial birds.

3.2.5.2. Post-challenge IHC results

There was more positive staining in the oviduct of post-challenge (PC) hens, compared to post-vaccination (PV) hens. Positive staining was also more extensive in the SPF hens than in the commercial hens (Table 3.7 and Fig. 3.9). The PC positive staining scores fluctuated, but generally they remained high with average scores of above 0.5 throughout the 10 days post-challenge period (Fig. 3.8). Positive staining in the uterus was more extensive (Fig. 3.17) than in the magnum and isthmus (Table 3.7 and Fig. 3.8, Fig. 3.9, Fig. 3.10 and Fig. 3.18). This pattern was maintained throughout the post-challenge period. However, there was no obvious difference in the distribution of positive staining following cloacal or eye vaccination in the SPF or commercial hens (Fig. 3.10). Viral tropism for the oviduct was more evident in the SPF hens than in the commercial hens (Fig. 3.9). 35 hens (18 commercial and 17 SPF), 28 (13 commercial and 15 SPF) and 22

(12 commercial and 10 SPF) hens of the challenged birds had NDV-associated specific positive staining in uterus, magnum and isthmus, respectively, irrespective of their antibody status or route of vaccination (Table 3.7).

Table 3.7 Extent of infection and distribution NDV antigen as detected by immunohistochemistry in the oviduct of La Sota-vaccinated, and GPMV-challenged SPF and commercial hens

COMMERCIAL HENS					**SPF HENS**				
Day Euth	***Sample No.***	***Uterus***	***Magnum***	***Isthmus***	***Day Euth***	***Sample No.***	***Uterus***	***Magnum***	***Isthmus***
2PC(6)	CV5 S2060-08	+	-	-	2PC(6)	CV1 S2058-08	+++	+++	-
2PC(6)	CV7 S2061-08	+	-	-	2PC(6)	CV3 S2059-08	+++	+++	+++
2PC(6)	EV6 S2064-08	+	++	+	2PC(6)	EV2 S2062-08	+++	+	-
2PC(6)	EV8 S2065-08	++	+	+	2PC(6)	EV4 S2063-08	+	-	-
4PC(7)	CV5 S2112-08	+	+	-	4PC(7)	CV1 S2110-08	++	++	+
4PC(7)	CV7 S2113-08	+	-	+	4PC(7)	CV3 S2111-08	Removed from the study		
4PC(7)	EV6 S2116-08	++	++	++	4PC(7)	EV2 S2114-08	+++	+	-
4PC(7)	EV8 S2117-08	++	++	+	4PC(7)	EV4 S2115-08	+++	+++	++
6PC(8)	CV5 S2167-08	++	++	-	6PC(8)	CV1 S2165-08	++	++	+
6PC(8)	CV7 S2168-08	++	+	-	6PC(8)	CV3 S2166-08	++	++	++
6PC(8)	EV6 S2171-08	+	-	-	6PC(8)	EV2 S2169-08	+++	+++	++
6PC(8)	EV8 S2172-08	+	+	+	6PC(8)	EV4 S2170-08	++	-	-
8PC(9)	CV5 S2175-08	+++	++	-	8PC(9)	CV1 S2173-08	+	+	-
8PC(9)	CV7 S2176-08	++	-	+	8PC(9)	CV3 S2174-08	++	+	+
8PC(9)	EV6 S2179-08	++	+	+	8PC(9)	EV2 S2177-08	+++	+++	++
8PC(9)	EV8 S2180-08	-	-	+	8PC(9)	EV4 S2178-08	Removed from the study		
10PC(10)	CV5 S2199-08	+++	+	+	10PC(10)	CV1 S2197-08	+++	+++	+++
10PC(10)	CV7 S2200-08	-	-	-	10PC(10)	CV3 S2198-08	++	+	-
10PC(10)	EV6 S2203-08	+	+	+	10PC(10)	EV2 S2201-08	++	++	+
10PC(10)	EV8 S2204-08	+	+	+	10PC(10)	EV4 S2202-08	-	-	-

- = IHC negative for NDV; + = 1 IHC positive cell or focal extracellular cluster of positive staining per 400× magnification; ++ = 2-5 IHC positive cells or focal extracellular clusters of positive staining per 400× magnification; +++ = >5 IHC positive cells or focal extracellular clusters of positive staining per 400× magnification.

Day Euth. = Day Euthanized; PC = Post-challenge; CV = Cloacal vaccination; EV = Eyedrop or ocular vaccination; S = Histopathology sample registration number
Birds CV3 S2111-08 and EV4 S2178-08 died of other causes (non-trial associated) and were therefore removed from the study.

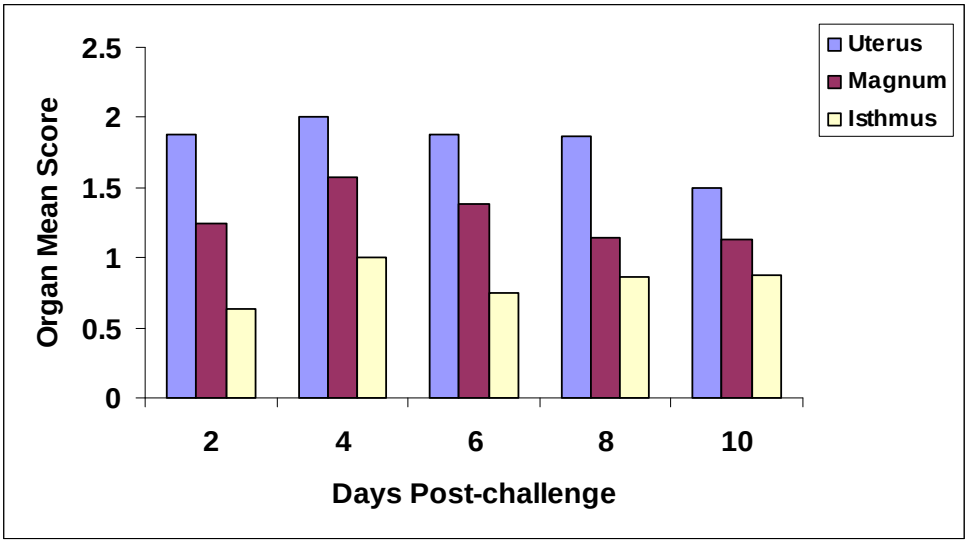


Fig. 3.8 Extent of infection and distribution of viral antigen in the oviduct of SPF and commercial hens challenged with GPMV.

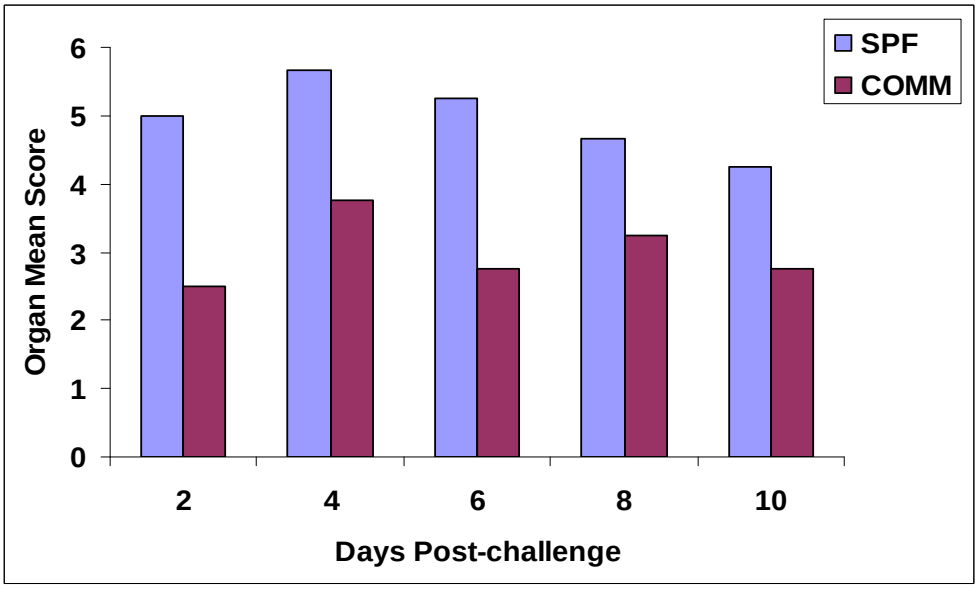


Fig. 3.9 Extent of viral infection and distribution of viral antigen in the SPF and commercial hens vaccinated with La Sota and challenged with GPMV.
SPF = Specific Pathogen-free hens; COMM = Commercial hens

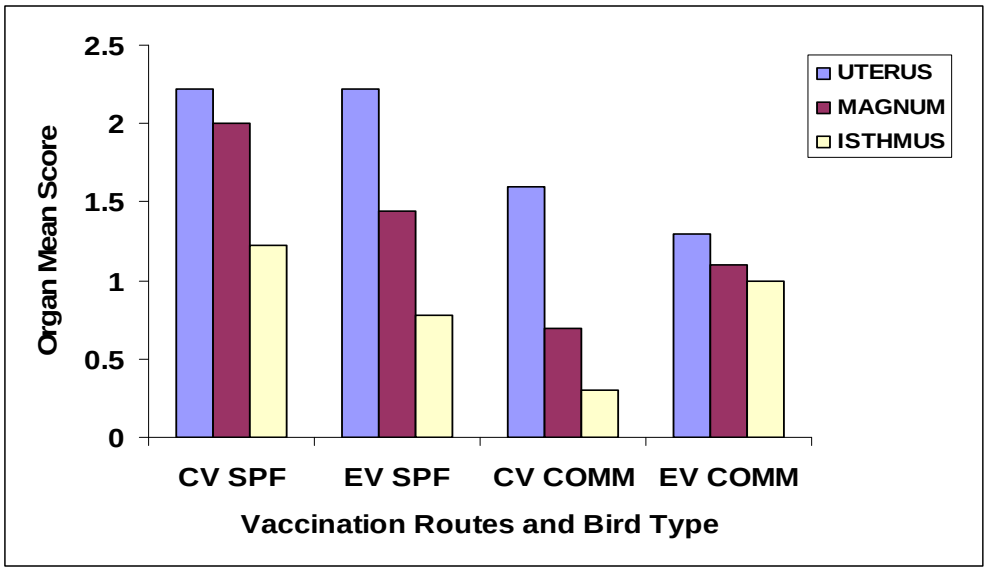


Fig. 3.10 Extent of infection and distribution of viral antigen in SPF and commercial hens vaccinated with La Sota by cloacal and eyedrop route and challenged with GPMV.
CV = Cloacal vaccination; EV = Eyedrop vaccination; SPF = Specific pathogen-free hens; COMM = Commercial hens.

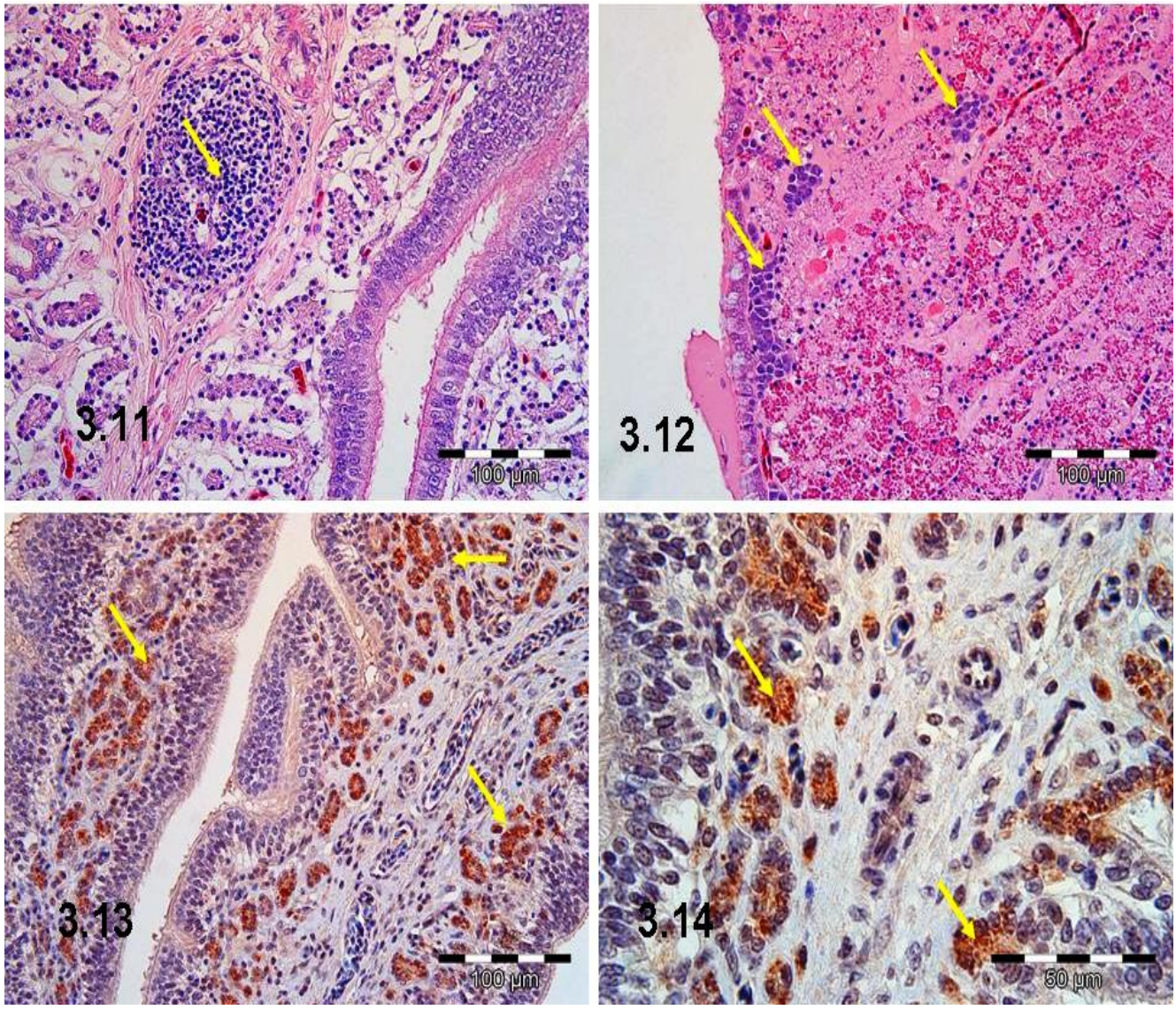


Fig. 3.11. Uterus; Hen No. CV3 S2166-08; SPF hen vaccinated with La Sota via cloacal route and challenged with a GPMV isolate; Day 6PC. Presence of a nodular lymphoid aggregate in the interstitium (arrow). Mayer's haematoxylin & Eosin (H & E).

Fig. 3.12. Magnum; Hen No. CV3 S2166-08; SPF hen vaccinated with La Sota via cloacal route and challenged with a GPMV isolate; Day 6PC. Plasmacytic infiltrations (arrows) in the interstitium. Mayer's haematoxylin & Eosin (H & E).

Fig. 3.13. Uterus; Hen No. EV2 S1991-08; SPF hen vaccinated with La Sota via eyedrop route; Day 8PV. Presence of finely granular NDV-specific positive staining in glandular epithelium (arrows). IHC, Polymer system, Mayer's haematoxylin counterstain.

Fig. 3.14. Uterus; Hen No. EV2 S1991-08; SPF hen vaccinated with La Sota via eyedrop route; Day 8PV. Higher magnification of NDV-specific positive staining in glandular epithelium (arrows). IHC, Polymer system, Mayer's haematoxylin counterstain.

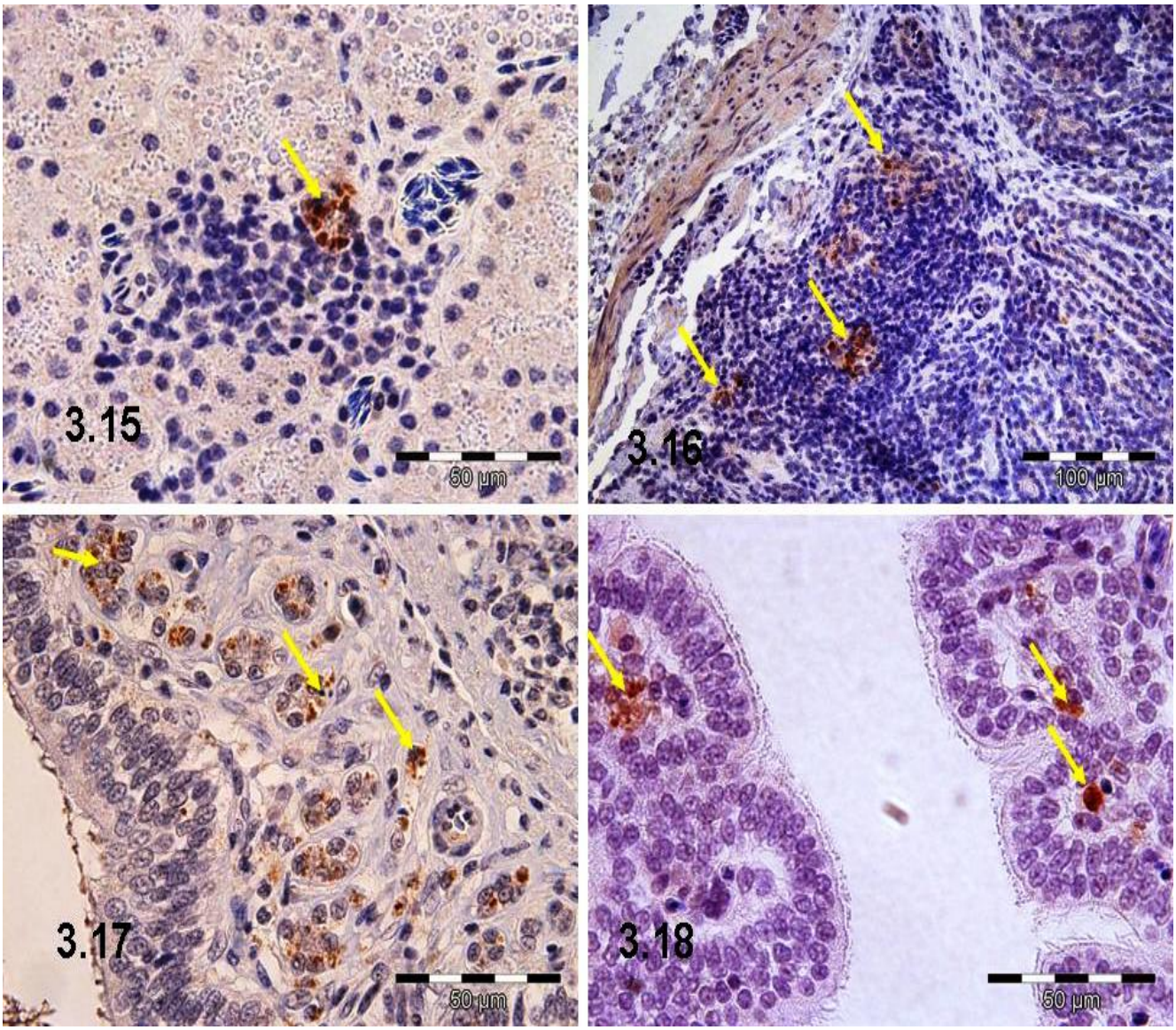
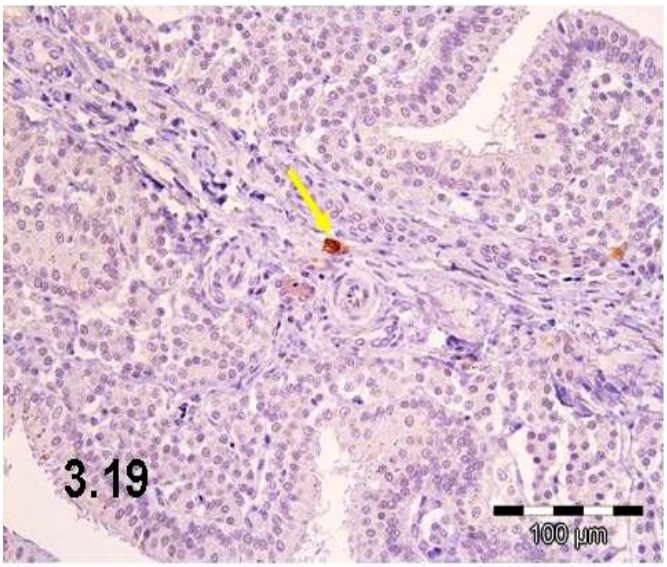


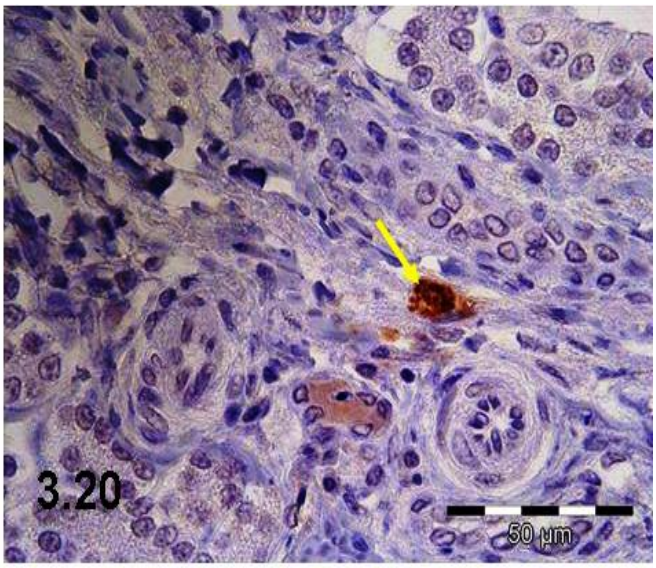
Fig. 3.15. Magnum; Hen No. CV7 S1990-08; Commercial hen vaccinated with La Sota via cloacal route; Day 8PV. Presence of NDV-specific positive staining within a lymphoid aggregate (arrow). IHC, Polymer system, Mayer's haematoxylin counterstain.

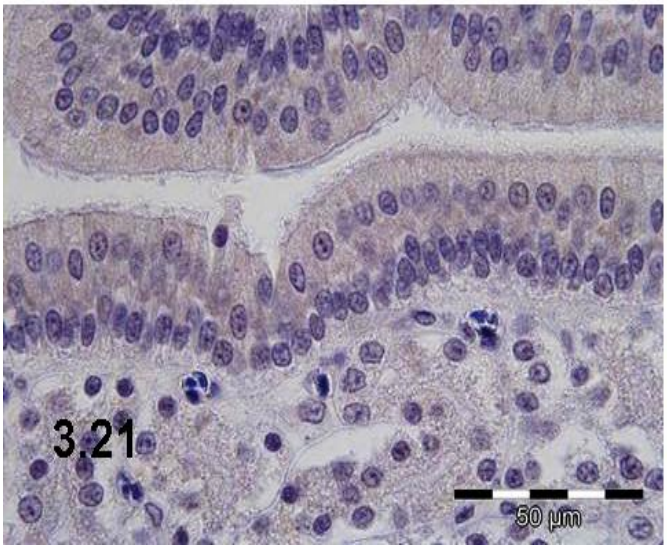
Fig. 3.16. Magnum; Hen No. CV3 S1996-08; SPF hen vaccinated with La Sota via cloacal route; Day 10 PV. Presence of positively staining NDV antigen within a lymphoid follicle (arrows). IHC, Polymer system, Mayer's haematoxylin counterstain.

Fig. 3.17. Uterus; Hen No. EV4 S2115-08; SPF hen vaccinated with La Sota by eyedrop route and challenged with a GPMV isolate; Day 4PC. Presence of NDV-positive staining in glandular epithelium (arrows). IHC, Polymer system, Mayer's haematoxylin counterstain.

Fig. 3.18. Isthmus; Hen No. CV1 S2197-08; SPF hen vaccinated with La Sota via cloacal route and challenged with a GPMV isolate; Day 10PC. Presence of ND viral antigen-specific positive staining in subepithelial mononuclear cells (arrows). IHC, Polymer system, Mayer's haematoxylin counterstain.







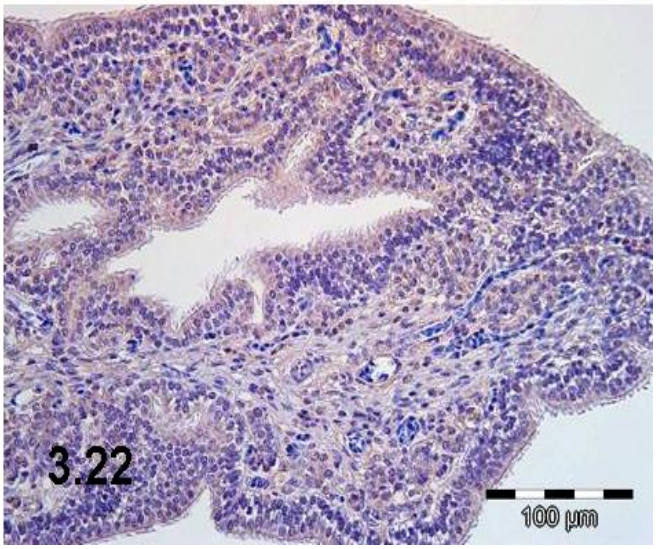


Fig. 3.19. Uterus; Hen No. CV5 S2199-08; Commercial hen vaccinated with La Sota via cloacal route and challenged with a GPMV isolate; Day 10PC. Presence of granular cell-associated NDV-specific positive staining in the connective tissue (arrow). IHC, Polymer system, Mayer's haematoxylin counterstain.

Fig. 3.20. Uterus; Hen No. CV5 S2199-08; Commercial hen vaccinated with La Sota via cloacal route and challenged with a GPMV isolate; Day 10PC. Higher magnification of cell-associated NDV-specific positive staining in the connective tissue (arrow). IHC, Polymer system, Mayer's haematoxylin counterstain.

Fig. 3.21. Uterus; Hen No. S1999-08; Unvaccinated and unchallenged SPF hen; Day 10PV. Negative tissue control. Absence of specific positive staining target cells. IHC, Polymer system, Mayer's haematoxylin counterstain.

Fig. 3.22. Uterus; Hen No. CV1 S2197-08; SPF hen vaccinated with La Sota via cloacal route and challenged with a GPMV isolate; Day 10PC. Negative reagent control. No specific positive staining was observed in target cells. IHC, Wesselsbron mAb and polymer system, Mayer's haematoxylin counterstain.

CHAPTER 4 – DISCUSSION

There was generally no difference and most of the fluctuations could be attributed to individual bird variations. Slight differences were observed in Fig. 3.5b which compared the post-challenge average NDV antibody titres of SPF birds vaccinated by cloacal and eye-drop routes. These differences could also be due individual birds variations as there were two birds per group. The average NDV antibody titres of both the SPF and the commercial birds bled on different days post-challenge has been presented in graphical form in Fig. 3.4. The graph (Fig. 3.4) was almost a straight line for the commercial birds, but showed a gradual downward trend from the average value of 14 to 10, indicating that the commercial birds reached their peak antibody titre of 15.5 on day 8 PV (Fig. 3.3), and challenge with virulent virus did not cause a substantial increase in antibody titre (Fig. 3.4). But there was a marginal increase in the average titre value from 11.25 on day 10PV to 14 on day 2PC before a gradual downward slide to the lowest value of 10.25 on day 10PC (Fig. 3.4).

The graph for the SPF birds was also almost a straight line with the average titre values fluctuating between 11 and 12. The drop in titre on day 8 PC was due to the low antibody titre (2^7) of bird CV3 S2174-08. This was probably due to the inability of bird CV3 S2174-08 to seroconvert optimally to the vaccination as well as to the challenge. Indeed, lots of ND viral antigen was apparent via IHC in all the sections of oviduct of this bird (Table 3.6), which lends credence to the suboptimal seroconversion theory in this case. Clearly this bird was not fully protected against virulent viral challenge, despite the fact that it did not develop or show visible clinical signs.

4.1 Introduction

Newcastle disease is a global disease of enormous economic importance. The virus is capable of infecting many avian species with a marked effect on the poultry industry, principally due to mortality but also due to the effects on the quality and quantity of meat and eggs produced by affected birds. Most countries where poultry is raised commercially and where the disease is endemic rely on vaccination to keep the disease under control (Alexander, 2001; Alexander, 2003; Alexander & Senne, 2008). However, the continued outbreaks of velogenic NDV in domestic poultry worldwide, even in fully-vaccinated birds (Burridge *et al*., 1975; Alexander, 2003; reviewed in van Boven *et al*., 2008) brings into question the efficacy of commercially available vaccines in protecting birds against challenge.

Where there is velogenic field challenge in apparently well-vaccinated laying hens, it has been found that there is seldom any significant increase in mortality associated with the challenge. Egg production is, however, frequently seriously depressed and often associated with abnormal egg-shell formation and white-shelled eggs (in brown egg layers). In order to try to limit these production losses, certain poultry producers in South Africa have resorted to cloacal application of live Newcastle disease vaccines before and during the laying period based on the assumption that superior immunity might be achieved in the oviduct through more direct application of the vaccine. Also the findings of Kapczynski & King (2005) and Czegledi *et al* (2006), that currently available vaccines induced better protection against viruses that were isolated in past epizootics than against

viruses that are currently circulating emphasizes the importance for continued research on vaccine efficacy, especially against newly-emerging strains of NDV.

Challenge models and immunohistochemical methods have widely been used to study the pathogenesis of NDV in different avian species and tissues. However, only Biswal & Morrill, (1954) and Rao *et al* (2002) assessed the pathogenesis of NDV in the reproductive tract of poultry. Biswal & Morrill (1954) looked at the clinicopathological presentation of the disease while evaluating the virulence of a California strain - 11914 NDV isolate in an experimental infection and another unknown strain from naturally infected birds; while Rao *et al* (2002) assessed the virulence of two vaccine strains and two field strains of NDV for the female reproductive tract of chickens using oviduct organ culture (OOC) prepared from precociously-induced oviducts in young chickens by oestrogen treatment.

In view of the above, two trial studies were carried out. A challenge study to determine the PD_{50} dose of Avinew Newcastle disease vaccine against two different strains of Newcastle disease virus and an immunohistochemical study of the pattern of distribution of La Sota vaccine and GPMV in the oviduct of vaccinated and challenged SPF and commercial hens to try to explain the effect of ND viruses in the production of poor quality eggs.

The results of the present studies are discussed below.

4.2 Dose dependant protection of ND vaccines against challenge with NDV isolates

In both studies, trial birds were protected from clinical disease and deaths from NDV challenge when vaccinated at the doses recommended by vaccine manufacturers.

The first study demonstrated that Avinew vaccine gave a similar level of protection against the effects of challenge with the two strains of ND virus used for the challenge. At the manufacturer's recommended dose of $10^{6.0}$ EID_{50} 100% protection from mortality was achieved against challenge with both GPMV and RCV, while 94.44% protection from mortality was achieved in the groups that received a vaccine dose of $10^{4.5}$ $EID_{50.}$ Birds that were vaccinated at a dose of $10^{3.0}$ EID_{50} had 13.89% protection against challenge with both GPMV and RCV. The protection in birds challenged with GPMV at the lowest vaccine dose of $10^{3.0}$ EID_{50} was poor but statistically significant ($P<0.05$) when compared to the control groups, while protection of RCV-challenged groups at $10^{3.0}$ EID_{50} was not statistically significant. At both higher doses there was a good protection which was statistically significant ($P<0.01$) when compared to the unvaccinated control birds and the lower dose of $10^{3.0}$ EID_{50}. The difference in the protection between the two higher doses of $10^{4.5}$ EID_{50} and $10^{6.0}$ EID_{50} was not statistically significant and both doses offered good protection from clinical disease and mortality, though the NDV antibody titres in the Avinew trial was not assayed. Nevertheless, Kapczynski & King (2005) demonstrated that a positive correlation exists between a higher dose of live vaccine and the presence of antibody titres and the subsequent protection offered post-challenge.

Since various modes of vaccination are used to administer diluted live vaccine in the field, it is possible for birds to receive doses that are lower than the recommended dose and that's why in this research doses lower than the recommended field doses were assessed. As the spread of lentogenic viruses may be limited (Al-Garib *et al.*, 2003a), it is important that care be taken in administering vaccines so that birds may get the dose required to produce high levels of protection despite the fact that a vaccine dose that is $10^{1.5}$ EID_{50} lower than the recommended field dose, still gives good protection.

4.3 Protection of chickens from clinical ND and mortality by vaccination

The above results showed the efficacy of the vaccine and its ability to protect against the clinical consequence of ND which includes clinical signs and death. This is in agreement with recent works on the efficacy of VG/GA vaccines (Avinew) done by (Beard *et al*., 1993; Silva *et al*., 2004; Perozo *et al*., 2004; 2008), all of which reported full protection against lethal NDV challenge at the recommended vaccination dose. This therefore contrasts with the concerns in the field and published reports (Liu *et al*., 2003; reviewed in van Boven *et al*., 2008) that ND vaccines may not produce adequate protection against velogenic challenge. Work done by (Beard *et al*., 1993; Silva *et al*., 2004; Perozo *et al*., 2004; 2008) using VG/GA and others (Liu *et al.,* 2003; Kapczynski and King, 2005; Miller *et al.,* 2007) using different vaccine against various NDV isolates all reported effective protection against challenge. After vaccination with ND La Sota vaccine, laying hens showed no clinical reaction to vaccination; even the immunologically naïve SPF birds. However, the temporary drop in egg production witnessed post-vaccination can be

attributed to some sort of reaction to the vaccine that had effects on FSH/LH secretion (hormonal control).

The absence of post-challenge clinical signs in the vaccinated birds could be attributed to the protection offered by vaccination, as most of the birds had high ND antibody titres subsequent to vaccination. This 100% protection from clinical disease demonstrated in the vaccinated layer hens, shows that both commercial and SPF hens can be protected from ND-related clinical disease when vaccinated with La Sota ND vaccine, by either the cloacal or ocular route, as none of the birds that were challenged after vaccination via either route developed clinical signs or died. Generally, La Sota vaccines are reported to confer greater protection than other ND vaccines such as Ulster 2C, B1 and F (lentogenic strain) (reviewed in Thornton *et al*., 1980; Rehmani, 1996). Work done on the VG/GA vaccine (Avinew) confirmed the vaccine's ability to protect birds from clinical disease and mortality against challenge with virulent NDV strains (Beard *et al*., 1993; Silva *et al*., 2004; Perozo *et al*., 2004; 2008). The protection result emanating from the present study concurs with quite a number of ND vaccine trials (Asplin, 1952; Parede & Young, 1990; Beard *et al*., 1993; Perozo *et al*., 2004; Kapczynski & King, 2005; Miller *et al*., 2007; Perozo *et al*., 2008), all of which demonstrated that the proper application of ND vaccines can protect birds against clinical signs and mortality from ND challenge.

The present study also confirmed that a single application of ND vaccine like La Sota can confer protection against clinical ND, since none of the immunologically naïve SPF birds manifested clinical signs or died from the challenge. This agrees with the report by

Rehmani (1996), where a single application of La Sota vaccine at 12 days of age was sufficient to offer reasonable protection until the chickens were 7 weeks old. In addition, the present study also showed that birds exposed to repeated vaccination are better protected against challenge, as was shown by the commercial birds that had histories of vaccinations and fewer number of NDV-specific positive staining in their oviduct. The results of the present study are also in agreement with those of Parede & Young (1990), who determined that in birds with high antibody titres (immune birds), clinical signs are mild or absent and there may not be any mortality after challenge with virulent field strains.

In both the experiments, the unvaccinated control birds were not protected and all died from the challenge. All the control birds in either experiment died within six day after challenge which met the OIE requirements for such challenge trials. The positive control birds on the other hand had clinical signs, mortalities and lesions that are consistent with that of velogenic NDV infection in non-immunized birds as reported by Biswal & Morrill (1954), McFerran McCracken (1988), Parede & Young (1990), Hamid *et al* (1991) and Ojok & Brown (1996). Torticollis was not observed but there was marked degeneration of the follicles, as has been reported by Biswal & Morrill (1954).

4.4 ND Viral infection and replication in vaccinated birds

The protection achieved in this study however, did not prevent the challenge viruses from infecting and replicating in the host tissues and organs as varied degree of gross pathology were encountered even in the apparently healthy challenged birds that were

euthanased and necropsied at the termination of the trial. IHC done on a few of these organs confirmed the presence of NDV-specific positive staining outside the oviduct as well as inside the oviduct. This agrees with the findings of some workers that vaccination of poultry against ND can only protect birds from the more serious consequence of virulent NDV infection (clinical signs and mortality) but not infection and replication of the virulent strains of the virus (Asplin, 1952; Utterback & Schwartz, 1973; Allan *et al*., 1978; Hamid *et al*., 1990; Parede & Young, 1990; Guittet *et al*., 1993; Alexander *et al*., 1999; Alexander, 2001; Senne *et al*., 2004; Kapczynski & King, 2005; Miller *et al*., 2007). The protection against challenge in the second study also did not prevent the challenged birds from developing necrohaemorrhagic lesions in the caecal tonsils. Similar macropathology has also been reported by Parede & Young (1990) and Hamid *et al* (1991) in high-antibody titre/immune birds. These findings of gross lesions in the challenged birds that did not show clinical signs, and the assertion that vaccination protects against clinical signs and death and not infection and replication of the viruses in the host tissues, were corroborated by the demonstration of viral antigens in the various sections of the oviduct of vaccinated birds in the second part of the study.

The ability of virulent ND strains to infect and replicate even in vaccinated birds as reported by some researchers (Allan *et al*., 1978; Hamid *et al*., 1990) and confirmed in this study by the presence of viral antigens in the oviduct of vaccinated birds, could result in pathological lesions. This probably explains the presence of macroscopic lesions in the "healthy challenged birds" that were euthanased at the end of the trial period. The inability of vaccines to fully protect against viral replication and shedding (though

shedding assessment was not done in this study) especially in natural infections in field situations according to Alexander (2001) presents a bigger problem as it may mask the possible introduction and spread of virulent virus which becomes endemic, but only becomes apparent when the level of immunity level is low.

4.5 The strain of ND virus used for challenge did not affect the level of protection

In the Avinew experiment where two viruses of different genotypes were assessed, no statistically significant differences was detected in the level of protection achieved by the Avinew vaccine against challenge with the two viruses. Indeed, contrary to the belief prior to commencement of this trial that the vaccine may protect less effectively against GPMV than against the "Classic" strain of the disease – it emerged that protection against the older virus was, if anything, slightly poorer. The difference was most probably linked to the slightly higher pathogenicity of the RCV strain (1.85 for GPMV versus 2.00 for the RCV strain). This was shown by the higher clinical scores and mortality rates in the groups challenged with RCV, most clearly shown at a virus dose of $10^{3.0}$ EID_{50}. Macroscopic pathology in the GPMV group was more pronounced than in the RCV group – possibly because birds survived the challenge longer and therefore had more time to develop typical lesions in the GPMV group. Though no statistically significant difference could be established in the protection offered against challenge with the two viruses, slight differences were observed.

Clinical scores were slightly higher for the RCV-challenged groups than the groups challenged with the GPMV virus. The higher clinical scores in the RCV-challenged

groups stemmed from the manifestation of clinical signs in 22 sick and 19 mortalities out of 53 birds as opposed to 17 sick and 14 mortalities out of 53 birds in the GPMV groups. (Fig. 3.1 and Table 3.1) The higher clinical scores resulted despite the fact that the vaccine appeared to delay the onset of clinical slightly in the RCV groups when compared to the GPMV groups. The higher mortality figures as well as the higher PD values for the RCV challenged groups suggested that the RCV is slightly more virulent than the GPMV. The difference in mortality was, however, confined to the lower dose of $10^{3.0}$ EID_{50} where five birds survived in the GPMV group.

Birds challenged with GPMV had macroscopically more organs involvement than birds challenged with RCV. This seems to suggest that the birds were better protected against challenge with GPMV than with RCV and therefore died more slowly thus allowing the infecting viruses ample time to produce more pathologic effects in the organs of affected birds as against the RCV-challenged birds that had per-acute deaths. The slight differences observed between the GPMV and RCV were probably due to pathogenicity rather than due to genetic differences. Generally no statistically significant difference could be detected between challenges with the two ND viruses.

4.6 The route of La Sota vaccination in laying hens did not affect the level of protection offered against challenge with GPMV

In the second experiment, intracloacal and intraocular routes of vaccination were compared to either confirm or disprove the opinion in the field which suggested that cloacal vaccination might provide “better” protection against infection and decreased egg

production than vaccinations applied via the eyes of birds. Though reports have shown that vaccination routes can influence the level of protection offered by the same vaccine against challenge (Kojnok et al., 1977; Rehmani, 1996), the study showed no clear difference in the protection of the oviduct between the two application routes. Slightly more NDV-specific positive staining was observed in the cloacally-vaccinated (CV) birds. This increased positive staining of viral antigens in the CV birds indicates that the vaccine virus can infect, replicate and spread within the tissues. This however did not translate into higher antibody titre and subsequent protection. The slightly higher post-challenge NDV-specific positive staining indicates the presence of ND viral antigens which could impede the infected cells from performing their specialized functions, and in this case, production of poor quality eggs could be the end results**.**

4.7 Susceptibility of oviduct to infection by NDV and its effect on egg production

The second experiment confirmed that the reproductive tract is targeted with both vaccine strain and field isolates of NDV, as has been previously reported by Biswal & Morrill (1954), and Rao *et al* (2002). These results indicate that vaccination with the La Sota vaccine had a transitory detrimental effect on egg production. However, the SPF hens were also much older (82 weeks) than the commercial hen (52 weeks) at receipt, and therefore did not lay very well. The temporary drop in egg production witnessed post-vaccination in the second study can therefore be attributed to the effects of the vaccine on the FSH/LH secretion (hormonal control) possibly through the stress-corticosterone pathway of the oviduct. La Sota vaccine has been reported to have a high “stress factor” as it may produce adverse effects (Mészáros, 1983; Allan & Borland, 1979) that could

cause a temporary drop in production in vaccinated birds. The quantity of positive immunostaining seen in the oviducts post-vaccination gradually increased from day 2PV to reach a maximum on day 10PV. This might indicate the pattern and rate at which the La Sota virus replicates and its subsequent distribution in the tissues of vaccinated birds. The demonstration of viral antigen in the oviducts in addition to the high antibody titres in commercial birds, as early as day 2PV, could be indicative of previous vaccinations. Perozo *et al* (2008) could only detected a La Sota vaccine virus on day 4 PV in tissues of SPF birds apart from trachea, while Rao *et al* (2002) only demonstrated a mesogenic vaccine virus (which is more virulent than the La Sota strain used in the present study) by immunostaining in the tissues of magnum and uterus of SPF birds by day 3 post-inoculation. These prior findings agree with the results of this study, where the La Sota strain was first detected in the uterus and magnum of vaccinated SPF birds on day 4 PV. Positive immunostaining was encountered in birds vaccinated by either cloacal or eyedrop route, further confirming the lack of difference between these two routes in terms of offering protection to vaccinated birds against challenge. This study also demonstrated that the uterus of laying hen is more susceptible to NDV than other sections of the oviduct, but the viral antigens were also detected in the magnum and the isthmus of the birds exposed to the La Sota vaccine.

The post-challenge viral staining was more numerous in the various sections of the oviduct than those seen in the oviducts of post-vaccinated hens. This is similar to the findings of Piacenti *et al* (2006) that velogenic viral antigens were demonstrated in abundance in lymphoid aggregates and/or epithelium as well as in a numbers of organs.

Just as in the PV oviduct, the uterine section was more susceptible than the other sections of magnum and isthmus. The magnum was also more susceptible than the isthmus region of the oviduct. The presence of the viruses in the magnum with their attendant effects could be responsible for the poor albumen quality of eggs laid by ND-infected birds. This was in agreement with the reports of Biswal & Morrill (1954) which demonstrated that the greatest functional damage in the oviduct of ND-infected birds was to the uterus. They reported marked pathologic changes in the glandularis with most of the closely packed glands separated by oedema, considerable shrinkage of the cytoplasm of the glandular cells as well as cystic, atrophic and necrotic uterine glands. Heterophilic and lymphocytic infiltrations were also reported. Oedema, morphological change in glandular cells and cellular infiltration as well as numerous viral antigens staining in the glandular regions of the uterus of some of the challenged birds were also observed in this study.

Rao *et al* (2002) reported the presence of NDV antigen in the lining and glandular epithelium of both the uterus and magnum of birds exposed to either vaccine or field isolates of NDV. The presence of especially the virulent or velogenic GPMV in the reproductive tract of challenged birds in the present study would have severe implications for the performance of the oviduct. Even lentogenic vaccine viruses have been reported by Rao *et al* (2002) to cause 100% ciliostasis by 3 days post-inoculation both in OOC (magnum) and in OOC (uterus) as well as deciliation, degeneration and necrosis of lining and glandular epithelium.

Field isolates of NDV have been reported to cause rapid and complete damage to the ciliated epithelium of the uterus and magnum (Rao *et al*., 2002) and it has been suggested that both the ciliated and/or non-ciliated cells in the shell gland epithelium are involved in the transport of Calcium ions (Ca^{2+}) into the shell gland fluid and the production of the porphrin pigment of the eggshells (Holm *et al*., 2003). The fact that NDV causes ciliostasis and other cilial damage might also account for the egg malformations encountered during ND infection. ND causes a) follicular degeneration resulting in inadequate production of steroid hormones which have been suggested to be responsible in determining the shell quality of eggs and b) poor quality albumen (Sjaastad *et al*., 2003).

However, in the present study, there were no significant morphological changes in the oviduct and consequently the absence of malformed eggs being laid by the infected bird. Therefore, the drop in egg production could be due to the effect of the infecting viruses on the ovarian follicle and possibly general body stress and biochemical changes that may have a negative feedback on hormonal production.

Generally, stresses (e.g. diseases) have been suggested to have the ability to prevent the secretion of LH (Chowdhury, & Yoshimura, 1999; Johnston & Gous, 2006) which is required for ovulation to occur. In addition, viral infections have been reported to cause stress reactions with the elevated production of endogenous glucocorticoids among which is corticosterone (Blalock, 1987). Cortisol, another glucocorticoids produced as a result

of stress during disease conditions, decrease Ca^{2+} absorption from the intestines and increase renal excretion of Ca^{2+} from the body (Eiler, 2004).

The continued excretion of Ca^{2+} from the body can lead to its extreme deficiency thereby resulting into what is termed "Pituitary cut-off mechanism", in which the release of both LH and FSH from the adenohypophysis is inhibited (Taylor, 1965). The inhibition of the release of the gonadotrophins reduces the rate of follicular growth and the subsequent production of oestrogen and progesterone. When this mechanism occurs during egg shell calcification, it produces a chain reaction that eventually results in a reduction in egg production and the production of shell-less eggs. The hormonal aspects regarding the causes of the drop in production were not looked at in this study.

Biochemical changes purportedly occur in viral-infected cells, inducing pathologies that are not visible to the naked eye or with the use of light or electron microscope. Therefore, according to Sharma & Adlakha (1995b); it would be unwise to ignore any cell showing evidence of viral replication, even if microscopic lesions are not evident.

Soft-shelled or shell-less eggs were also laid in the second experiment of this study. This concurs with the reported findings by Biswal & Morrill (1954) and McFerran & McCracken (1988). Also dramatic falls in egg production have been reported as a consistent feature of infection with all pathotypes of ND (Biswal & Morrill, 1954; Al-Garib *et al*., 2003b) as the degeneration of follicles leads to arrest of ovulation and subsequent Oviposition (Biswal & Morrill, 1954).

The distribution of NDV antigen (which may also signify replication) in the oviduct confirmed that the reproductive tract of even vaccinated birds is susceptible to viral infection and possibly viral replication. Clusters of finely granular intracytoplasmic positive staining occurred in lining and glandular epithelium, fibroblasts in the interstitium and mononuclear cells (macrophages, monocytes and lymphocytes). However, target cells in the present study could not always be identified with certainty, because double-staining immunodetection techniques were not applied to facilitate the identification of cells. However, several workers have reported similar target cells for ND infection and replication (Lai & Ibrahim, 1982; Kotani *et al*., 1987; Lockaby *et al*., 1993; Lam & Vasconcelos, 1994; Lam *et al*., 1996; Ojok & Brown, 1996; Al-Garib *et al*., 2003b; Oldoni *et al*., 2005). The presence of ND viral antigen in the aforementioned cells is clearly integral to the pathogenesis of the disease, since infection is likely to result in alterations in the physiological activity of these cells and consequently the organ. Other workers have reported variable pathology in the oviduct of ND-infected birds, ranging from infiltration of inflammatory cells and the formation of lymphoid aggregates, to degenerative changes in ciliated epithelium, atrophy and necrosis of glandular epithelium and oedema of submesothelium in most of the tissues (Biswal & Morrill, 1954; Rao *et al*., 2002).

However, apart from the effects of NDV on the ovary and oviduct of the challenged birds in this study, the percentage (%) lay of the SPF birds was generally low when compared to the commercial birds. Possibly reasons include: The SPF birds were much older (82 weeks) than the commercial birds (52 weeks) when they were received; and secondly, 10

SPF birds were confirmed not to be in lay at necropsy they had small and flaccid oviduct and an inactive ovary characterized by cluster of small and immature ova.

CHAPTER 5 – CONCLUSION

Results from the Avinew trial did not detect any statistically significant difference in protection offered by the vaccine against the GPMV strain in comparison to the "classical" challenge strain. The vaccine protected birds from developing clinical disease and mortality against challenge with both viruses. This indicated good cross-protection by the Avinew vaccine against the two challenge viruses despite belonging to different virus lineage within avian paramyxovirus serotype 1 (APMV-1). This finding does not support the contention that there exists a genetic difference between the lineage 5d/VIId strain (GPMV) and previous strains of NDV that is sufficient to cause an antigenic shift, or that the GPMV may be more virulent than previous strains. The protection offered by Avinew vaccine against challenge with GPMV and RCV was found to be dose-dependant with $10^{6.0}$ EID_{50} producing a 100% protection; $10^{4.5}$ EID_{50} vaccine dose gave a 94.44% protection while at $10^{3.0}$ EID_{50} vaccine dose, only 13.89% of the birds were protected from the consequences of the challenge. The computed protective dose (PD_{50} and PD_{90}) for the Avinew vaccine were $10^{3.51}$ and $10^{4.38}$ for GPMV and $10^{3.79}$ and $10^{4.43}$ for RCV respectively.

In the second experiment, the route of vaccination (cloacal and eyedrop) was found to have no effect on the level of protection against challenge in laying hens as both groups vaccinated with La Sota were protected against challenge with the virulent GPMV.

However, numerous NDV-specific positive staining viral antigen was demonstrated by immunostaining in the various sections of the oviduct in both cloacal and ocularly-vaccinated hens. There was no clear-cut difference in the amount of NDV-specific positive stained viral antigen demonstrated in the oviduct of the cloacal and ocularly-vaccinated SPF or in the commercial hens which indicates that vaccination did not stop either the vaccine or challenge viruses from replicating in the oviduct. Also protection offered by both the vaccines did not stop the challenge viruses from infecting and producing macropathological lesions in other organs of the trial birds, as gross lesions in a number of organs were manifest even in birds that did not show any visible clinical signs before euthanasia. This therefore disproves the report that cloacal vaccination offers a stronger and long lasting immunity and protection than the ocular route.

The susceptibility of the oviduct to colonisation by both the lentogenic La Sota vaccine and the virulent GPMV isolate was established as NDV-specific positive staining of viral antigens was demonstrated in all sections of the oviduct that were examined. The uterus was found to be more susceptible to infection than the magnum; and the magnum more susceptible than the isthmus. The NDV-specific positive staining of viral antigens was also demonstrated in the cytoplasm of epithelial cells, subepithelial cells and the cytoplasm of mononuclear cells within the stroma of the various sections of the oviduct. Such positive staining were also observed in lymphoid nodules, lymphoid aggregates and within connective tissues.

Most researchers have ascribed the causes of the production of malformed eggs to structural damage to the oviduct. However, no obvious morphological damage was observed in the post-vaccinated oviduct, but glandular dilation, interstitial oedema, lymphocytic and plasmacytic infiltration were observed in the various tissues of the oviduct post-challenge.

The cause of egg production drops in the post-vaccinated hens (where no visible lesion or structural damage was observed) was attributed to stress-induced hormonal changes associated with the ND vaccination as La Sota vaccine is reported to have a "high stress" factor which can affect production (Mészáros, 1983; Allan & Borland, 1979). The neuroendocrine response to disease and stress induced by viral challenge has been reported to lead to the activation of the hypothalamic-pituitary-adrenal axis and the hypothalamic-pituitary-gonadal system with the subsequent peripheral secretion of cortisol and corticosterone that affects the metabolism and availability of calcium ions required for use in egg calcification (Blalock, 1987; Eiler, 2004; reviewed in Borghetti *et al*., 2009) with the resultant production of poor quality and malformed eggs. This could be another possible cause for the production of eggs with poor quality albumen and shells, in addition to structural damage that may be caused by the infecting NDV in the shell gland and the albumen-secreting section (magnum). The fact that ND viruses can infect well-vaccinated flocks and replicate within the tissues remains a danger to the poultry industry. The development of improved vaccines against ND that can more effectively reduce the replication of virulent virus during infection will be essential for the long term control of this disease.

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APPENDIX 1A

V043/07

University of Pretoria

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Private Bag X04
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0110

Tel: +27 12 529 8043
Fax: +27 12 529 8300

24 August 2007

Dr SPR Bisschop
Department Production Animal Studies
shahn.bisschop@up.ac.za

Dear Dr Bisschop

PROTOCOL V043/07: CHALLENGE STUDY TO DETERMINE THE PD 50 DOSE OF AVINEW NEWCASTLE DISEASE VACCINE AGAINST TWO DIFFERENT STRAINS OF NEWCASTLE DISEASE

I am pleased to inform you that the abovementioned protocol was approved and signed by the **Research Committee Chairman**, and that you may now commence with your project.

Kind regards

NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

Copy: Prof GH Rautenbach
HOD: Production Animal Studies
gert.rautenbach@up.ac.za

Prof PN Thompson
Departmental Research Co-ordinator
peter.thompson@up.ac.za

APPENDIX 1B

V017-08

University of Pretoria
Animal Use and Care Committee
Private Bag X04
0110 Onderstepoort
Tel +27 12 529 8434
Fax +27 12 529 8300

7 April 2008

Dr SPR Bisschop
Poultry Reference Centre
Production Animal Studies
Faculty of Veterinary Science
(shahn.bisschop@up.ac.za)

Dear Dr Bisschop

V017-08 - Pathogenesis of Goose Paramyxovirus in experimentally infected specific Pathogen-Free and Commercial Hens in South Africa *(DG Bwala)*

The abovementioned protocol was approved by the Animal Use and Care Committee at its meeting held on 31 March 2008.

Kindly note that the protocol has to be approved by the Research Committee as well, before you may commence with the project.

Please contact this office should you have any questions.

Best regards

EMostert
Elmarie Mostert
AUCC Contact Person

Copy: Mrs N Tromp (Niesje.tromp@up.ac.za)
DG Bwala (dgbwala@yahoo.com)

APPENDIX 2A

I
University of Pretoria
Poultry Reference Centre
Faculty of Veterinary Science
University of Pretoria
Private Bag X04
Onderstepoort
0110
Republic of South Africa

Method number	PAS/PRC/035
Date Issued	October, 2007

Method title:

Recording daily observations in PD_{50} trail

Purpose:

To monitor and score observations of the birds challenged with the test virus and, using the data generated to calculate the PD_{50} of the trail vaccine by standard statistical method

Reference:

-Code of Federal Regulation
-European Pharmacopoeia monograph
-OIE Manual of diagnostic tests and vaccine for terrestrial animals

Reagents:

Chemicals:	Classification

Equipment:

Equipment	Consumables
	-Exercise book -Pen -Permanent markers -Polythene bags (380 x 600mm-075mic bags)

5/13/2009	Name:	Date:
Compiled by:	Dr. Bwala, DG	October, 2007
Authorized by:	Dr. SPR Bisschop	

LOGO

Poultry Reference Centre
Faculty of Veterinary Science
University of Pretoria
Private Bag X04
Onderstepoort
0110
Republic of South Africa

Method number	PAS/PRC/035
Date Issued	October, 2007

Method:

	Description	Responsibility
	-Chicks are observed two times daily for 10days after challenge. At each observation, the birds are scored:- ***0 = Normal*** ***1 = Sick*** ***2 = Dead*** -Dead individual birds must be scored as 2 at each of the remaining daily observations after its death. -Dead birds are placed in individual polythene bags, labeled and kept at -20^{o}C awaiting posting according to SOP PAS/PRL/013. Scores (data) generated from the daily readings are used to calculate the PD_{50} using the formula of **Reed & Muench, 1938**.	Technician & Veterinarian

5/13/2009	**Name:**	**Date:**
Compiled by:	**Dr. Bwala, DG**	October, 2007
Authorized by:	**Dr. SPR Bisschop**	

APPENDIX 2B

Poultry Reference Centre

University of Pretoria
Private Bag X04
Onderstepoort
0110
Republic of South Africa

Procedure number	SOP 0104
Date Issued	February 2007

Procedure title:

Euthanasia of Chickens

Purpose:

To describe procedures for the humane euthanasia of chickens. Procedures are devised with the aim of minimizing the exposure of experimental animals to suffering and pain, while ensuring that trial results obtained are clear, unambiguous and able to withstand careful third party scrutiny.

Reference:

Standard ref's

Equipment:

Equipment	Consumables
1) CO_2 Euthanasia a) Euthanasia chamber (rubber bin). b) CO_2 tank fitted with appropriate pressure regulator. c) Rubber connector pipe. 2) Cervical Dislocation a) Scissors or burdizzo may be used depending on size of animal.	1) Barbiturate Overdose a) Syringe and needle appropriate for size. b) Sodium pentobarbital or commercial euthanasia solution. 2) CO2 Euthanasia a) CO_2

	Name:	Date:
Compiled by:	Dr. S.P. R. Bisschop	February 2007
Authorized by:		

Poultry Reference Centre

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Procedure number	SOP 0104
Date Issued	February 2007

Procedure :

Description	Responsibility
1. Asphyxiation using CO_2 This is the preferred method of euthanasia, especially when relatively large numbers of birds must be destroyed. a. The regulator on the CO_2 cylinder is set to the maximum flow rate and CO_2 gas is piped into a plastic rubbish bin from a cylinder for a mimimum of 2 minutes. b. After 2 minutes birds are lowered carefully into the bin. Birds must lose consciousness within 30 seconds of exposure to CO_2 and death should follow approximately 3 minutes later. No more than four adult birds should be euthanased at once. c. All birds are confirmed dead by the absence of a cardiac pulse in the gap in the sternum below the birds' neck, before removal from the bin. d. Birds move from euthansia to the post mortem room or into the dedicated bins for carcass disposal. (SOP pending)	Post mortem room assistant " " "
2. Barbiturate Overdose This method is used routinely when birds need to be euthanased away from the post mortem area, particularly in trials in the isolation unit where potentially dangerous micro-organisms are involved. a. A suitable overdose (approximately 0.5 - 1ml/1kg bodymass) of "Euthanase" is drawn into a syringe. b. The birds are suitably restrained. c. The "Euthanase" is administered to birds either intravenously via the brachial vein or intracranially via the foramen magnum.	Veterinarian " "

	Name:	Date:
Compiled by:	Dr. S.P. R. Bisschop	February 2007
Authorized by:		

University of Pretoria
Private Bag X04
Onderstepoort
0110
Republic of South Africa

Procedure number	SOP 0104
Date Issued	February 2007

d. All birds are confirmed dead by the absence of a cardiac pulse in the gap in the sternum below the birds' neck. In the event there is still a pulse present 2 minutes after administration of the initial dose, a further dose may be administered. e. Once confirmed dead, birds are placed into a plastic bag of the appropriate size and moved to the post mortem area. (SOP pending) f. Record quantity of barbiturate used in drug register. 3. <u>Cervical Dislocation</u> This method of euthanasia is only when methods 1 and 2 cannot easily be carried out. The most common situation would be a circumstance where a bird in the isolation unit meets the criteria for euthanasia but no veterinarian is present to euthanase it with barbiturates. Under these circumstances it is appropriate for the responsible technician to euthanase the bird by cervical dislocation. The neck vertebrae are dislocated and separated, thus severing the spinal chord and causing massive haemorrhage through rupture of the carotid arteries. The procedure results in very rapid death of the bird, but should only be carried out by an experienced operator who should make every effort to ensure the bird dies quickly. a. The head may be separated from the upper neck by a rapid pull and twist motion. b. Cervical vertebrae are crushed using either the handles of a pair of surgical scissors (birds 0-10 days of age) or a burdizza (adult birds). All birds are confirmed dead by the absence of a cardiac pulse in the gap in the sternum below the birds' neck. c. Once confirmed dead, birds are placed into a plastic bag of the appropriate size and moved to the post mortem area. (SOP pending)	Technician " "

	Name:	Date:
Compiled by:	Dr. S.P. R. Bisschop	February 2007
Authorized by:		

APPENDIX 3

	CALIFORNIA ANIMAL HEALTH & FOOD SAFETY LABORATORY UNCONTROLLED		Page 1 of 4
			Revision #: 1
			Status: Uncontrolled
Document #:	DHIS-02-716	Effective date:	July 17, 2008
Document Title:	Paramyxovirus Type-1 IPX Stain	Supersedes:	DHIS-02-716-0
Authorized by:	Mark Anderson	Author:	Mike Manzer
Controlled hard copies of this document are marked on the first page with a colored stamp			

PURPOSE

To detect paramyxovirus in paraffin-embedded tissue sections with an antigen-specific antibody and immunoperoxidase stain.

SCOPE

PMV bound antibodies are visualized after a peroxidase conjugated polymer catalyses a colored substrate-product reaction. Since the performance of certain reagents vary with sources and lots, their optimal use limits inclusion of exact times and concentrations in the Test Method Instructions. Optimal times and concentrations are recorded in the laboratory IHC Records referred to in the References and Related Documentation section.

DEFINITIONS AND ACRONYMS

1. AEC – 3-amino-9-ethylcarbazole
2. PMV – Paramyxovirus
3. IHC – Immunohistochemistry
4. IPX – Immunoperoxidase
5. PBS – Phosphate buffered saline
6. TBS – Tris buffered saline
7. Lab grade water is distilled or deionized water.

SPECIMEN INFORMATION

Any formalin-fixed, paraffin-embedded tissue sectioned at 3-5 microns and mounted on charged slides. Two slides are usually required for each tissue block to be tested. One slide is labeled with the specific primary antibody (PMV) and the other with the nonspecific/negative antibody (Neg PMV). This ensures that slides are properly paired-up for evaluation of questionable staining. The negative slide is routinely included in each run; however, the case coordinators may decide otherwise. Slides are also labeled with the relevant accession number, block number, primary antibody dilution, and run date.

REAGENTS AND MEDIA

About 250 uls of solution is needed per slide for the immunoautostainer, and about 100 uls for manual applications.

1. Hydrogen Peroxide, 3%
   - Hydrogen peroxide, 30% . . . . . 20 ml
   - Methanol, 100% . . . . . 180 ml
2. Rinse Buffer (use one type throughout procedure)
   a. TBS-Tween Buffer
      - TBS 1L
      - Tween 20 . . . . . 0.5 ml

   b. PBS-BRIJ Buffer
      - PBS . . . . . 1 L
      - BRIJ 35 . . . . . 2.5 ml

Document Title:	Paramyxovirus Type-1 IPX Stain			Page 2 of 4
Document #:	DHIS-02-716	Author:	Mike Manzer	Revision #: 1
Status:	Uncontrolled	Authorized by:	Mark Anderson	

REAGENTS AND MEDIA Continued

3. Blocking Solution
   3% Normal Horse Serum
   Normal horse serum . . . . . 3 ml
   Rinse buffer . . . . . 100 ml
4. Antibody Diluent
   3% Normal Horse Serum
   Normal horse serum . . . . . 3 ml
   Rinse buffer . . . . . 100 ml
5. Scott's Tap Water Substitute
   Sodium bicarbonate . . . . . 8 gm
   Magnesium sulfate . . . . . 40 gm
   Lab grade water . . . . . 4 L

For the following solutions, review the IHC records for current sources, lots, optimal dilutions, and incubation times. Suggested sources and use are noted in the Appendices section.

6. Antigen Retrieval Solution
7. Primary Antibody Solutions
   Dilute the specific and nonspecific antibodies in separate reagent tubes of antibody diluent.
8. Secondary Detection Solution
9. Chromogen Substrate Solution

SUPPLIES

Micropipettes, reagent tubes, charged slides, 96° C water bath, Mayer's hematoxylin, coverglass

SPECIAL SAFETY PRECAUTIONS

1. AEC – toxic, avoid contact with skin and mucus membranes.
2. Hydrogen peroxide – oxidizer, corrosive, avoid contact with skin and mucus membranes.
3. Xylene – toxic, avoid inhaling fumes and contact with skin and mucus membranes.
4. See MSDS for additional information.

EQUIPMENT CALIBRATION AND MAINTENANCE

N/A

QUALITY CONTROL

Any formalin-fixed, paraffin-embedded tissue from confirmed or presumptive case or reference material that stains positive by this technique. Current and past positive controls are recorded in the IHC Run log. Labels on control slides include the word "Control", in addition to the information in the Specimen Information section above. At the case coordinator discretion, a case slide need not be paired up with a nonspecific case slide. However, each run will include two duplicate positive tissue control slides; one receives the specific antibody and the other the nonspecific antibody. Results are valid when agent specific sites in the control tissue stain 2+ to 3+ with the specific antibody and 0 to 1+ with the nonspecific antibody.

TEST METHOD INSTRUCTIONS

1. Review the IHC records for current reagent sources, lots, dilutions and incubation times.
2. Deparaffinize and hydrate sections to lab grade water, quenching endogenous peroxidase with 3% hydrogen peroxide for 5-10 minutes after the last absolute alcohol in the deparaffinization series.
3. Rinse slides in running deionized water for 3-5 minutes.
4. Place slides in antigen retrieval solution, then place container and slides in a 96° C water bath for 40 minutes.
5. Place container in cold running tap water for 10 minutes.

Document Title:	Paramyxovirus Type-1 IPX Stain			Page 3 of 4
Document #:	DHIS-02-716	Author:	Mike Manzer	Revision #: 1
Status:	Uncontrolled	Authorized by:	Mark Anderson	

TEST METHOD INSTRUCTIONS Continued

6. Rinse slides in running deionized water for 3-5 minutes.
7. Rinse slides in rinse buffer for 3-5 minutes.
8. Apply blocking solution to sections and incubate for 20-30 minutes.
9. Without rinsing, apply primary antibody solutions to their respective slides and incubate for 20-30 minutes.
10. Rinse sections with rinse buffer for 3-5 minutes.
11. Apply secondary detection solution to sections for 20-30 minutes.
12. Rinse sections with rinse buffer for 3-5 minutes.
13. Apply chromogen to sections for 10 – 20 minutes.
14. Rinse slides in running deionized water for 3-5 minutes.
15. Place slides in Mayer's hematoxylin for 15-90 seconds.
16. Rinse slides in deionized water for 3-5 minutes.
17. Blue slides in tap water for 1-2 minutes. Scott's tap water substitute may be used in place of tap water.
18. Rinse slides in running deionized water for 3-5 minutes.
19. When using AEC chromogen, apply aqueous mounting media to sections, allow to dry, and then coverslip using synthetic mounting media. Sections with Nova Red do not require aqueous mounting media and can be immediately cover slipped with synthetic mounting media.

CALCULATIONS

N/A

EXPECTED VALUES

1. Specific stain – red to brownish red with AEC, or bright red to fuchsin with Nova Red
2. Non-specific stain – no color to light pink
3. Nuclei – blue

INTERPRETATION OF RESULTS

N/A

METHOD LIMITATIONS

The PMV clone 10-5E6 is a mouse IgG_1 monoclonal antibody specific to a phosphoprotein found on the nucleocapsid of paramyxovirus type-1.

POST-ANALYTICAL PROCEDURES

1. Print run date on case and control slides.
2. Complete IHC Run log.

REFERENCES AND RELATED DOCUMENTATION

1. References
   a. Boenisch, T. Handbook of Immunochemical Staining Methods. 3rd Ed. DakoCytomation, Carpinteria, California, 2001.
   b. McGinnes, L, et al. The Protein and the Nonstructural 38K and 29K Proteins of Newcastle Disease Virus Are Derived from the Same Open Reading Frame. Virol 164:256-264, 1988.
   c. Peeples, M. E. Differential Detergent Treatment Allows Immunofluorescent Localization of the Newcastle Disease Virus Matrix Protein within the Nucleus of Infected Cells. Virol 162: 255-259, 1988.
   d. Roach, C. M. A Synergistic, Combination Pretreatment Applied To IHC Using Routinely Processed Tissue Samples. DakoCytomation, Carpinteria, CA, 2003.

Archived

Document Title:	Paramyxovirus Type-1 IPX Stain			Page 4 of 4
Document #:	DHIS-02-716	Author:	Mike Manzer	Revision #: 1
Status:	Uncontrolled	Authorized by:	Mark Anderson	

REFERENCES AND RELATED DOCUMENTATION Continued

2. IHC Records
    a. IHC Run Log for Diagnostics and Reagent QC
    b. IHC Reagent QC Notes
    c. Summary of IHC Reagent QC Notes
    d. IHC Reagent Orders

APPENDICES

1. Suggested Reagent Source and Use
    a. Antigen Retrieval Solution
        i. Combined Target Retrieval
            Protease Type XIV, Sigma catalog # P5147 . . . 2 mg
            Citra Target Retrieval Solution, 1X, Dako . . . 200 ml
    b. Specific Primary Antibody
        i. PMV, clone 10-5E6
            a. CAHFS, starting dilution for optimization 1:25
    c. Nonspecific Primary Antibody
        i. Mouse IgG
            a. Vector Labs, catalog # I-2000, match dilution to specific primary antibody.
    d. Secondary Detection Solution
        i. Horseradish Peroxidase Labeled Polymer, anti-Mouse
            a. DakoCytomation, catalog # K4001, ready to use.
    e. Chromogen Substrate Solution
        i. AEC (use one type and source)
            a. DakoCytomation, catalog # K3464, ready to use.
            b. Vector Labs, catalog # SK-4200
                1. To 5 mls of lab grade water, add 2 drops of Buffer Stock Solution and mix well.
                2. Add 3 drops of AEC Stock and mix well.
                3. Add 2 drops of Hydrogen Peroxide Solution and mix well.
                4. Discard before expiration date or when precipitate develops.
        ii. Nova Red
            a. Vector Labs, catalog # SK-4800
                1. To 5 mls of lab grade water add 3 drops of Reagent 1 and mix well.
                2. Add 2 drops of Reagent 2 and mix well.
                3. Add 2 drops of Reagent 3 and mix well.
                4. Use within 15 minutes.

Uncontrolled

Appendix 4 – Reconstituting 1000mls of 1X Target Retrieval Solution

- Distilled Water ---------- 900mls
- Dako Target Retrieval Solution 10X Concentration, Ref. S1699---------- 100mls
- Sigma Protease, Type XIV, Bacterial, from *Streptomyces griseus* (4.5units/mg solid) #P5147-5G ---------- 10mg
- Mixed on a stirrer with magnetic bar at 750 rpm for about 5-10 minutes ensuring that the Protease is completely dissolved.

Appendix 5 – Preparing 2000mls working PBS – Phosphate Buffered Saline (pH 7.6) with Bovine Serum Albumin

- Distilled water (fresh) ---------- 2000mls
- Sodium chloride crystals extra pure (Nacl) ---------- 17.42g
- Potassium dihydrogen phosphate (KH_2PO_4) ---------- 0.52g
- Di-Sodium hydrogen phosphate, 12 hydrate ($Na_2HPO_4.12H_2O$) ---------- 5.80g
- Mix on a stirrer with magnetic bar for all solids to dissolve completely
- Adjust pH with Nacl or HCL to a Ph of 7.6.
- Add 4gms of Bovine Serum Albumin, Fraction V (Roche Diagnostic GmbH, Germany, - Ref. 10 735 094 001) and mix on a stirrer until dissolved

Appendix 6 – Preparation of 200mls of 2% Milk Powder Solution

- Distilled water ---------- 200mls
- Elite Fat-Free Instant Milk Powder (Clover S.A Pty, Ltd South Africa H2038-3-2-500G) ---------- 4gms
- Mixed on a stirrer with a magnetic bar for milk to completely dissolve
- Filter the milk using filter paper and keep refrigerated.

APPENDIX 7

Case: 829/08

Date received: 2008-04-15

Client Name/Site/House: PRL #26 GPMV

Order No: Bwala

UNIVERSITY OF PRETORIA

Poultry Reference Centre

Private Bag X04. Onderstepoort 0110, Tel 012 529-8224 Fax 012 529-8306

ONDERSTEPOORT — **Numerical Summary - Titer Group Breakdown** — **Interpretation**

08/04/16

Case	Assay	Date	AMean	CV	Count	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
SPRCT26COM8062UNT	NDV	08/04/16	21727	36.3	3										1						1		1
SPRCT26COM7062UNT	NDV	08/04/16	25015	33.6	3												1					1	
SPRCT26COM6062UNT	NDV	08/04/16	6789	28.1	3						1		1	1									
SPRCT26COM5062UNT	NDV	08/04/16	11798	33.4	3								1			1	1						
SPRCT26SPF4083UNT	NDV	08/04/16	46	0	2	2																	
SPRCT26SPF3083UNT	NDV	08/04/16	69	65.3	3	3																	
SPRCT26SPF2083UNT	NDV	08/04/16	63	54	2	2																	
SPRCT26SPF1083UNT	NDV	08/04/16	109	15.2	3	3																	
					22																		

APPENDIX 8

POST-VACCINATION OVIDUCT SAMPLES				POST-CHALLENGE OVIDUCT SAMPLES			
Bird Type	Bird No.	Vac.	Day Euth.	Bird Type	Bird No.	Vac.	Day Euth.
COMM	CV5 S1947-08	CV	2PV	SPF	CV1 S2058-08	CV	2PC
COMM	CV7 S1948-08	CV	2PV	SPF	CV3 S2059-08	CV	2PC
SPF	EV2 S1949-08	EV	2PV	COMM	CV5 S2060-08	CV	2PC
SPF	EV4 S1950-08	EV	2PV	COMM	CV7 S2061-08	CV	2PC
COMM	EV6 S1951-08	EV	2PV	SPF	EV2 S2062-08	EV	2PC
SPF	CV1 S1952-08	CV	4PV	SPF	EV4 S2063-08	EV	2PC
SPF	CV3 S1954-08	CV	4PV	COMM	EV6 S2064-08	EV	2PC
COMM	CV5 S1953-08	CV	4PV	COMM	EV8 S2065-08	EV	2PC
COMM	CV7 S1955-08	CV	4PV	SPF	CV1 S2110-08	CV	4PC
SPF	EV2 S1956-08	EV	4PV	COMM	CV5 S2112-08	CV	4PC
SPF	EV4 S1957-08	EV	4PV	COMM	CV7 S2113-08	CV	4PC
COMM	EV6 S1958-08	EV	4PV	SPF	EV2 S2114-08	EV	4PC
COMM	EV8 S1959-08	EV	4PV	SPF	EV4 S2115-08	EV	4PC
SPF	CV1 S1961-08	CV	6PV	COMM	EV6 S2116-08	EV	4PC
SPF	CV3 S1962-08	CV	6PV	COMM	EV8 S2117-08	EV	4PC
COMM	CV5 S1963-08	CV	6PV	SPF	CV3 S2166-08	CV	6PC
COMM	CV7 S1964-08	CV	6PV	COMM	CV5 S2167-08	CV	6PC
SPF	EV2 S1965-08	EV	6PV	COMM	CV7 S2168-08	CV	6PC
SPF	EV4 S1966-08	EV	6PV	SPF	EV2 S2169-08	EV	6PC
COMM	EV6 S1967-08	EV	6PV	SPF	EV4 S2170-08	EV	6PC
COMM	EV8 S1968-08	EV	6PV	COMM	EV6 S2171-08	EV	6PC
SPF	CV1 S1987-08	CV	8PV	COMM	EV8 S2172-08	EV	6PC
SPF	CV3 S1988-08	CV	8PV	SPF	CV1 S2173-08	CV	8PC
COMM	CV5 S1989-08	CV	8PV	SPF	CV3 S2174-08	CV	8PC
COMM	CV7 S1990-08	CV	8PV	COMM	CV5 S2175-08	CV	8PC
SPF	EV2 S1991-08	EV	8PV	COMM	CV7 S2176-08	CV	8PC
SPF	EV4 S1992-08	EV	8PV	SPF	EV2 S2177-08	EV	8PC
COMM	EV6 S1993-08	EV	8PV	COMM	EV6 S2179-08	EV	8PC
COMM	EV8 S1994-08	EV	8PV	COMM	EV8 S2180-08	EV	8PC
SPF	CV1 S1995-08	CV	10PV	SPF	CV1 S2197-08	CV	10PC
SPF	CV3 S1996-08	CV	10PV	SPF	CV3 S2198-08	CV	10PC
COMM	CV5 S1997-08	CV	10PV	COMM	CV5 S2199-08	CV	10PC
COMM	CV7 S1998-08	CV	10PV	COMM	CV7 S2200-08	CV	10PC
SPF	EV2 S2000-08	EV	10PV	SPF	EV2 S2201-08	EV	10PC
SPF	EV4 S2001-08	EV	10PV	SPF	EV4 S2202-08	EV	10PC
COMM	EV6 S2002-08	EV	10PV	COMM	EV6 S2203-08	EV	10PC
COMM	EV8 S2003-08	EV	10PV	COMM	EV8 S2204-08	EV	10PC